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From Cell Biology to Tissue Engineering

Overexpression of lactate dehydrogenase A in cholangiocarcinoma is correlated with poor prognosis

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Summary. Lactate dehydrogenase A (LDHA), a key metabolic enzyme, plays a crucial role in the final step of anaerobic glycolysis. Overexpression of LDHA is observed in many human malignancies in association with tumor progression. The purpose of this study was to investigate LDHA expression pattern during carcinogenesis, its clinico-pathological association, and evaluate the prognostic value of LDHA in CCA patients. LDHA expression was investigated using immunohistochemistry technique in both hamster- (n=60) and human-CCA tissues (n=82). Plasma LDH from healthy control (n=40) and CCA patients (n=29) were determined using an enzymatic based assay. The association of LDHA expression with clinicopathological findings and prognostic value were evaluated by statistical analysis. In the CCA hamster model, an increase of LDHA expression was associated with the progression of CCA-genesis. Higher LDHA overexpression was associated with shorter survival of CCA patients. Multivariate analysis indicated that LDHA expression including histological type were independent prognostic risk factor of patient's survival. However, there was no difference in plasma LDH level between CCA patients and healthy controls. LDHA expression is involved in cholangio-carcinogenesis. Overexpression of LDHA can be a marker of poor prognosis in CCA patients and it might be a potential target for CCA treatment.

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Introduction

Cholangiocarcinoma (CCA), a cancer of bile duct epithelium, is a rare malignancy worldwide. However, CCA has its highest incidence and is a leading cause of morbidity and mortality in Southeast Asian countries, especially in Thailand (Sripa and Pairojkul, 2008). This cancer is still a major public health problem in Northeastern Thailand (Andrews et al., 2008). Epidemiological (Haswell-Elkins et al., 1994) and experimental animal studies (Thamavit et al., 1978) indicated that a liver fluke, *Opisthorchis viverrini* (Ov) infection is the major risk factor for CCA development in this area.

The difficulty of dealing with CCA is the availability of early diagnosis and effective treatment (Sirica, 2005). So far there is no specific tumor marker for early detection of CCA. Surgical resection is still the only curative treatment for CCA (Khan et al., 2012). However, the majority of CCA-patients are unresectable due to the late stage of detection (Blechacz and Gores, 2008). Moreover, this tumor is often resistant to conventional chemo- and radio-therapy resulting in poor prognosis (Rizvi and Gores, 2013). Therefore, identification of a specific tumor marker and development of effective treatment for CCA are still urgently required.

Metabolic alteration is one of the ten cancer

hallmarks as described by Hanahan and Weinberg (Hanahan and Weinberg, 2011). Preferential anaerobic glycolysis, named Warburg effect, usually occurs in cancers (Warburg, 1956). This is not only for energy generation but also for production of intermediates in macromolecule biosynthesis such as protein, lipid and nucleotide synthesis to support rapid growth and proliferation of cancer cells (Gatenby and Gillies, 2004). Thereby, glucose transporters and key enzymes in the glycolytic pathway such as hexokinase, pyruvate kinase and lactate dehydrogenase are found up-regulated in several cancers (Miao et al., 2013). These three key enzymes are intensively studied as potential biomarker and target molecules for cancer treatment. Since LDH plays an important role in carcinogenesis (Le et al., 2010), we attempted to explore its role in CCA.

LDH enzymes are encoded by two genes, *LDHA* and *LDHB*, which generate two types of subunits, M and H. Since active LDH enzymes are the tetramers of various combination of M and H, there are 5 LDH isoforms; LDH-1 (4H), LDH-2 (3H, 1M), LDH-3 (2H, 2M), LDH-4 (1H, 3M), and LDH-5 (4M) (Maekawa and Sugano, 1998). LDH5 is also called LDHA which preferentially converts pyruvate to lactate in the final step of glycolysis. Overexpression of LDHA has been reported in several cancers such as gastric-, colorectal-, lung-, and pancreatic cancers (Miao et al., 2013; Rong et al., 2013). Association of LDHA expression level with tumor stage, tumor size, histological grade and survival has been reported in renal and gastric cancer patients (Girgis et al., 2014; Kim et al., 2014).

In the present study, the possible involvement of LDHA in carcinogenesis was investigated in the Ov-induced CCA hamster model. In addition, the clinical implication of LDHA expression as a prognostic marker was investigated in CCA patients.

Materials and methods

Tissue samples

Animal tissues

Formalin-fixed paraffin embedded samples were obtained from Ov-induced CCA-hamster model. Syrian golden hamsters (n=60) were divided into 4 groups based on the treatments as follows; untreated normal control group, Ov-infected group, N-nitrosodimethylamine (NDMA)-treated group and Ov-NDMA treated group in which 5 animals each were sacrificed at 1, 3, and 6 months (Boonmars et al., 2009). The protocol of the study was approved by the Ethics committee for Animal Research of Khon Kaen University (AEMDKKU1/2558).

Human specimen

Formalin-fixed paraffin embedded samples (n=82) and tissue-unmatched-plasma (n=69) were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand. Written informed consent was obtained from each subject and the protocol has been reviewed and approved by the Ethics Committee for Human Research of Khon Kaen University (HE571283 and HE531320) based on the Declaration of Helsinki and ICH-Good Clinical Practice Guidelines. All tissues were histologically proven to be CCA. Clinico-pathological data of CCA patients were retrieved from clinical and pathological records, including age, gender, histological types of CCA, tumor size, tumor staging, and survival.

LDHA immunohistochemistry

The immunohistochemistry was performed according to the standard protocol (Taylor, 2006). The tissue slides were incubated with 1:1600 diluted sheep anti-LDHA polyclonal antibody (Abcam, Cambridge, USA; cat. no. ab9002) at room temperature overnight. Then, tissue sections were incubated with 1:100 diluted HRP-conjugated rabbit polyclonal antibody to sheep IgG (Abcam; cat. no. ab6747) for 1 hour at room temperature. The peroxidase activity was developed using diaminobenzidine tetrahydroxychloride solution (DAB; Dako, Glostrup, Denmark) as the substrate and counterstained with hematoxylin.

LDHA expression levels were evaluated using an immunostaining score. The frequency score of LDHA expression was graded according to the percentage of positively stained tumor cells: (<10%=0, 10-25%=1; 26-50%=2; and >50%=3). The intensity of LDHA expression was scored as: negative=0, weak=1, moderate=2 and strong=3. The immunohistochemistry indices of LDHA expression (frequency X intensity) were categorized using the cut-off point=6, into low (<6) and high (≥6) expression.

Plasma LDH levels in CCA patients

Blood samples were collected in a tube containing anticoagulant, 3.2% sodium citrate. After centrifugation at 1000x g, 4°C, for 20 minutes, plasma samples were collected and kept at -20°C before analysis. Plasma LDHA level was determined using the enzymatic assay auto-machine (SYNCHRON LX20PRO, BECKMAN COULTER, USA) according to the manufacturer's protocol.

Statistical analysis

Data were analyzed using SPSS Statistic 17.0 software (SPSS, Inc., Chicago, IL). Chi-square (χ^2) test was used to investigate association between LDHA expression and clinico-pathological findings. Survival analysis was conducted using Kaplan-Meier curves (Log-rank test). Multivariate analysis was performed by Cox regression to estimate the prognosis significant

independent factors. P value <0.05 was considered to be statistically significant.

Results

LDHA expression was up-regulated during cholangiocarcinogenesis in Ov-induced CCA-hamster model

The relationship between LDHA expression and cholangiocarcinogenesis was investigated in the Ov-associated CCA hamster tissues. LDHA was not expressed in normal bile duct of untreated control group (Fig. 1A) and NDMA group (Fig. 1C) at any time point examined. Marginal to low LDHA expression was observed in the bile duct hyperplasia of the untreated group at 3 and 6 monthsand in the NDMA group at 1 and 3 months. The strikingly high expression of LDHA was seen in Ov infected group (Fig. 1B), mostly in the bile duct hyperplasia. As was expected, the highest expression of LDHA was observed in CCA tissues of the Ov plus NDMA-treated group (Fig. 1D). The results in Fig. 1 showed that LDHA expression was increased with carcinogenesis steps, from normal to hyperplasia towards cancer. The percentage of positive LDHA expression and histological classification in these 4 groups of treatment are summarized in Table 1. Almost all hyperplasia of each group and 100% of cancer area of the Ov plus NDMA group showed LDHA expression. When the data at 1, 3, and 6 months of the cancer group, Ov plus NDMA-treated group, were analyzed based on histological classification, the level of LDHA expression was increased significantly from normal bile duct to

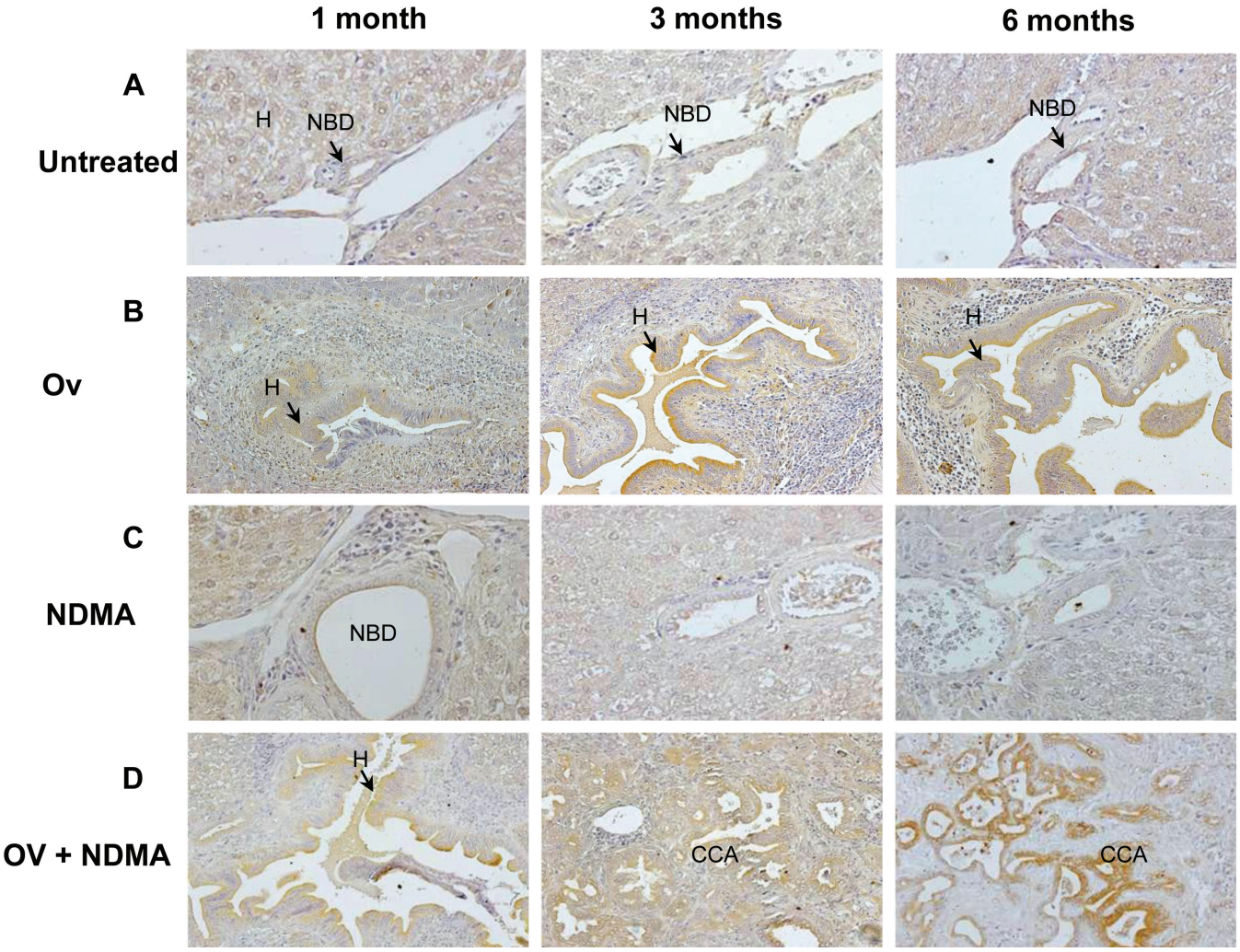

Fig. 1. Immunohistochemistry staining for LDHA expression in hamster-CCA tissues (**A-D**). LDHA expression is related to carcinogenesis of CCA. **A.** Untreated group, no staining of LDHA expression in normal bile duct (NBD). **B.** Ov infected group, high expression of LDHA in bile duct hyperplasia (HD). **C.** NDMA treated group, staining of LDHA expression in normal bile duct. **D.** Ov + NDMA group, highest LDHA expression in tumor area at 3 and 6 months. x 200.

hyperplasia towards cancer (Fig. 2).

LDHA expression was associated with a shorter overall survival of CCA patients

The association of immunohistochemical expression level of LDHA and clinico-pathological findings were examined using 82 CCA tissues with clinical data of the patients. LDHA was expressed in both cytoplasm and nuclei of the cancer cells. In most cases, cytoplasmic reactivity was stronger than the nuclear staining. Higher LDHA expression was observed in CCA area compared to normal bile duct in the adjacent tissues of the same case. The percentage of LDHA expression was 90% (75/82) in CCA areas and 10.5% (8/76) in adjacent tissues containing normal bile duct. The representative immunohistochemical staining of LDHA in the normal bile duct, bile duct hyperplasia, and CCA of humans are illustrated in Fig. 3.

According to the immunohistochemistry index, LDHA expression levels were categorized into two groups, low (<6) and high (≥6) expression. The results of univariate analysis of LDHA expression and clinico-pathological findings of CCA patients were shown in Table 2. The patients with a survival time of less than 30 days and those with incomplete information of pathology were excluded from the analysis. No significant differences among the variables tested with LDHA expression were found.

Survival analysis performed according to the Kaplan-Meier analysis method showed a significant correlation between LDHA expression levels and survival (P=0.019) (Fig. 4). Univariate analysis indicated

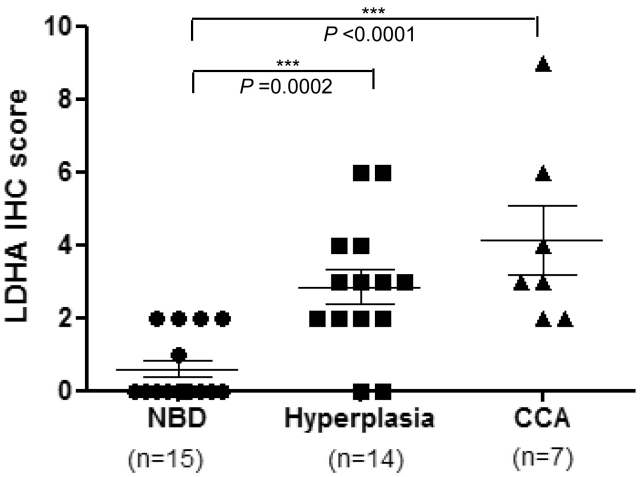

Fig. 2. LDHA expression based on histological classification; normal bile duct, hyperplasia, and CCA in Ov-associated CCA hamster tissues.

Table 2. LDHA expression and clinico-pathological findings of CCA patients.

Clinical characteristics		LDHA expression		
	No. of patients	Low n (%)	High n (%)	P-value
Age (y) (n=82)				0.280
<56	40	17 (42.5)	23 (57.5)	
≥56	42	23 (54.8)	19 (45.2)	
Gender (n=82)				0.482
Male	55	25 (45.5)	30 (54.5)	
Female	27	15 (55.6)	12 (44.4)	
Histological type (n=82)				1.000
Non papillary	55	27 (49.0)	28 (50.9)	
Papillary	27	13 (48.1)	14 (51.9)	
T stage(n=82)				0.104
T1	6	4(66.6)	2 (33.4)	
T2	10	7 (70.0)	3 (30.0)	
T3	33	18 (54.5)	15 (45.5)	
T4	33	11 (33.3)	22 (66.7)	
N stage(n=72)				0.098
N0	38	23 (68.4)	15 (46)	
N1	34	13 (39)	21 (61)	
M stage (n=75)				0.516
M0	65	34 (52.3)	31 (47.7)	
M1	10	4 (40.0)	6 (60.0)	
Tumor stage (n=82)				0.112
I+II	11	8 (72.7)	3 (27.3)	
III+IV	71	32 (45.1)	39(54.9)	
Tumor size (cm) (n=82)				0.278
<7	38	16 (42.1)	22 (57.9)	
≥7	44	24 (54.5)	20 (45.5)	

The association between LDHA expression and clinico-pathological findings were analyzed using Chi-square (χ^2) test. * P<0.05.

Table 1. LDHA expression and histological classification in hamster liver tissues.

Group of experiments	1M			3M			6M		
	NBD	Hyper	CCA	NBD	Hyper	CCA	NBD	Hyper	CCA
Untreated	0/5 (0)	-	-	0/5 (0)	1/1 (100)	-	0/5 (0)	2/2 (100)	-
Ov	1/5 (20)	4/4 (100)	-	1/5 (20)	5/5 (100)	-	2/5 (40)	5/5 (100)	-
NDMA	0/5 (0)	2/3 (67)	-	0/5 (0)	1/4 (25)	-	0/5 (0)	0/3 (0)	-
Ov+ NDMA	2/5 (40)	4/5 (80)	-	2/5 (40)	5/5 (100)	3/3 (100)	0/5 (0)	3/5 (60)	4/4 (100)

All data are expressed as n/N (%), n=number of positive cases of LDHA expression, N=total cases with the presence of histological classification. NBD: Normal bile duct, Hyper: Hyperplasia, CCA: Cholangiocarcinoma.

that histological type, M stage,and LDHA expression levels were dependent factors of survival (Table 3). Multivariate analysis indicated that histological type, and LDHA expression were independent risk factors to poor prognosis of CCA (Table 3).

Plasma LDH levels in healthy control and CCA patients

Plasma LDH levels from healthy controls (n=40) and CCA patients (n=29) were measured using an automated enzymatic assay. There was no statistically significant difference in plasma LDH levels between healthy controls (136.9±56.0 U/L) and CCA patients (142.4±73.1 U/L) as shown in Fig. 5.

Discussion

In the present study, we first investigated whether LDHA expression is associated with CCA-genesis using an Ov-induced CCA hamster model. The results clearly showed that immunohistochemical LDHA expression

Table 3. Univariate and multivariate analysis of LDHA expression and clinico-pathological findings in CCA patients (n=82).

Characteristics	Groups	HR (95% CI)	P -value
Univariate analysis			
Age (y)	≥56 vs.<56	1.05 (0.68-1.63)	0.831
Gender	Male vs. Female	0.93 (0.58-1.48)	0.743
Histological type	Non papillary vs. Papillary	2.23 (1.34-3.73)	0.002*
T stage	T_4 vs. $T_1/T_2/T_3$	1.06 (0.85-1.31)	0.635
N stage (n=72)	N_1 vs. N_0	1.05 (0.77-2.03)	0.362
M stage (n=75)	M_1 vs. M_0	2.57 (1.29-5.12)	0.007*
Tumor stage	III+IV vs. I+II	1.29 (0.68-2.46)	0.436
Tumor size (cm)	≥7 vs.<7	1.24 (0.80-1.94)	0.341
LDHA	High ≥6 vs. Low <6	1.69 (1.08-2.62)	0.020*
Multivariate analysis			
Histological type	Non papillary vs. Papillary	2.20 (1.17-4.14)	0.014*
LDHA	High ≥6 vs. Low <6	2.05 (1.05-3.99)	0.036*

Univariate and Multivariate analysis was performed by Cox regression. *: P value <0.05. All parameters are included in multivariate analysis, but only parameters that reach statistical significant are illustrated.

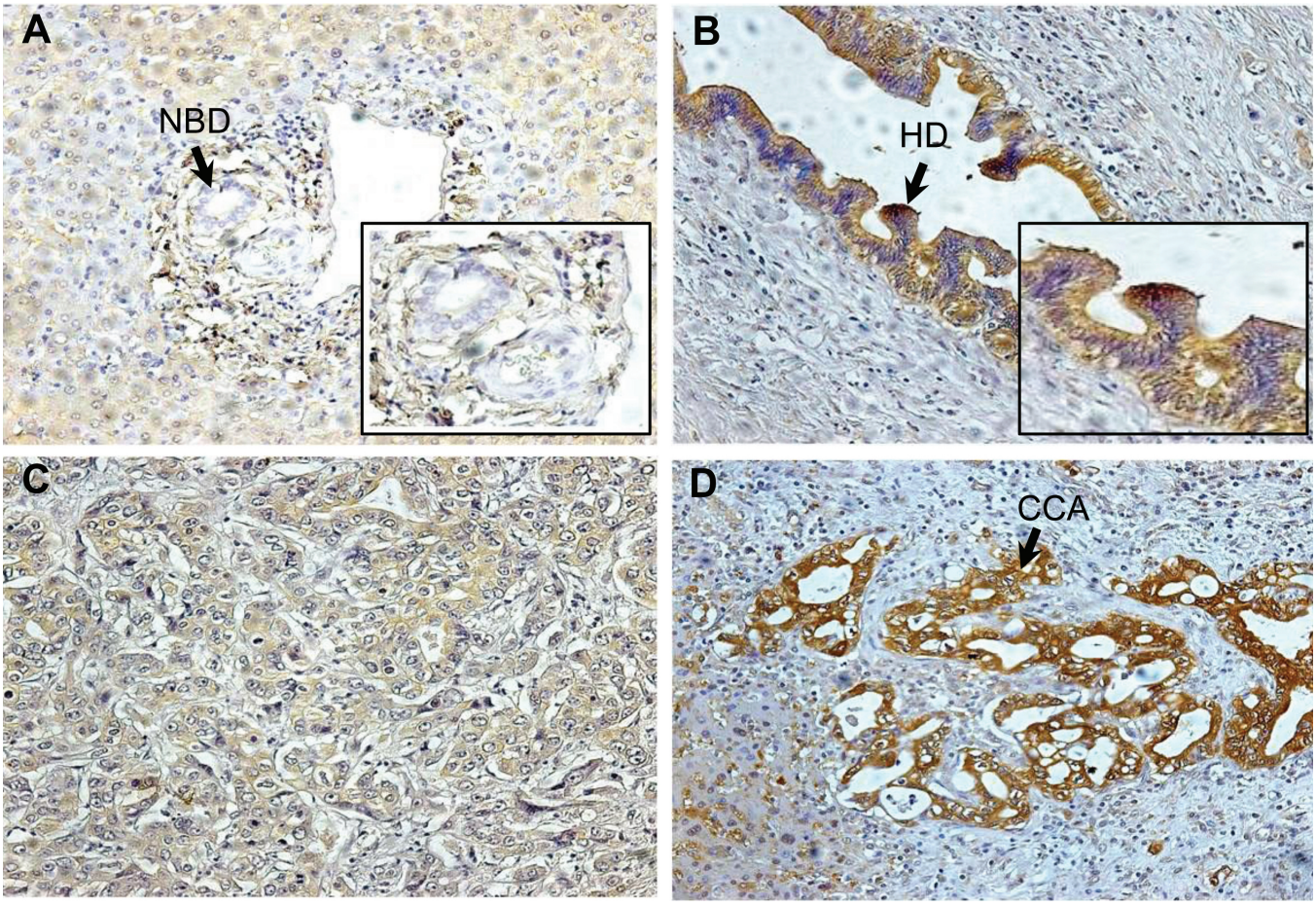

Fig. 3. Immunohistochemistry staining of LDHA expression in human-CCA tissues (n=82). **A.** Low LDHA expression in normal bile duct. **B.** High LDHA expression in bile duct hyperplasia. **C.** Low LDHA expression in tumor area. **D.** High LDHA expression in tumor area. x 200.

became detectable as early as 1 month of experimental CCA induction and its level was associated with CCA development. Then, we examined LDHA expression in human CCA tissues and found that the majority (90%) of human CCA tissues exhibited aberrant expression of LDHA. The higher level of LDHA expression was significantly associated with shorter survival of the patients.

In the hamster model experiments, the normal bile duct epithelium did not express LDHA. LDHA expression was initially observed in the bile duct epithelial cells of hyperplasia lesion, which was especially prominent in the Ov infected group. CCA development after administration of hepatocarcinogen, NDMA, to Ov-infected hamsters was observed at 3 and 6 months post-treatment. The highest expression of LDHA was spotted in these CCA tissues. These findings indicate that LDHA expression increased along with the CCA-genesis. The involvement of LDHA in tumor initiation and progression was demonstrated previously in lymphoma and pancreatic cancer (Le et al., 2010; Rong et al., 2013), suggesting that LDHA expression in carcinogenesis is a common event of various cancers.

It should be noted that Ov infection caused injury/inflammation, which induced cell proliferation of the bile duct epithelium. Ov adult worms produce both physical and chemical stimuli to induce the host's immune/inflammatory response (Sripa et al., 2012). Growth factors and cytokines produced by immune/inflammatory cells activate LDHA gene transcription (Matrisian et al., 1985) which might result in LDHA expression. In the present study, increased LDHA expression was observed in bile duct hyperplasia and CCA areas and the immunoreactivity signal of LDHA expression was seen in both cytoplasmic and nuclear compartments, supporting that of previous studies in prostate and gastric cancers (Kim et al., 2014; Koukourakis et al., 2014). Overexpression of LDHA was reported in other cancers such as colon cancer (Koukourakis et al., 2005), pancreatic cancer (Rong et al., 2013) and even cholangiocarcinoma of Ov-non-endemic area (Yu et al., 2014). Thus, LDHA overexpression is a common feature of many tumors and may be important for cancer development.

In the present study, a high level of LDHA expression was associated with shorter survival of CCA-patients. The association of LDHA overexpression with shorter survival was reported in prostate (Giatromanolaki et al., 2006), lung (Koukourakis et al., 2014), colon (Koukourakis et al., 2003), and stomach (Zhao et al., 2015) cancers. This may suggest that aggressive cancer requires a high level of LDHA activity. The functional role of LDHA in promoting cell growth was reported in CCA cell lines. Inhibition of LDHA gene expression using shRNA induced apoptosis and suppressed tumor growth (Yu et al., 2014). Furthermore, LDHA inhibitors such as FX11, oxamate, galloflavin could reduce tumor cell proliferation *in vitro* (Rong et al., 2013; Zhao et al., 2015; Han et al., 2015) and suppress tumor volume *in vivo* (Koukourakis et al., 2005).

To determine whether LDHA expression can be used for diagnosis and prognosis for CCA, plasma LDH levels of CCA-patients and healthy controls were investigated. The results show that the plasma LDH level of CCA patients was not significantly different from that of healthy controls. Our result agreed with that of the previous study in hepatobiliary cancer (Wulaningsih et al., 2015). In contrast, elevated serum LDH level was observed in patients with breast cancer (Brown et al., 2012) and ovarian cancer (Koukourakis et al., 2009). Moreover, serum LDH has been reported to be a prognostic and predictive value in patients with

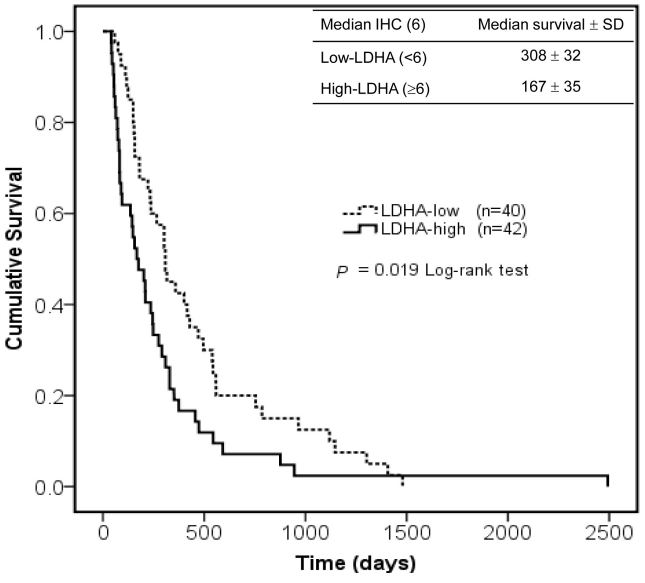

Fig. 4. Relationship between LDHA expression and overall survival. Dashed line: low LDHA expression; simple line; high LDHA expression.

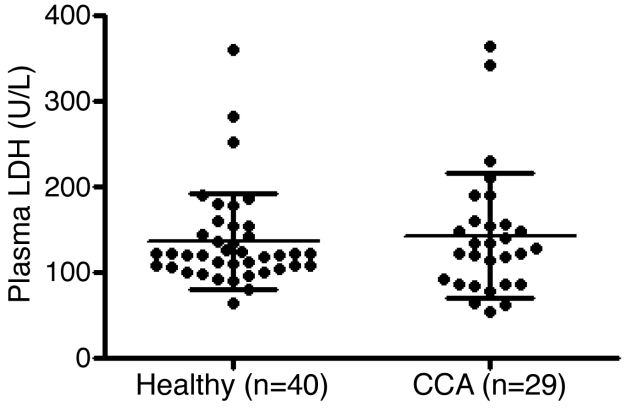

Fig. 5. Plasma LDH levels: No significant differences in LDH levels between healthy controls and CCA patients (P=0.72).

renal cell carcinoma (Armstrong et al., 2012), melanoma (Weide et al., 2013), and lymphoma (Endrizzi et al., 1982). More intensive study with a greater number of CCA patients is still a challenging task to determine the diagnostic value of plasma LDH levels.

Conclusion

Our findings indicated that LDHA is aberrantly expressed in bile duct epithelia with hyperplasia and CCA cells. LDHA expression was associated with CCA-genesis. Patients with CCA of high LDHA expression had a shorter survival than those with low expression, which can probably be used as a prognostic marker for poor patient outcome. As LDHA is highly expressed in CCA but not normal bile duct cells, reduction of LDHA expression and/or suppression of its activity in combination with currently used drugs might be an alternative choice of treatment for CCA-patients with high LDHA expression.

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