RESEARCH ARTICLE

Association of CYP39A1, RUNX2 and Oxidized Alpha-1 Antitrypsin Expression in Relation to Cholangiocarcinoma Progression

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Abstract

Cytochrome P450 (CYP) enzymes are a large family of constitutive and inducible mono-oxygenase enzymes that play a central role in the oxidative metabolism of both xenobiotic and endogenous compounds. Several CYPs are involved in metabolism of oxysterols, which are cholesterol oxidation products whose expression may be dysregulated in inflammation-related diseases including cancer. This study focused on CYP39A1, which can metabolize 24-hydroxycholesterol (24-OH) that plays important roles in the inflammatory response and oxidative stress. We aimed to investigate the expression status of CYP39A1 and its transcription factor (RUNX2) in relation to clinical significance in cholangiocarcinoma (CCAs) and to determine whether 24-OH could induce oxidative stress in CCA cell lines. Immunohistochemistry showed that 70% and 30% of CCA patients had low and high expression of CYP39A1, respectively. Low expression of CYP39A1 demonstrated a significant correlation with metastasis. Our results also revealed that the expression of RUNX2 had a positive correlation with CYP39A1. Low expression of both CYP39A1 (70%) and RUNX2 (37%) was significantly related with poor prognosis of CCA patients. Interestingly, oxidized alpha-1 antitrypsin (ox-A1AT), an oxidative stress marker, was significantly increased in CCA tissues in which CYP39A1 and RUNX2 were down regulated. Additionally, immunocytochemistry showed that 24-OH could induce ox-A1AT in CCA cell lines. In conclusion, our study revealed putative roles of the CYP39A1 enzyme in prognostic determination of CCAs.

Keywords: CYP39A1 - RUNX2 - oxidized alpha-1 antitrypsin - cholangiocarcinoma

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Introduction

Cholangiocarcinoma (CCA) is a tumor characteristic of bile duct epithelial cells which is related with a poor prognosis as well as responding poorly to current therapies (de Groen et al., 1999; Vatanasapt et al., 2002). The incidence of CCA burden greatly increases worldwide such as in the United Kingdom, United States, Western Australia and Thailand (Khan et al., 2008). Diverse exposure to risk factors for CCA result in varying geographic incidences with parasitic infections, such as liver fluke, Opisthorchis viverrini (O. viverrini), chronic viral hepatitis and cirrhosis including hepatitis C virus (HCV), hepatitis B virus (HBV), liver cirrhosis, and hepatolithiasis (Shin et al., 2010). A major mechanism linking between inflammation and CCA is related to the generation of free radicals such as superoxide anion ($O2^{-}$•), nitric oxide (NO) and other reactive oxygen (ROS) and nitrogen species (RNS) which cause DNA damage and the alteration of gene expressions in both a hamster animal model and humans (Jaiswal et al., 2000; Pinlaor et al., 2003; Thanan et al., 2008; Yongvanit et al., 2012b). Regarding to the molecular mechanism underlining CCA progression, there are some studies have demonstrated aberrant expression of potential genes involving in CCA (Kunlabut et al., 2012; Namwat et al., 2012; Thongchot et al., 2014). In addition, several studies show that oxidative DNA damage plays a role in not only DNA mutation but also genetic instability and epigenetic changes which could be involved in all steps of inflammation-related carcinogenesis (Murata et al., 2012).

The cytochrome P450 enzymes (CYP), a family of mono-oxygenase enzymes that metabolize endogenous substances including vitamins, steroids, fatty acids and prostaglandins also play a crucial role in metabolizing xenobiotics to be in either detoxified forms or to be

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harmful reactive intermediates, including carcinogens (Simpson, 1997). Certain CYP enzymes, for instance CYP2A6 which is a major liver enzyme that biotransforms N-nitrosodimethyl amine (NDMA) into a carcinogen was previously shown to have increased activity in subjects infected with *O. viverrini* (Satarug et al., 1996). Increased CYP2A6 expression and activity were also found in chronic inflammation-related disease including hepatocellular, bronchogenic, esophageal carcinomas and CCA (Crawford et al., 1998; Raunio et al., 1998; Godoy et al., 2002; Yongvanit et al., 2012a). Decreased activity of CYP enzymes has been found to be associated with oxidative stress, for example, CYP2E1 in CCA (Yongvanit et al., 2012a) and HCC (Ho et al., 2004). CYP39A1, a steroid 7α-hydroxylation enzyme, is capable of metabolizing 24-hydroxycholesterol (24-OH) which is a cholesterol oxidation product (Li-Hawkins et al., 2000). In cancer, it has been reported that CYP39A1 was hypermethylated in ovarian tumor (Huang et al., 2009b).

On the other hand, 24-hydroxycholesterol (24-OH) plays important roles in the inflammatory response and oxidative stress. An experimental study reported an adverse effect of 24-OH on primary human neural cells via the significant induction of inflammatory gene expressions such as HSP70, COX-2, cPLA2 and beta-APP genes when compared to cholesterol stimulation (Alexandrov et al., 2005). Additionally, 24-OH significantly induces ROS production determined in primary porcine retinal pigment epithelial cells. (Joffre et al., 2007).

It is accepted that inflammation and oxidative stress play significant roles in inflammatory diseases including liver fluke-associated CCA (Yongvanit et al., 2012b). We recently determined oxidized alpha-1 antitrypsin (ox-A1AT) expression in human serum of CCA patients. Our results identified ox-A1AT as a potential risk indicator for opisthorchiasis-associated CCA which could be used as an oxidative stress biomarker in *O. viverrini*-associated CCA (Jamnongkan et al., 2013). In the current study, we hypothesize that there is an alteration of CYP39A1 expression in CCA, which might enhance the tumor progression. The gene expression pattern of CYP39A1 was investigated in order to address clinico-pathological associations. The underlying mechanism(s) of alteration of CYP39A1 expression governed by its transcription activator RUNX2 was investigated. In addition, ox-A1AT was also investigated in CCA to show the correlation of oxidative stress in alteration of CYP39A1 expression. We also confirmed whether 24-OH could induce ox-A1AT, an oxidative stress marker in CCA cell line.

Materials and Methods

Human CCA specimens

The 30 paraffin-embedded tissues collected from primary tumors of CCA patients were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand during 2004-2011. Informed consent was obtained from each patient prior to surgery and the Ethics Committee for Human Research at Khon Kaen University approved the research protocols (HE521209 and HE531163).

Immunohistochemical analysis

The expression of CYP39A1, RUNX2 andox-A1AT in paraffin-embedded tissues was determined by immunohistochemical staining. The sections were deparaffinized and rehydrated by submerging respectively in xylene and ethanol with stepwise decreasing concentrations. For antigen retrieval, the sections were boiled in Tris-EDTA buffer (pH 9.0) and left to cool down for 20 min. Tissue sections were treated with 0.3% H2O2 in phosphate-buffered saline (PBS) to block the endogenous peroxidase activity, subsequently treated with 10% skim milk for non-specific binding blocking. The sections were incubated with primary antibodies against human CYP39A1 (1:200, rabbit polyclonal antibody; Sigma chemical, USA), RUNX2 (1:200, mouse monoclonal antibody; Abcam, USA) and oxA1AT (1:100, mouse monoclonal antibody; Ikagaku Co. Ltd, Kyoto, Japan) at 4ºC for overnight incubation. Subsequently, sections were incubated with peroxidase-conjugated Envision™ secondary antibody (DAKO,Glostrup, Denmark) at room temperature for 1h. Peroxidase activity was observed using 3, 3'-diaminobenzidine tetrahydrochloride (DAB) as a substrate. The sections were counterstained with hematoxylin, dehydrated with stepwise increasing concentrations of ethanol, cleared with xylene and mounted with permount solution. Protein staining was evaluated by calculating a total immunostaining index (IHC index) as the product of a frequency and intensity score by two independent observers blinded to the protein type under analysis. The proportion score described the estimated fraction of positive stained tumor cells (0= none; 1= 1-25%; 2= 26-50%; 3= 51-75%; 4≥76%). The intensity score represented the estimated staining intensity (0=negative staining; 1= weak; 2= moderate; 3= strong) (Thanan et al., 2012a). These scores were calculated by multiplying the frequency score and intensity score. The mean of IHC index was defined as the cut-off value of low and high expression.

Cell lines and cell culture

M214 CCA cell line was cultured in Ham F'12 (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin and incubated at 37°C in a humidified incubator maintained with an atmosphere of 5% CO2. Subculture was done when the cell reached the confluent stage and the media were changed once every two to three day.

Immunocytochemical analysis

Immunocytochemical analysis was used to determine whether 24-OH could induce oxidative stress condition in CCA cell line. Cells were plated on slide chamber and treated with 24-OH (Enzo Life Sciences, Inc.USA) at concentrations of 6.25 and 12.5 mM. After incubation, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% (v/v) Triton X-100. Fixed cells were then incubated with antibodies specific for ox-A1AT followed by secondary antibodies conjugated with Alexa

Table 1. Immunihistochemical Analysis and Clinicopathological Data of CCA Patients

Variable	No. of patients	CYP39A1 expression (%) Low 21 (70%)	CYP39A1 expression (%) High 9 (30%)	P value	RUNX2 expression (%) Low 11 (37%)	RUNX2 expression (%) High 19 (63%)	P value	Ox-A1AT expression (%) Low 23 (77%)	Ox-A1AT expression (%) High 7 (23%)	P value
Age (Year)										
< 56	15	10 (67%)	5 (33%)	1	6 (40%)	9 (60%)	1	13 (87%)	2 (13%)	0.39
> 56	15	11 (73%)	4 (29%)		5 (33%)	10 (67%)		10 (67%)	5 (33%)	
Gender										
Female	12	10 (83%)	2 (17%)	0.249	8 (67%)	4 (33%)	0.009*	8 (67%)	4 (33%)	0.392
Male	18	11 (61%)	7 (39%)		3 (17%)	15 (83%)		15 (83%)	3 (17%)	
Histological grading										
Non-papillary	13	9 (69%)	4 (31%)	1	4 (31%)	9 (69%)	0.708	10 (77%)	3 (23%)	1
Papillary	17	12 (71%)	5 (29%)		7 (41%)	10 (59%)		13 (76%)	4 (24%)	
Metastasis stage										
Non-metastasis	16	8 (50%)	8 (50%)	0.017*	4 (25%)	12 (75%)	0.257	14 (87%)	2 (13%)	0.204
Metastasis	14	13 (93%)	1 (7%)		7 (50%)	7 (50%)		9 (64%)	5 (36%)	

*P<0.05 was considered statistically significant

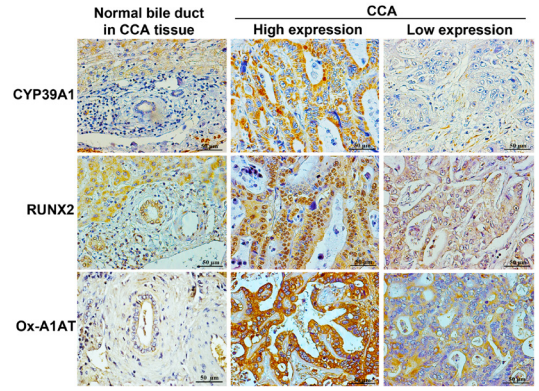

Figure 1. Immunohistochemical analysis of CYP39A1, RUNX2 and ox-A1AT in normal bile duct, which shows negative staining, whereas in CCA tissue shows positive immunoactivity that could be grouped into high and low expression. Original magnification is ×400 for normal bile duct and CCA tissues. Scale bars=50μm

Table 2. Correlation between Expression of CYP39A1 and its Regulator, RUNX2 and an Oxidative Protein Marker, ox-A1AT in Tumor Tissues of CCA Patients Demonstrated by Immunohistochemical Staining

Factors	CYP39A1	RUNX2	Ox-A1AT
CYP39A1	1	0.498	-0.361
Pearson correlation Sig. (2-tailed)	-	0.005	0.05
N	30	30	30
RUNX2			
Pearson correlation	0.498	1	-0.562
Sig. (2-tailed)	0.005*	-	0.001*
N	30	30	30
ox-A1AT			
Pearson correlation	-0.361	-0.562	1
Sig. (2-tailed)	0.050*	0.001*	-
N	30	30	30

*P<0.05 was considered statistically significant

fluor 555 for 1 hr. Preparations were mounted on slides with DAPI for nuclear staining and examined using a fluorescence microscope with a 40x objective lens.

Statistics

Statistical analysis was performed using SPSS software version 17 (IBM Corporation, NY, USA). The association of protein expression in CCA tissues and patients' clinico-pathological parameters assessed by Fisher's exact test was analyzed. The survival analysis was performed using Kaplan-Meier with Log-rank test. Correlations between CYP39A1, RUNX2 and ox- A1AT level in all groups were determined by Pearson's correlation analysis. A P-value less than 0.05 was considered statistically significant.

Results

BExpressions of CYP39A1, RUNX2 and ox-A1AT in human CCA tissues in relation to clinicopathological features

As shown in Table 1, results of immunohistochemical staining revealed high expressions of CYP39A1, RUNX2 and ox-A1AT were 30% (9/30), 63% (19/30) and 23% (7/30) of cases, respectively. In Figure 1, a strong expression of those proteins was detected in tumor cells and hepatocytes. RUNX2 was detected only in nuclei of CCA cells. The low expression of CYP39A1, RUNX2 and ox-A1AT was 70% (21/30), 37% (19/30) and 77% (23/30) of cases, respectively. Normal bile duct epithelia residing in non-tumor adjacent tissues showed weak staining in CYP39A1, RUNX2 and ox-A1AT for most cases. Among liver sections of 30 patients with intrahepatic CCA examined, 18 (60%) cases were male and 12 (40%) cases were female. The age of patients ranged from years 37-73 old (median age=56.5 years). In this study, the CCA histological types were classified as the papillary type of 57% (17/30) cases and tubular type of 43% (13/30) cases. CCA metastatic stage were classified as the metastasis of 47% (14/30) cases and non-metastasis of 53% (16/30) cases, Fisher's exact test showed a significant negative correlation between high CYP39A1 expression with tumor metastasis (p=0.017). Age, sex and histological grade did not show any association with CYP39A1 and ox-A1AT (Table 1). Notably, high expression of RUNX2 was significantly correlated with male gender (p=0.009). In Table 2, CYP39A1 expression was positively correlated with RUNX2 expression (r=0.498, p=0.005) but negatively correlated with ox-A1AT (r=-0.361, p=0.050) and RUNX2 expression was negatively correlated with ox-A1AT expression (r=-0.562, p=0.001).

Expressions of CYP39A1, RUNX2 and ox-A1AT and survival in CCA patients

The Kaplan-Meier method with log rank test showed

Table 3. Multivariate analysis by the COX proportional hazard regression model

Variable	Adjusted Hazard ratio	95% confidence interval	P value	Adjusted Hazard ratio	95% confidence interval	P value	Adjusted Hazard ratio	95% confidence interval	P value
CYP39A1				RUNX2			ox-A1AT		
Low	1	0.108-0.933	0.037	1	0.071-0.732	0.013	1	1.081-10.479	0.036
High	0.318			0.228			3.365		
Age (Year)									
< 56	1	0.368-2.518	0.938	1	0.467-3.064	0.71	1	0.280-2.156	0.628
> 56	0.963			1.196			0.777		
Gender									
Female	1	0.391-2.285	0.9	1	0.159-1.562	0.232	1	0.332-2.091	0.697
Male	0.945			0.499			0.833		
Histological grading									
Non-papillary	1	0.889-5.916	0.086	1	0.866-6.587	0.092	1	0.705-5.129	0.204
Papillary	2.293			2.389			1.902		
Metastasis stage									
Non-metastasis	1	0.424-2.842	0.848	1	0.760-3.998	0.189	1	0.782-3.976	0.171
Metastasis	1.097			1.744			1.764		

*P<0.05 was considered statistically significant

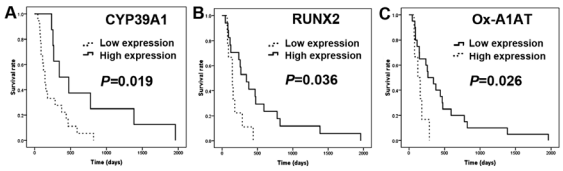

Figure 2. Comparison of survival in CCA tissue of patients, whose tumors showed high and low expression of CYP39A1 (log-rank test p=0.019) A); RUNX2 (log-rank test p= 0.036) B); and ox-A1AT (log-rank test p=0.026) C). Patients with low expression of the three proteins show the higher survival days than those with high expressions

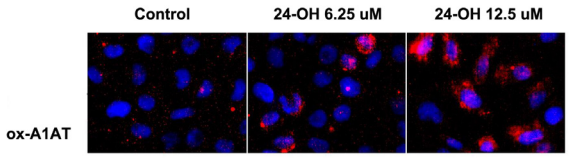

Figure 3. The immunofluoresence assay was performed to investigate the level of ox-A1AT switching in 24-OH-induced M214 cell lines. M214 cell lines were treated with 0 µM as control A), 6.25µM B) and 12.5µM C) respectively. The production of ox-A1AT increased after induction with increasing concentration of stimuli when compared to untreated

that CCA patients who had low expression of CYP39A1 and RUNX2 had significantly shorter survival (p=0.019 and p=0.036, respectively) when compared with patients who had high expression of those proteins (Figure 2). In contrast, low expression of ox-A1AT in CCA patients had significantly longer survival (p=0.026). Multivariate analysis was performed using the Cox proportional hazards model to investigate the independent value of each factor to predict overall survival. In Table 3, the result showed that CYP39A1 (hazard ratio, 0.318; 95% confidence interval, 0.108-0.933; p=0.037) and RUNX2 (hazard ratio, 0.228; 95% confidence interval, 0.071-0.732; p=0.013) remained significantly independently protective with the prognostic model whereas ox-A1AT (hazard ratio, 3.365; 95% confidence interval, 1.081-10.479; p=0.036) were independent prognostic risk factors

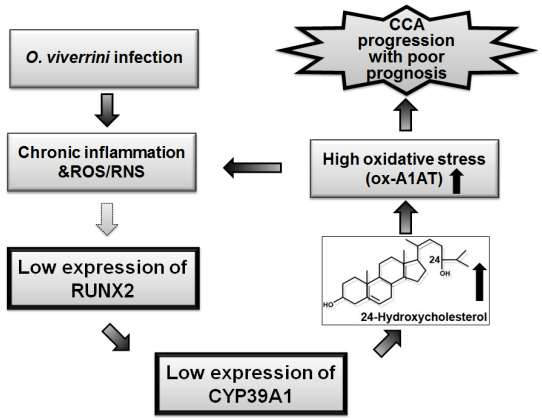

Figure 4. Proposed mechanism linking the altered expressions of RUNX2 and CYP39A1, that affects 24-OH level and oxidative stress in relation to CCA progression

for overall survival.

24-OH-induced ox-A1AT expression in CCA cell line

After 24 h incubation, 24-OH-induced M214 CCA cells significantly increased ox-A1AT, oxidative stress marker expression as dose dependent manner determined by immunocytochemistry analysis (Figure 3). This evidence suggests that 24-OH is involved in inflammatory-associated CCA.

Discussion

The cytochrome enzyme CYP39A1 (oxysterol 7α-hydroxylase) preferentially catalyzes the 7α-hydroxylation of 24-OH (Li-Hawkins et al., 2000) and the presence of CYP39A1 has been reported only in the liver (Bjorkhem et al., 1998). The major brain cholesterol metabolite 24-OH (Lutjohann et al., 1996) which is eliminated in the liver is catalyzed by CYP39A1 and CYP7A1 (Bjorkhem et al., 2006). It has been reported to be involved in the pathogenesis of Alzheimer's disease, which has an accumulation of oxidative brain damage (Bjorkhem et al., 2006). In cancer, CYP39A1 has been found to be hypermethylated in ovarian cancers as well as being related to poor prognosis (Huang et al., 2009). To

date the presence of CYP39A1 or its metabolite 24-OH has not been elucidated in CCA. In this study, we revealed that CYP39A1 showed low expression in most of CCA patients. Interestingly, CCA patients with high CYP39A1 expression in their tumor tissues had longer survival than patients with low expression. In addition, high expression of CYP39A1 was well-correlated with non-tumor metastasis status. These data suggest that CYP39A1 might be a promising protective-prognostic factor and altered expression of CYP39A1 might play roles in CCA progression, possibly leading to the accumulation of 24-OH. The molecular mechanism by which CYP39A1 expression is regulated and its role in CCA genesis and progression as well as the level of 24-OH in CCA requires further comprehensive investigation.

We demonstrated that CYP39A1 showed a positive correlation with RUNX2. In addition, patients with high RUNX2 expression had longer survival than those with low expression, suggesting a role in the prognostic indication for CCA. In contrast to ox-A1AT, patients with high ox-A1AT expression had shorter survival than those with low expression. It has been known that RUNX2 controls genes involved in sterol/steroid metabolism, including Cyp11a1, Cyp39a1, Cyp51, Lss, and Dhcr7, discovered in murine osteoprogenitor cells (Teplyuk et al., 2009). In addition, the tumor suppression property of RUNX2 has been reported in breast cancer cell lines in blocking the effect of estradiol, resulting in inhibiting tumor growth (Chimge et al., 2012). Notably, we found that high expression of RUNX2 in CCA was significantly related with male than female gender. Previously, estrogen level in male CCA patients' sera was shown to increase and correlated with clinical presentations (Hunsawong et al., 2012).

The oxidative damage occurs frequently in CCA. CCA patients who had high oxidative stress via protein damage formation significantly correlated with poor prognosis while low oxidative stress could be related with good prognosis (Thanan et al., 2012b). An oxidative damage marker, ox-A1AT, has been found to be highly expressed in CCA tissues (Jamnongkan et al., 2013). Our result demonstrated that ox-A1AT was highly expressed in CCA tissues and correlated with shorter patient survival. In addition, low CYP39A1 and RUNX2 expressions were positively correlated with high expression of ox-A1AT in CCA tissues. This implies that patients who present with low CYP39A1 and RUNX2 expression can have poorer prognosis than those who have high expressions due to the oxidative damage in CCA tissues which may enhance tumor progression.

In conclusion, significant roles of CYP39A1 and RUNX2 were confirmed in CCA clinical samples, suggesting that low expressions of those proteins were significantly related with high oxidative stress and correlated with poor prognosis and they could serve as a prognostic indicator of CCA. The cause and effect of 24-OH accumulation driven by the aberrant expression of these investigated proteins is depicted in Figure 4. The underlying mechanism by which CYP39A1 and RUNX2 are involved in the genesis of CCA requires further investigated.

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