DOCTORAL THESIS

Significance of HMGA2 expression as independent poor prognostic marker in perihilar and distal cholangiocarcinoma resected with curative intent

（肝門部周囲胆管癌，遠位胆管癌におけるＨＭＧＡ２発現の予後予測因子としての意義）

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Consensus Statement

Significance of HMGA2 expression as independent poor prognostic marker in perihilar and distal cholangiocarcinoma resected with curative intent

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ABSTRACT

Background: Extrahepatic cholangiocarcinoma requires invasive surgery and is associated with poor prognosis; thus, a prognostic biomarker is highly needed. Extrahepatic cholangiocarcinoma is subclassified into two types based on their location, namely perihilar and distal. Perihilar cholangiocarcinoma requires lobectomy as curative surgical resection, whereas the distal requires pancreatoduodenectomy. HMGA2 overexpression is reported to correlate with progression, aggressiveness, dissemination and poor prognosis in several types of cancers. Although its association with extrahepatic cholangiocarcinoma has been reported, none of the previous studies assessed its significance in each subtype.

Methods: We assessed the expression of HMGA2 protein in surgical specimens after curative intent surgery in 80 patients including 41 with perihilar cholangiocarcinoma and 39 with distal cholangiocarcinoma by immunohistochemistry. We then examined its association with clinicopathological findings and patient survival outcomes.

Results: We found that HMGA2 was expressed in 51% (21 of 41) of perihilar cholangiocarcinoma and 41% (16 of 39) of distal cholangiocarcinoma samples. In perihilar cholangiocarcinoma, we found significant correlations between expression and vascular invasion and perineural invasion. In distal cholangiocarcinoma, we found that protein levels correlated with tumor grade. Univariate and multivariate analyses demonstrated that HMGA2 expression was an independent poor prognostic factor for patients with both subtypes of disease.

Conclusions: Our results revealed that HMGA2 expression as an independent prognostic marker for both perihilar and distal cholangiocarcinoma that were resected with curative intent.

Introduction

Extrahepatic cholangiocarcinoma (EHCC) consists of perihilar and distal cholangiocarcinomas according to the anatomical location of tumors. Both types are characterized by high malignancy with invasion and metastasis at early stages, resulting in poor prognosis. Curative surgical resection is recommended only for localized tumors; however, the 5-year survival rate after resection

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with negative surgical margins for perihilar and distal cholangiocarcinoma are 25–40% [1–6] and 27–44% [1,7], respectively.

The division of EHCC subtypes into perihilar and distal has been primarily motivated by different approaches to operative resection, and the biological and clinical differences between both subtypes have been reported in several papers [8–11]. Therefore, perihilar and distal cholangiocarcinoma should be viewed as independent entities because of their distinct biology and management.[12] Surgical resection entailing pancreatoduodenectomy is recommended for distal cholangiocarcinoma, whereas lobectomy with bile-duct resection is used for perihilar cholangiocarcinoma. Additional lobectomy or pancreatoduodenectomy and/or vascular resection are occasionally performed to achieve curative resection. Despite these highly invasive surgical procedures for each type, recurrence after surgery is frequent and the efficacy of chemotherapy is limited. It is thus essential to identify prognostic biomarkers to stratify patients, in addition to elucidating the underlying molecular mechanisms and potential targets for treatment.

Many studies have suggested that epithelial-to-mesenchymal transition (EMT) contributes to the early-stage dissemination of cancer cells and is pivotal for invasion and metastasis [12–14]. High-mobility group A2 (HMGA2) is an architectural transcription factor that belongs to the high mobility group AT-hook gene family. It can modulate gene expression, replication, and DNA repair by binding to the minor groove of AT-rich regions of DNA by altering the chromatin structure [15]. It also has the ability to recruit other transcriptional regulators and bind numerous protein complexes located on enhancer sites comprising the enhanceosome [16]. HMGA2 is highly expressed during embryogenesis but is silenced in most normal adult tissues [17–19]. Recently, HMGA2 was reported to be overexpressed in various human cancers and to correlate with progression, aggressiveness, dissemination, and poor patient prognosis [20–30].

A recent study also reported a significant association between HMGA2 expression and prognosis in extrahepatic cholangiocarcinoma [30]. However, it was not clear if this association exists for both perihilar and distal forms of the disease. Furthermore, because nearly half of their cohort had palliative surgery or did not have surgical treatment, this association was not clear within patients treated with curative intent surgery. In this study, we evaluated the association between HMGA2 protein expression and clinicopathologic features and prognosis in a cohort consisting of patients treated by surgery with curative intent. We also evaluated each EHCC subtype, namely perihilar and distal cholangiocarcinoma. This enabled us to assess the clinical significance of HMGA2 in each subtype.

Material and methods

Patients and sample collection

Surgical specimens were obtained from 80 patients with EHCC (41 with perihilar cholangiocarcinoma and 39 with distal cholangiocarcinoma) who underwent surgical resection with curative intent and were diagnosed with adenocarcinoma at the department of Gastroenterological Surgery at Yokohama City University Graduate school of Medicine between January 2009 and December 2016. Perihilar cholangiocarcinoma and distal cholangiocarcinoma was diagnosed according to UICC classification. Perihilar cholangiocarcinoma was defined as tumor located in the extrahepatic biliary tree proximal to the origin of the cystic duct, and distal cholangiocarcinoma was defined as tumor located in the extrahepatic bile duct distal to the insertion of the cystic duct. None of the patients received preoperative therapy. Written informed consent was obtained from each subject. This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Yokohama City University Hospital (B18020005).

Immunohistochemistry

Paraffin-embedded tissues of pancreatic cancer and cholangiocarcinoma were sliced at a thickness of 4 μm using a microtome and were blocked by immersing the slides in a solution of absolute methanol containing 0.3% hydrogen peroxide for 30 min at room temperature. Antigens were retrieved by autoclaving the slides at 121 °C for 15 min in a tris-EDTA buffer (pH 9). Slides were peroxidase-blocked using 3% (w/v) hydrogen peroxide solution for 15 min and then incubated with the primary antibody overnight at 4 °C. Rabbit monoclonal HMGA2 antibody (ab207301, 1:1000 dilution, Abcam, 330 Cambridge Science Park, Cambridge, United Kingdom) was used. Immunohistochemical reactions were visualized using HIstoFine (Nichirei, Tokyo, Japan) and DAB (Dako, Carpinteria, CA, USA) kits. Finally, the sections were counterstained with hematoxylin and examined.

Statistical analysis

The chi-square test or Fisher's exact test was used to compare categorical variables and a Mann-Whitney *U* test was used to compare continuous variables. Overall survival (OS) and disease-free survival (DFS) curves were constructed using the Kaplan–Meier method and compared using the log-rank test. The Cox proportional hazards regression model was used for univariate and multivariate analyses. $P < 0.05$ indicated statistical significance. All statistical analyses were conducted using R statistical software version 3.5.0.

Results

Patients and surgical procedure

We analyzed 80 patients with EHCC who underwent surgical resection without preoperative therapy. Patients consisted of 41 perihilar cholangiocarcinoma and 39 distal cholangiocarcinoma cases. The clinical characteristics of both groups are shown in Supplementary Tables 1 and 2 Most patients were male (90% and 85%, respectively). Of 41 perihilar cholangiocarcinoma patients, 32 (78%) were subjected to hepatectomy and the other nine (22%) underwent bile duct resection. Furthermore, of the 32 patients with hepatectomy, seven had hepatopancreatoduodenectomy because they were diagnosed with perihilar cholangiocarcinoma located in the left or right hepatic bile duct, which extended to the intrapancreatic bile duct. Of the nine patients with bile duct resection, seven had been diagnosed with perihilar cholangiocarcinomas that were located mainly in the common hepatic duct and extended to the intrapancreatic bile duct; therefore, we performed pancreatoduodenectomy with bile duct resection. However, postoperative pathological findings revealed that the tumor did not extend to the intrapancreatic bile duct. All distal cholangiocarcinoma patients were treated by pancreatoduodenectomy including three patients with hepatopancreatoduodenectomy. Of all 41 perihilar cholangiocarcinoma patients, 14 (34%) patients had microscopic positive margins, including nine cases of carcinoma in situ. Of the 39 distal cholangiocarcinoma patients, five (13%) had microscopic positive margins including one case of carcinoma in situ in the distal cholangiocarcinoma group.

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Immunohistochemical staining for HMGA2

The expression pattern of HMGA2 was evaluated by immunohistochemistry based on tumor sections from perihilar and distal cholangiocarcinoma patients. Positive staining was detected only in the nuclei of tumor cells. We evaluated the expression level of HMGA2 protein based on the area of nuclear staining in tumor cells per field of view. We classified the results as Grade 0 for cases without any staining, Grade 1 for those with less than 20% staining, Grade 2 as 20%–50%, and Grade 3 as more than 50%. We defined samples with Grade 2 or Grade 3 staining as HMGA2-positive and defined those of Grade 0 or Grade 1 as HMGA2-negative (Fig. 1). Based on this, 51% of perihilar cholangiocarcinoma tumors and 41% of distal cholangiocarcinoma tumors were positive for HMAG2.

Correlations between HMGA2 expression and clinicopathological characteristics

Table 1 and Table 2 show the correlations between patient clinicopathological characteristics and HMGA2 expression. For perihilar cholangiocarcinoma, HMGA2 expression was related to the presence of vascular invasion and perineural invasion ($P = 0.020$, $P = 0.048$, respectively). In contrast, HMGA2 expression was significantly related to tumor differentiation for distal cholangiocarcinoma. Poorly differentiated adenocarcinoma was more often observed with HMGA2-positive tumors than with negative samples ($P = 0.013$). In addition, patients with HMGA2-positive distal cholangiocarcinoma tumors were subjected to postoperative chemotherapy at a high frequency ($P = 0.049$).

Survival analysis stratified by HMGA2 expression

Next, we examined the correlation between HMGA2 levels and OS or DFS in patients with perihilar and distal cholangiocarcinoma. OS curves stratified by HMGA2 expression revealed that patients with HMGA2-positive tumors had significantly worse prognosis for perihilar cholangiocarcinoma ($P = 0.02$; Fig. 2A). The 5-year survival rates for HMGA2-positive and negative cases were 32.5% and 62.5%, respectively. DFS curves also revealed significantly worse prognosis for patients with HMGA2-positive tumors for perihilar cholangiocarcinoma ($P = 0.0008$ a; Fig. 2B). For distal cholangiocarcinoma, the OS rate of patients with HMGA2-positive tumor was also significantly lower than that of patients with HMGA2-negative tumors ($P = 0.01$; Fig. 3A). The 5-year survival rates of HMGA2-positive and negative distal cholangiocarcinoma were 24.3% and 58.8%, respectively. DFS curves for distal cholangiocarcinoma patients also revealed significantly worse prognosis ($P = 0.009$; Fig. 3B). Regarding the pattern of initial recurrence, liver recurrence was observed in patients with HMGA2-positive tumors at a higher frequency for both subtypes. Specifically, for perihilar cholangiocarcinoma, 39.3% (6/17) of recurrent patients with HMGA2-positive tumors had initial liver recurrence, whereas this rate was 18.2% (2/11) with HMGA2-negative tumors. For distal cholangiocarcinoma, initial liver recurrence occurred in 50.0% (5/10) of recurrent patients with HMGA2-positive tumors and in 12.5% (1/8) of recurrent patients with HMGA2-negative tumors (Supplementary Table 3).

Prognostic factors for perihilar and distal cholangiocarcinoma

According to univariate analysis of OS, in addition to HMGA2 expression, positive surgical margin, the presence of lymph node metastasis, tumor differentiation (poorly differentiated adenocarcinoma), the presence of lymphatic invasion, and the presence of vascular invasion were significant poor prognostic factors for perihilar cholangiocarcinoma. Multivariate analysis using the Cox proportional hazard model indicated that HMGA2 expression and tumor differentiation were independent predictive factors

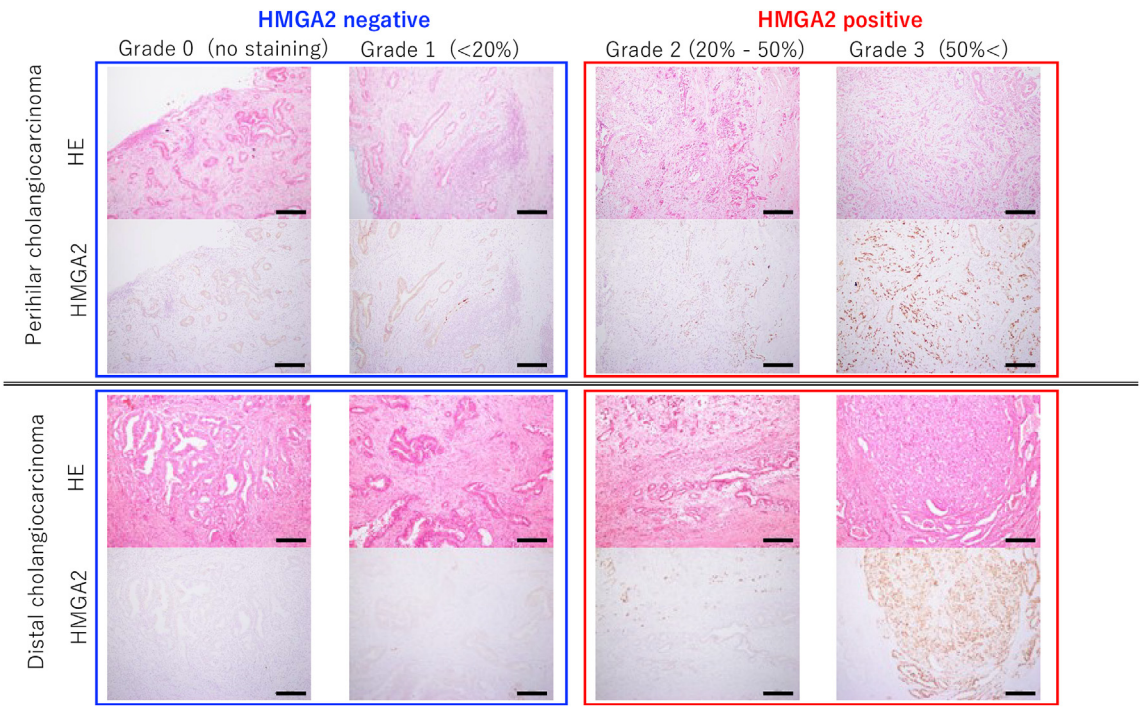

Fig. 1. Immunohistochemistry (IHC) staining for HMGA2 in perihilar and distal cholangiocarcinoma samples. Original magnification, × 100. Bar, 200 μm. Positive nuclear staining was detected only in tumor cells. The positive staining rate was defined based on four grades by the area containing positive nuclear-stained tumor cells. Grade 0 or 1 was defined as HMGA2-negative, whereas Grade 2 or 3 was defined as HMGA2-positive.

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Table 1
Clinical data of perihilar cholangiocarcinoma patients in HMGA2 positive and negative (n = 41).

Characteristic		HMGA2 + (n = 21)	HMGA2 - (n = 20)	P value
Age	mean (range)	73 (56–85)	70 (40–86)	0.240
Gender	Male	20	17	0.343
	Female	1	3	
CA19–9 (U/mL)	>37	13	16	0.306
	37≧	8	4	
CEA (ng/mL)	>5	5	4	1.000
	5≧	16	16	
Surgical margin	+	9	5	0.326
	-	12	15	
Lymph node metastasis	+	12	7	0.215
	-	9	13	
Differentiation	por	4	4	1.000
	well/mod	17	16	
Lymphatic invasion	+	8	6	0.744
	-	13	14	
Vascular invasion	+	18	10	0.020
	-	3	10	
Perineural invasion	+	21	16	0.048
	-	0	4	
Liver invasion	+	5	1	0.184
	-	16	19	
Portal vein invasion	+	6	7	0.744
	-	15	13	
Arterial invasion	+	2	2	1.000
	-	19	18	
UICC stage	≧IIIA	16	11	0.197
	IIIA>	5	9	
Adjuvant chemotherapy	+	12	11	1.000
	-	9	9	

Table 2
Clinical data of distal cholangiocarcinoma patients in HMGA2 positive and negative (n = 39).

Characteristic		HMGA2 + (n = 16)	HMGA2 - (n = 23)	P value
Age	mean (range)	67 (56–83)	72 (58–94)	0.161
Gender	Male	14	19	1.000
	Female	2	4	
CA19–9 (U/mL)	>37	8	6	0.179
	37 ≧	8	17	
CEA (ng/mL)	>5	4	3	0.415
	5≧	12	20	
Surgical margin	+	2	3	1.000
	-	14	20	
Lymph node metastasis	+	7	8	0.740
	-	9	15	
Differentiation	por	6	1	0.013
	well/mod	10	22	
Lymphatic invasion	+	3	2	0.631
	-	13	21	
Vascular invasion	+	7	4	0.146
	-	9	19	
Perineural invasion	+	9	12	1.000
	-	7	11	
Pancreatic invasion	+	8	6	0.179
	-	8	17	
Portal vein invasion	+	3	0	0.061
	-	13	23	
Arterial invasion	+	1	1	1.000
	-	15	22	
UICC stage	≧IIA	12	12	0.192
	IIA>	4	11	
Adjuvant chemotherapy	+	12	9	0.049
	-	4	14	

(P = 0.03) for patients with perihilar cholangiocarcinoma (Table 3).

For distal cholangiocarcinoma, similar results were observed. Specifically, HMGA2 expression, the presence of lymph node metastasis, and tumor differentiation were significant poor prognostic factors. Multivariate analysis indicated that HMGA2 expression and lymph node metastasis were independent predictive factors (P = 0.012) for patients with distal cholangiocarcinoma (Table 3). Three patients with distal cholangiocarcinoma had portal vein invasion, and all of them had an extremely poor prognosis. One patient died of cancer 5.7 months after surgery, the second patient died of cancer 10.6 months after surgery, and the third patient died of another disease 7.8 months

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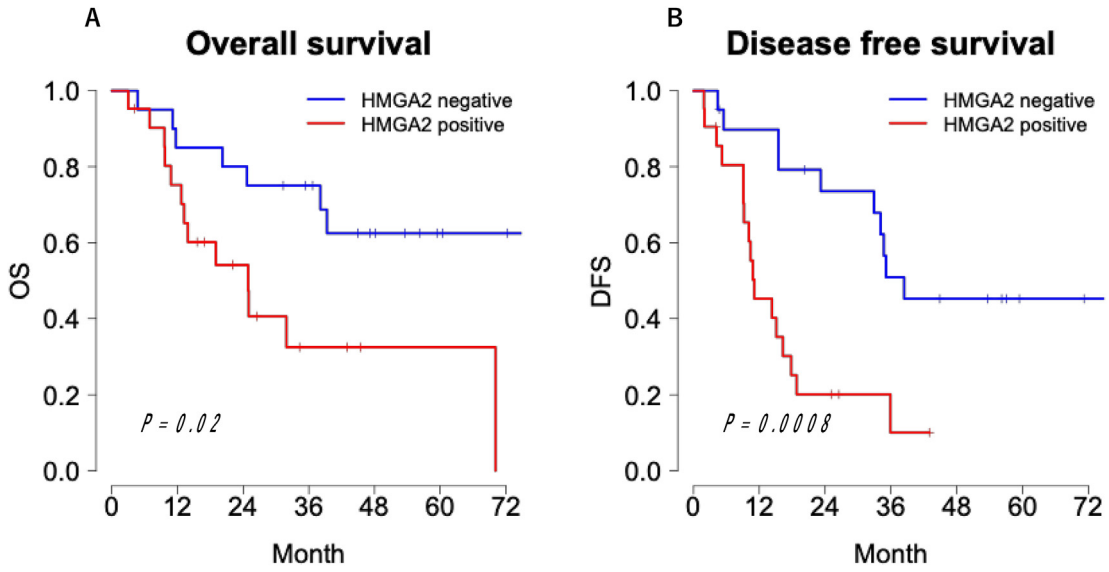

Fig. 2. Kaplan–Meier plot for overall survival (A) and disease-free survival (B) of patients with perihilar cholangiocarcinoma stratified by the expression of HMGA2. Both OS and DFS of perihilar cholangiocarcinoma cases with HMGA2 positive were significantly worse than the case with HMGA2 negative.

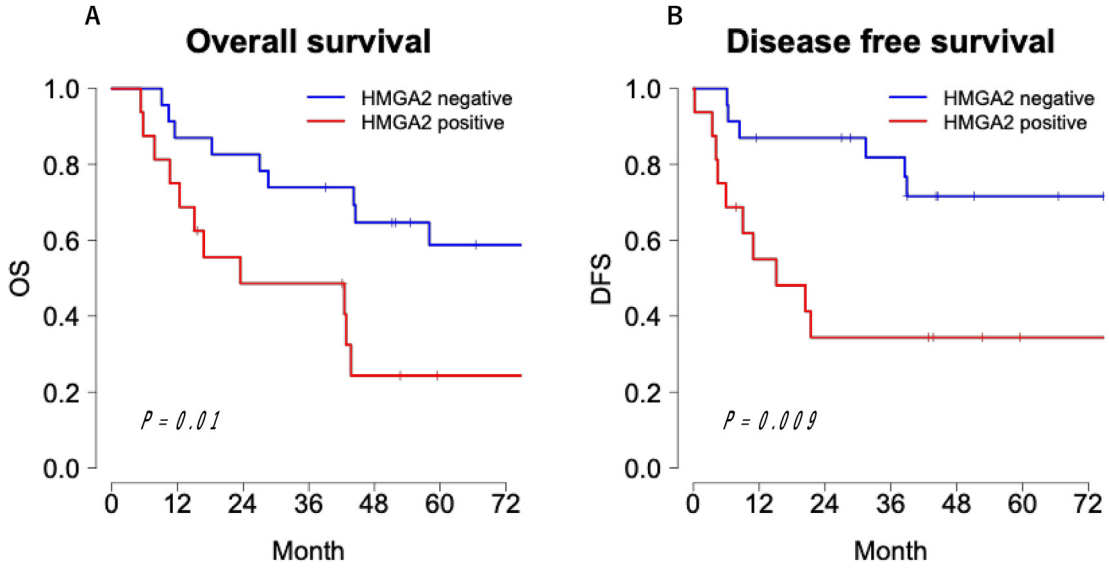

Fig. 3. Kaplan–Meier plot of overall survival (OS) (A) and disease-free survival (DFS) (B) for patients with distal cholangiocarcinoma stratified based on the expression of HMGA2. OS and DFS of distal cholangiocarcinoma HMGA2-positive cases were significantly worse than those for HMGA2-negative cases.

after surgery. To avoid the strong bias, we excluded "portal vein invasion" from multivariate analysis.

Discussion

In this study, we revealed that HMGA2 is a key prognostic marker for both types of EHCC, namely perihilar and distal cholangiocarcinoma, treated with surgical resection. HMGA2 expression was observed in 51% of perihilar cholangiocarcinomas and 41% of distal cholangiocarcinomas and was associated with poor prognosis. It was previously reported that the expression of HMGA2 is correlated with increasing tumor grade [20–23], vascular invasion [24], lymphatic invasion [25,26], and perineural invasion [25]. A high frequency of lymph node metastasis [20,21,25–27] and distant metastasis [28,29] was also observed in various cancers with HMGA2 expression in many studies. Based on our data, HMGA2 expression was correlated with vascular invasion and perineural invasion in perihilar cholangiocarcinoma and with tumor grade in distal cholangiocarcinoma. These results are consistent with the results of previous studies on other malignancies [20–25], suggesting that HMGA2-positive tumors have aggressive, malignant potential in many cancer types including perihilar and distal cholangiocarcinoma.

Survival analyses further showed the high malignancy of HMGA2-expressing perihilar and distal cholangiocarcinoma. The prognosis of patients with HMGA2-positive tumors was significantly worse than that of patients with HMGA2-negative tumors. Lymph node metastasis, surgical margin, and tumor grade were previously reported to be prognostic factors of perihilar and distal cholangiocarcinoma [31,32]. Based on our perihilar cholangiocarcinoma data, they were also identified as prognostic factors with lymphatic invasion and perineural invasion by univariate

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Table 3
Univariate and multivariate analysis of OS in perihilar and distal cholangiocarcinoma patients.

		Perihilar cholangiocarcinoma (n = 41)					Distal cholangiocarcinoma (n = 39)				
Variable		No of patients	Univariate Analysis		Multivariate Analysis		No of patients	Univariate Analysis		Multivariate Analysis	
			Hazard Ratio (95% CI)	P value	Hazard Ratio (95% CI)	P value		Hazard Ratio (95% CI)	P value	Hazard Ratio (95% CI)	P value
Age	>75	26	1.028 (0.404	0.953			28	1.603 (0.638–4.08)	0.311		
	75≧	15	−2.615)				11				
Gender	Male	37	0.640 (0.207	0.435			33	1.966 (0.455–9.488)	0.356		
	Female	4	−1.977)				6				
CA19−9 (U/mL)	>37	13	1.160 (0.466	0.760			13	0.976 (0.389–2.450)	0.958		
	37≧	8	−3.015				8				
CEA (ng/mL)	>5	5	1.174 (0.426	0.755			5	1.083 (0.361–3.244)	0.887		
	5≧	16	−3.233)				16				
Surgical margin	+	14	2.571 (1.030	0.036			34	1.400 (0.409–4.793)	0.590		
	-	27	−6.421)				5				
Lymph node metastasis	+	19	3.852 (1.487	0.003			15	2.733 (1.125–6.642)	0.021	3.016 (1.220	0.017
	-	22	−9.977)				24			−7.456)	
Differentiation	por	8	4.555 (1.733	0.001	8.894 (2.768	<0.001	7	2.926 (1.104–7.758)	0.024		
	well/ mod	33	−11.973)		−28.578)		32				
Lymphatic invasion	+	14	3.216 (1.341	0.006			5	1.134 (0.331–3.882)	0.841		
	-	27	−7.716)				34				
Vascular invasion	+	28	6.566 (1.511	0.004			11	1.448 (0.555–3.779)	0.447		
	-	13	−28.522)				28				
Perineural invasion	+	37	233115300 (1-Inf)	0.074			21	0.650 (0.269–1.572)	0.335		
	-	4					18				
Liver invasion	+	6	3.047 (1.190	0.555			14	2.010 (0.825–4.899)	0.117		
	-	35	−7.802)				25				
Portal vein invasion	+	13	1.884 (0.795	0.144			3	21.444 (4.133	<0.001		
	-	28	−4.467)				36	−111.254)			
Arterial invasion	+	4	1.525 (0.477	0.474			2	1.225 (0.163–9.196)	0.843		
	-	37	−4.867)				37				
HMGA2	+	21	3.047 (1.190	0.015	5.234 (1.767	0.003	16	2.956 (1.201–7.270)	0.014	3.246 (1.296	0.012
	-	20	−7.802)		−15.496)		23			−8.133)	
Adjuvant chemotherapy	+	18	1.037 (0.442	0.936			18	1.012 (0.421–2.435)	0.978		
	-	23	−2.548)				21				

analysis, and tumor grade was the only factor identified as an independent prognostic factor with HMGA2 expression. For distal cholangiocarcinoma, lymph node metastasis and tumor grade were significant prognostic factors based on univariate analysis and lymph node metastasis was an independent prognostic factor with HMGA2 expression.

In this study, we used surgically-resected samples. The detection of HMGA2 expression in perihilar and distal cholangiocarcinoma is helpful to predict patient prognosis. In addition, if HMGA expression can be assessed in tumors using biopsy samples, this could help to decide treatment strategies including preoperative treatment.

It was reported that HMGA2 plays an important role in EMT in various malignancies [33–35], which was shown to confer invasive and metastatic characteristics. EMT is highly regulated through several HMGA-mediated signaling pathways such as TGFβ [14,34,36], Wnt/β-catenin [37,38], IL6/Stat 3 [39], and MAPK [40]. The Let 7 miRNA family is known to inhibit HMGA2 [41–43] indicating that it could be exploited as a new nucleic acid-based therapeutic.

In conclusion, we found that HMGA2 expression is as independent prognostic factor for perihilar and distal cholangiocarcinoma. This could thus be a useful biomarker to predict disease outcomes and decide treatment strategies.

CRediT authorship contribution statement

Tomoaki Takahashi: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Hideya Kawaji:** Conceptualization, Methodology, Writing - review & editing. **Yasuhiro Murakawa:** Conceptualization, Methodology, Writing - review & editing. **Yoshihide Hayashizaki:** Conceptualization, Methodology, Writing - review & editing, Supervision. **Takashi Murakami:** Methodology, Resources. **Yasuhiro Yabushita:** Methodology, Resources. **Yuki Homma:** Methodology, Resources. **Takafumi Kumamoto:** Methodology, Resources. **Ryusei Matsuyama:** Methodology, Resources. **Itaru Endo:** Conceptualization, Methodology, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejso.2020.08.005.

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論文目録

Ⅰ 主論文

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Ⅱ 副論文

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Ⅲ 参考文献

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