

RESEARCH ARTICLE

Altered Expression of Oxidative Metabolism Related Genes in Cholangiocarcinomas

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Abstract

Cholangiocarcinoma (CCA) is a rare but highly fatal cancer for which the molecular mechanisms and diagnostic markers are obscure. We therefore investigated the kinetic expression of isocitrate dehydrogenase-1 (IDH1), isocitrate dehydrogenase-2 (IDH2) and homogentisate 1,2-dioxygenase (HGD) during the tumorigenesis of *O. viverrini* infection-associated CCA in an animal model, and confirmed down-regulation of expression in human cases of opisthorchiasis-associated CCA through real time PCR. Kinetic expression of HGD, IDH1 and IDH2 in the animal model of *O. viverrini* infection-induced CCA was correlated with human CCA cases. In the animal model, expression of HGD was decreased at all time points ($p < 0.01$) and expression of both IDH1 and IDH2 was decreased in the CCA group. In human cases, expression of HGD, IDH1 and IDH2 was decreased more than 2 fold in 55 cases (70.5%), 25 cases (32.1%) and 24 cases (30.8%) respectively. The present study suggests that reduction of HGD, IDH1 and IDH2 may be involve in cholangiocarcinoma genesis and may be useful for molecular diagnosis.

Keywords: Liver fluke - cholangiocarcinogenesis - (IDH1) - IDH2 - (HGD) - oxidative metabolism related genes

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Introduction

Infection of *Opisthorchis viverrini* can induce tumorigenesis of cholangiocarcinoma (CCA), which has been demonstrated by the epidemiological investigations and animal experiments (Thamavit et al., 1987; IARC, 1994; Sithithaworn et al., 2003; Sriamporn et al., 2004; IARC, 2011). As one of the risk factors, combining local traditional eating custom of fermented food which contains potent carcinogen, for example, N-nitrosodimethylamine (NDMA), opisthorchiasis-associated CCA has become a public health problem in endemic region, especially in the northeastern of Thailand where the highest prevalence of CCA in the world was observed (Jongsuksuntigul et al., 2003; Sriamporn et al., 2004; Sripa et al., 2008). Many efforts have been made to reveal the mechanism of tumorigenesis of CCA induced by *O. viverrini* infection. Although it is not still unknown completely, NOS derived from inflammation caused by the infection is considered to be one of factors to cause the tumorigenesis. It has been demonstrated that *O.*

viverrini caused the accumulation of NOS (Srianujata et al., 1984; Srivatanakul et al., 1991; Pinlaor et al., 2004b). The accumulated NOS may induce the DNA damage, such as, epigenetic changes and genomic instability, which may initial tumorigenesis. The oxidative DNA damage was observed during *O. viverrini* infection, for example, 8-nitroguanine and 8-oxo-7,8-dihydro-20-deoxyguanosine (8-oxodG), an indicator of oxidative DNA damage, mediating iNOS dependent DNA damage in intrahepatic bile duct epithelium (Pinlaor et al., 2004a; Kawanishi et al., 2006). Under a physiological condition, there is a constant endogenous production of ROS. To control the balance between production and removal of ROS, there are a series of protective molecules, defined as antioxidant defenses, including superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase, glutathione, cysteine and thioredoxin etc (McCord et al., 1969; Chelikani et al., 2004; Hayes et al., 2005; Valko et al., 2007; Raza, 2011; Miki & Funato, 2012). Our previous cDNA microarray indicated that in the animal model of *O. viverrini* infection-induced CCA,

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the expressions of many antioxidative enzyme related genes were down-regulated, for example, IDH (isocitrate dehydrogenase), HGD (homogentisate 1,2-dioxygenase), Gsta (glutathione S-transferase), GST (glutathione synthetase) and Prdx6 (peroxiredoxin 6) (Wu et al., 2011). Therefore, we speculate that the down-regulated expressions of these antioxidative enzymes may play the important roles in the tumorigenesis of opisthorchiasis-associated CCA.

The IDH1 and IDH2 are the enzymes to catalyze the oxidative decarboxylation of isocitrate to alpha-ketoglutarate, contributing to NADPH production by generating NADPH from NADH⁺. NADPH is required for the synthesis of glutathione, which protects cells from redox stress. HGD is one of the enzymes required for the catabolism of the aromatic amino acids phenylalanine and tyrosine, and has oxidoreductase activity to catalyze oxidation-reduction (redox) reaction. So far, most of the studies on IDH were focused to the mutation of the genes in gliomas, the tumors of central nervous system (Hartmann et al., 2009; Qi et al., 2011; Kim et al., 2012). Little has been reported on the down-regulation of IDH in other cancers and its relationship with tumorigenesis, and a few reports are found on these enzymes in human *O. viverrini* related and non-*O. viverrini* related CCAs (Chan-On et al., 2013; Jiao et al., 2013). Therefore, in the present study, we investigated the kinetic expression of IDH1, IDH2 and HGD during the tumorigenesis of *O. viverrini* infection-induced CCA in animal model, and confirmed the gene regulation of the expression in the human CCA in endemic area of *O. viverrini* infection.

Materials and Methods

Parasite and infection

An animal model for *O. viverrini* infection-induced CCA has been well established (Thamavit et al., 1987;1993). Infection only or feeding NDMA-containing water only does not cause formation of CCA, while infection plus feeding NDMA-containing water can induce the development of CCA.

Metacercariae of *O. viverrini* were collected as the previously described (Boonmars et al., 2008; Boonmars et al., 2009a; Boonmars et al., 2011). In brief, naturally infected cyprinoids fish captured from a fresh water reservoir in an endemic area of Khon Kaen, Northeast Thailand, were minced and digested with pepsin-HCl, filtrated and then washed with saline. Afterwards the *Opisthorchis* metacercaria which was 204 x 145 µm, oval-shaped, with large, black excretory bladders – were identified under a dissecting microscope.

Eighty Syrian hamsters (6 weeks) were divided in to 4 groups (n=20 per group): (1) normal group; (2) infection group: 20 hamsters were infected with 50 metacercariae; (3) NDMA group: 20 hamsters were maintained with drinking water containing 12.5 ppm of NDMA (Wako, Japan) for 2 months; and (4) infection plus NDMA group: 20 hamsters were infected with 50 metacercariae and maintained with drinking water containing 12.5 ppm of NDMA for 2 months. Hamster livers (five hamsters at each time point for each group) were collected at 1, 2, 3 and 6

month(s) post infection (p.i.) for total RNA isolation and histological observation.

Histopathological changes

The hamster liver of each group was processed as the previous study (Boonmars et al., 2011). In brief, liver was fixed with 10% neutral formalin and cleared with xylene. And then was paraffinized embedded. Liver sections were deparaffinized in xylene, rehydrated through a descending alcohol series, washed with distilled water and stained with Harris's hematoxylin followed by eosin. Finally, the sections were dehydrated through an alcohol series, cleared in xylene, and mounted on slides using Permount resin. The liver sections were observed and digitized under a light microscope (Olympus BX51; Tokyo, Japan) for pathological grading.

Collection of human CCA and adjacent tissues

Seventy eight pairs of liver samples from CCA patients coming from heavy epidemical area of opisthorchiasis were provided by the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand. Each pair of sample included the CCA tumor tissue and the adjacent normal tissues from same patient were histologically confirmed. The clinicopathological features of the CCA patients were represented by status, histological type, histological grade, stage and metastasis based on the criteria of the Liver Cancer Study Group of Japan, and the International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC). The samples were defined pathologically and histologically (Table 1). Total RNA was isolated from the tumor and adjacent normal tissues, and subjected to the real time PCR for the quantity of expression level as described below. The utilization of the specimens in the present study was approved by the Human Ethics Committee of the Khon Kaen University (Ethical Clearance No. HEKKU501153) and Gifu University.

RNA isolation

Total RNA was extracted from the tissues (200 mg) at the same region (hilar region of liver) from the four groups mentioned above (normal, infection, NDMA and infection plus NDMA) using TRIZOL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. The isolated RNA was treated with DNase (RQ1 RNase-Free DNase, Promega, Co., Madison, WI, USA). The treated RNA was purified using conventional method and dissolved in RNase-free water.

Quantitative real time PCR

Quantitative real-time RT-PCR was performed to determine the expressions of IDH1, IDH2 and HGD using the Thermal Cycler Dice Real Time System (TAKARA, Japan). Because there was not genetic information on hamster IDH1 and IDH2, we first determined the sequences of hamster of these two genes with the primers developed based on the mouse sequence, and then designed the primers for hamster IDH1 (forward gggtatggtcccttggtatga and reverse gatgtctcctgtcctttctgta), IDH2 (forward tgccttgatgggaagacaa and reverse gcaaagatgctggcgatagg)

and HGD (forward ttgcttccaacaggatgtctc and reverse atggtcaaggccactgcat). The primer for hamster G3PDH (forward gacatcaagaagggtggaagca and reverse catcaagggtggaagagtggga) was designed based on the previous published paper (Boonjaraspinyo et al., 2012).

The primers for human IDH1, IDH2, HGD and GUSB were designed based on the published sequences in GenBank, IDH1: forward ggaacccaaaagggtgacatacc and reverse caaactgggactgtactgcttg; IDH2: forward caaaatggcttccacccaaa and reverse catcgtaggcttctcagtatggt; HGD: forward gatgctgactgcttggagaagg and reverse cactttgtgaccgccagactta and GUSB: forward atggaagaagtggtgcgtacg and reverse aaggatttgggtgagcga.

Total RNA was isolated and purified as mentioned above. Reverse transcription was performed using a Prime Reverse Transcriptase (TAKARA BIO Inc., Ootsu, Shiga, Japan) according to the manufacturer's instructions. Real time PCR was performed as previous described (Boonmars et al., 2009b). In brief, optimal conditions for all investigated genes were established using SYBR Premix Ex Taq Kit (TAKARA BIO) according to manufacturer's instructions. Twenty μ l of the reaction solution consisted of 2 μ l of the template (appropriate dilution was determined by genes), 10 μ l of SYBR Premix Ex Taq, 0.8 μ l of 5 μ M of each primer. PCR amplification was performed as follows: predenature for 1 cycle at 95 $^{\circ}$ C for 30s, and 40 cycles at 95 $^{\circ}$ C for 5s, 60-62 $^{\circ}$ C for 30s. Melting curve analysis was done at 65 $^{\circ}$ C to 95 $^{\circ}$ C with 0.1 $^{\circ}$ C/second temperature transition.

Specific external controls were constructed for target genes. The PCR fragment of each gene was cloned into a pT7Blue T-Vector (Novagen, Inc., Madison, WI, USA). The recombinant plasmids were introduced into competent cells of *Escherichia coli* JM 109. The plasmid DNA was isolated from *E. coli* using a FlexiPrep Kit (Amersham

Pharmacia Biotech Inc., Piscataway, NJ, USA). Ten-fold serial dilutions (101 to 107 copies/ 2 μ l) of the plasmids were used to generate standard curves for each gene.

Differences in the amount of cDNA from different samples were normalized by quantification of the housekeeping gene G3PDH and expression levels were represented as the copy number of target gene /106 G3PDH copies. The values are expressed as mean \pm SD.

Results

Histopathology

The histological changes was similar to our previous study (Boonmars et al., 2011). In brief, the OV infected group was observed the inflammatory cell aggregation at all-time points (1, 2, 3 and 6 months) of observation. The NDMA treatment group was observed the inflammatory cell aggregation at all-time points and bile duct proliferation at 2 and 3 months and cholangiocarcinoma at 6 months. The group of infection plus NDMA treatment was observed inflammatory cell aggregation at all-time points of observation. Bile duct proliferation was observed at 2 and 3 months and the CCA area was observed at 6 months.

Kinetic expression of HGD, IDH1 and IDH2 in the animal model of *O. viverrini* infection-induced CCA

The expression kinetics of HGD, IDH1 and IDH2 during the development of CCA was determined by real time PCR, in the hamster groups of normal, infection only, NDMA-containing water feeding only, and infection plus NDMA-containing water feeding at 1, 2, 3 and 6 months after infection. As shown in (Figure 1), infection only caused down-regulation of HGD expression. The expression of HGD was decreased at 1 month p.i. ($p < 0.05$), lowest at 2 months p.i. ($p < 0.01$), and returned

Table 1. The Correlation of Expressions of HGD, IDH1 and IDH2 with Clinicopathology in 78 CCA Patients

Clinicopathological parameter	n	Expression of HGD mRNA			Expression of IDH1 mRNA			Expression of IDH2 mRNA		
		≥ 3 folds	< 3 folds	P	≥ 3 folds	< 3 folds	P	≥ 3 folds	< 3 folds	P
Age										
< 60	50	33	17	0.279	15	35	0.413	12	38	0.796
≥ 60	28	15	13		6	22		6	22	
Gender										
Male	50	32	18	0.55	14	36	0.774	15	35	0.052
Female	28	16	12		7	21		3	25	
Status										
Dead	50	35	15	0.040*	11	39	0.19	10	40	0.389
Alive	28	13	15		10	18		8	20	
Histology type										
Tubular type	21	12	9	0.549	7	14	0.73	6	15	0.78
Papillary type	48	29	19		23	36		10	38	
Mixed type	9	7	2		2	7		2	7	
Histology grade										
Well differentiation	34	15	19	0.005*	10	24	0.663	7	27	0.647
Moderate and poor	44	33	11		11	33		11	33	
Stage										
I, II	25	10	15	0.007*	5	20	0.344	4	21	0.308
III, IV	53	38	15		16	37		14	39	
Metastasis										
No metastasis	41	17	24	0.000*	13	28	0.945	9	32	0.804
Metastasis	37	31	6		12	25		9	28	

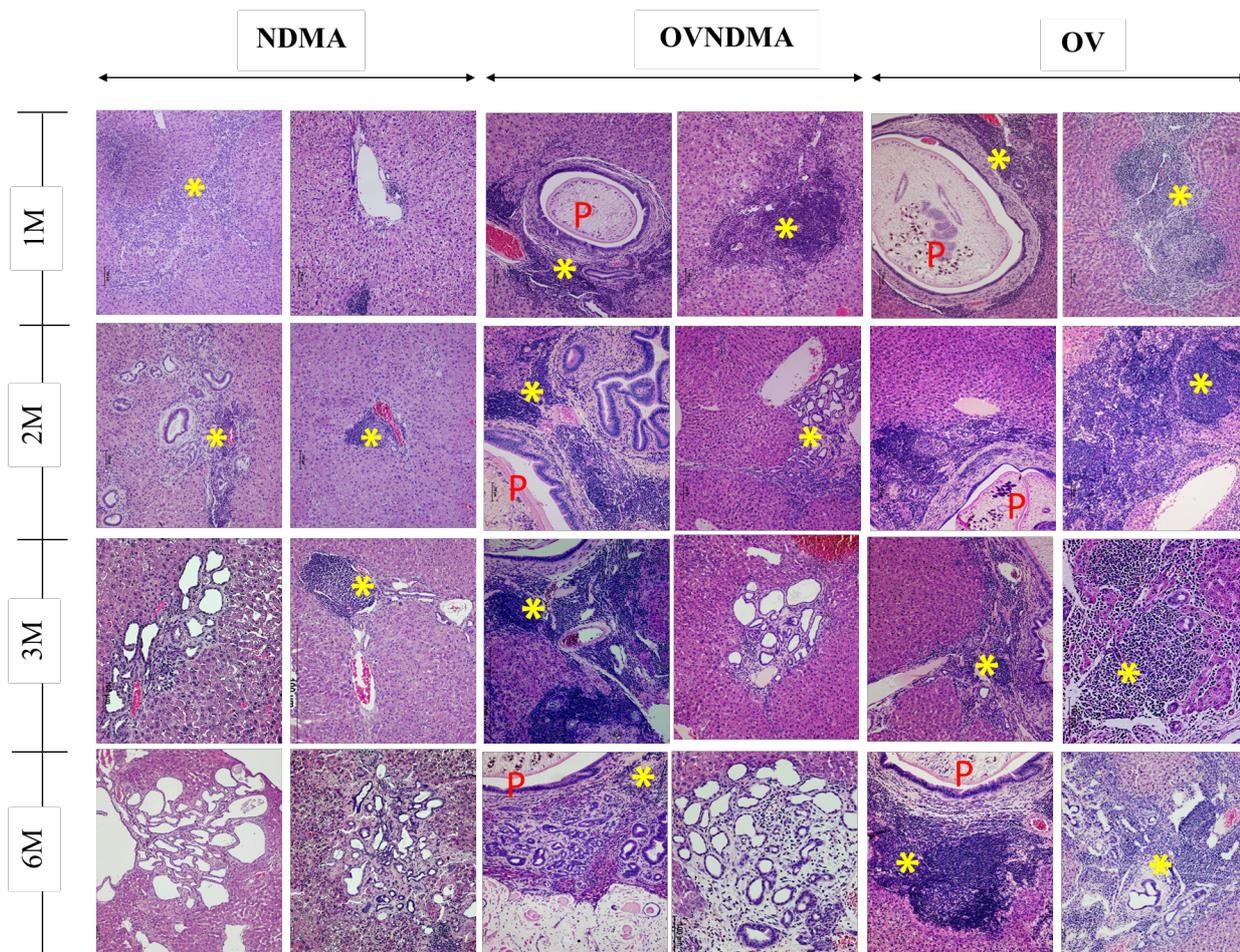


Figure 1. The Representative Histopathological of Hamster CCA Model

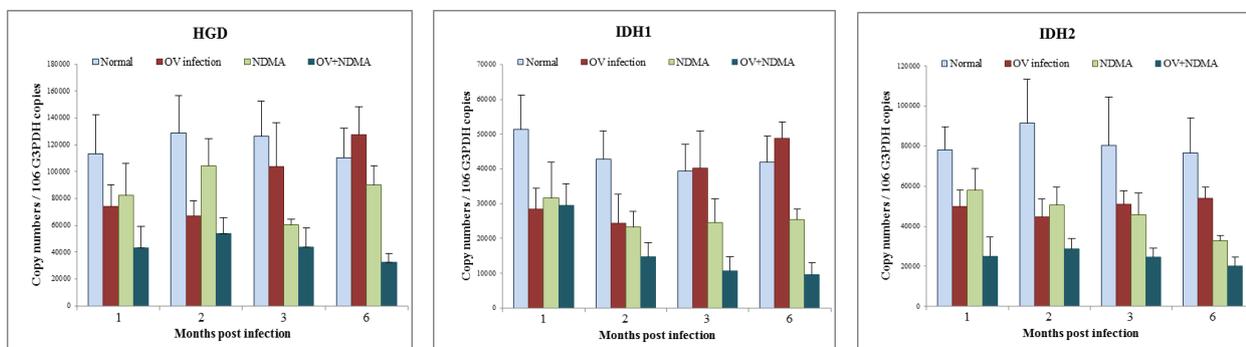


Figure 2. The Expressions of HGD, IDH1 and IDH2 in Hamster CCA Model

normal level at 3 and 6 months p.i. ($p>0.05$). Much low level of expression in infection plus NDMA treatment was observed at all-time points ($p<0.01$). The expression was decreased slightly in the group of NDMA treatment alone, but much higher than those in the group of infection plus NDMA treatment. The expressions of both IDH1 and IDH2 were decreased in the group of infection plus NDMA treatment, as shown in (Figure 2). Infection only caused the decrease of expression of IDH1 at 1 and 2 months p.i., while there was no significant difference at 1 and 6 months p.i. The decreased expression of IDH2 was observed in the group of infection only at all time points.

Expression of HGD, IDH1 and IDH2 in the human cases of CCA

To confirm the expression of these 3 oxidative

metabolism related genes in human CCA cases, we determined the expression of these genes in tumor tissue and adjacent tissue in same CCA case. Totally 78 cases were examined. The expression of HGD was decreased more than 2 folds in 55 cases (70.5%), ranging from -2.1 to -50.5 folds with an average of -17.3 folds, and there was one case which showed the expression more than 2 folds. The expression of IDH1 was decreased more than 2 folds in 25 cases (32.1%), ranging from -2.1 to -20.7 folds with an average of -8.1 folds, and there were 11 cases which showed the expression more than 2 folds (14.1%). The expression of IDH1 was decreased more than 2 folds in 24 cases (30.8%), ranging from -2.1 to -21.1 folds with an average of -7.9 folds, and there were 17 cases which showed the expression more than 2 folds (21.8%) as shown in Figure 3.

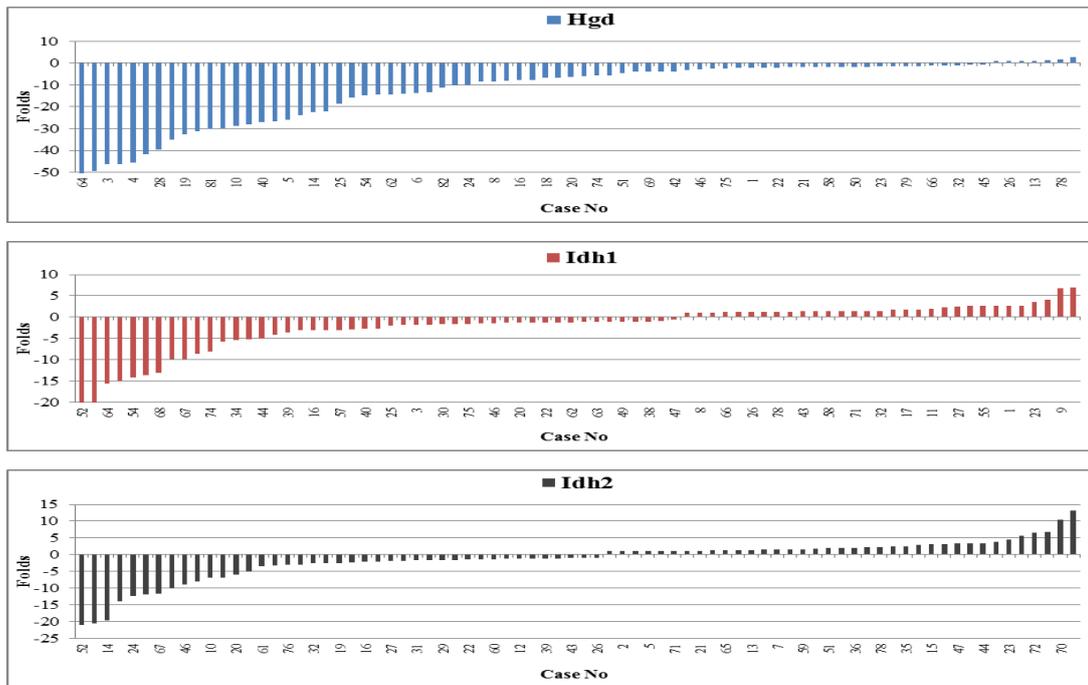


Figure 3. The Expressions of HGD, IDH1 and IDH2 in Human CCA

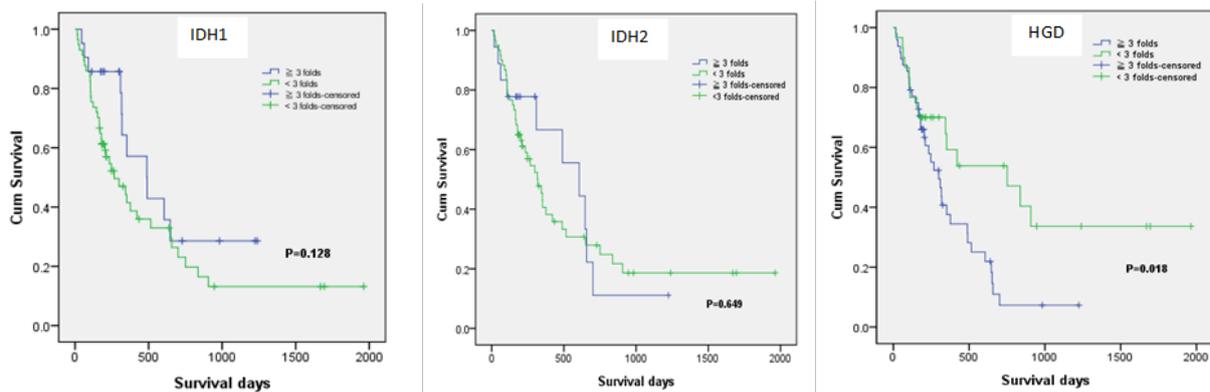


Figure 4. Survival Days

The correlation of expressions of HGD, IDH1 and IDH2 with clinicopathology

The correlation of HGD, IDH1 and IDH2 expressions at mRNA levels with all CCA patient's clinicopathological parameters was analyzed. As shown in Table 1, the expression levels were significantly correlated with the clinicopathology. Those patients with high HGD mRNA expression (≥ 3 folds) showed more dead ($p=0.040$), more severe histopathology at grade III and IV ($p=0.005$), advanced stage of CCA ($p=0.007$), high grading stage and metastasis ($p=0.000$) than those with low expression (< 3 folds). IDH1 and IDH2 expression had no clinicopathological relation with age, gender, status, histological type, histological grading, staging and metastasis.

Survival time

Figure 4 showed the down or up regulation of IDH1 and IDH2 had no effect on the survival rate but down regulation of HGD in human CCA shows that < 3 fold down regulation of the HGD had higher survival rate than others.

Discussion

In animal model, *O. viverrini* infection induces inflammation cells filtration in the tissues around parasite. The inflammation is most obvious at 1-2 months post infection whereas the kinetic expression of all genes in infection animal and NDMA treatment group were not corresponding to the inflammation situation (Figure.1 and 2). However, the three IDH isoenzymes are important play role in the electrons exchange of metabolites between the mitochondria and the cytosol for cellular defense against oxidative damage as a source of NADPH. IDH1 and IDH2 found in cytoplasm and mitochondria respectively. Both IDH1 and IDH2 are important for shuttling electrons between the mitochondria and the cytosol (Sazanov et al., 1994; Minard et al., 1999; Joseph et al., 2006; Reitman et al., 2011). Recently, IDH1 and IDH2 are suppose to be the proto-oncogene (Reitman et al., 2011) so several reports show that in various cancers found IDH1 and IDH2 mutation such as acute myeloid leukemia (Dang et al., 2010), central chondrosarcoma and central and periosteal chondromas (Amary et al., 2011), prostate carcinoma (Kang et al., 2009), colorectal cancer (Sjöblom

et al., 2006) and there are some reports show that found IDH1 and IDH2 mutation was good prognosis and high survival rate for glioblastomas (Hartmann et al., 2009; Yan et al., 2009; SongTao et al., 2012). Moreover, Borger et al. (2012) found 23% of the mutation of IDH1 and IDH2 in intrahepatic cholangiocarcinoma. According to above reasons thus, IDH1 and IDH2 expressions in normal condition or uninfected normal hamster control was remained in high level in all time points whereas in abnormal conditions, *O. viverrini* infection, NDMA administration and *O. viverrini* infection plus NDMA, were decreased (Figure 2). It may cause of the IDH1 and IDH2 mutation and electron transport abnormality in the liver including intrahepatic cholangiocarcinoma (Jiao et al., 2013) and mutated non- *O. viverrini* related CCA (Chan-On et al., 2013). HGD gene is homogentisate 1, 2-dioxygenase that provides instructions for making an enzyme called homogentisate oxidase, which is generally active in the liver and kidneys. This enzyme involve in a step-wise process that breaks down two protein building amino acids, phenylalanine and tyrosine, which play a role in making certain hormones, pigments, and brain chemicals called neurotransmitters. Most of these mutations change single amino acids used to build the homogentisate oxidase enzyme by changing its structure. There are reported that HGD mutations found in alkaptonuria (Al-sbou et al., 2012). This is the first report of down regulation of HGD in human and hamster CCA and moreover, the present data shows that < 3 fold down regulation of the HGD had higher survival rate than others (Figure 3). The present study suggests that the animal CCA models induced by liver fluke and NDMA is correlated to the human CCA. The down regulation of IDH1, IDH2 and HGD genes was observed in both hamster CCA development at 1, 2 and 3 months p.i. and the lowest expression was observed at 6 months p.i. (Figure 2). This was similar in most human CCA cases evidenced by down regulation of IDH1, IDH2 and HGD genes (Figure 3). Even through, the IDH1, IDH2 and HGD expression in group of infected *O. viverrini* alone or NDMA alone are not involved in any liver inflammations, induced by the *O. viverrini* infection or NDMA but also may involve in the structure change (mitochondria and cytosol) of hepatocyte which may involve in cholangiocarcinogenesis and be useful for CCA diagnosis however, further study is needed.

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