RESEARCH ARTICLE

High Expression of HIF-1α, BNIP3 and PI3KC3: Hypoxia-Induced Autophagy Predicts Cholangiocarcinoma Survival and Metastasis

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Abstract

Hypoxia and autophagy are known to facilitate tumor progression. We here aimed to investigate the role of hypoxia-associated autophagy in cholangiocarcinoma (CCA) survival and metastasis. Immunostaining of hypoxic-responsive proteins (HIF-1 α and BNIP3) and a key regulator of autophagy (PI3KC3) were examined in CCA tissues and their expression levels were compared with clinicopathological parameters. A hypoxia mimicking condition (CoCl₂ treatment) was also tested regarding CCA cell functions. Our results showed that HIF-1 α (66%), BNIP3 (44%) and PI3KC3 (46%) showed strong staining in human CCA tissues. Positive expression of HIF-1 α (p=0.033), BNIP3 (p=0.040) and PI3KC3 (p=0.037) was significantly correlated with lymph node metastasis. HIF-1 α was well associated with BNIP3 (r=0.3, p<0.01) and PI3KC3 (r=0.2, p<0.01). The survival rates of patients who were positive with HIF-1 α (p=0.047) or co-expressed HIF-1 α and BNIP3 (p=0.032) or HIF-1 α and PI3KC3 (p=0.043) were significantly greater than in the negative groups. CCA cells treated with CoCl₂ showed an increase in HIF-1 α , BNIP3, PI3KC3 and LC3-II, with increased cell migration and pFAK levels. These data suggest that hypoxia associated autophagy enhances CCA metastasis, resulting in a poor prognosis of CCA.

Keywords: HIF-1a - BNIP3 - PI3KC3 - metastasis - cholangiocarcinoma

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Introduction

Cholangiocarcinoma (CCA), a bile duct cancer, arises from biliary epithelium within either the intrahepatic or the extrahepatic biliary tract (Patel, 2006). Recent epidemiological studies show that the incidence and mortality rates of CCA are increasing worldwide (Patel, 2006). Surgical intervention is the only promising curative treatment in the early stage of disease; unfortunately, the prognosis of advanced CCA is extremely poor and chemotherapy is the only feasible treatment for this stage of disease (Thongprasert et al., 2005). Therefore, the hallmark of molecular pathways regulating CCA progression need to be further addressed in order to provide new prognostic markers and possible therapeutic targets to improve clinical outcomes.

Hypoxia is a common feature of solid tumors that alters imperative pathways resulting in an aggressive behavior in an extensive variety of tumors. Recent studies suggest that tumor hypoxia induced by oxygen depletion contributes to chemotherapeutic resistance of CCA cell lines (Seubwai et al., 2012). In addition, it has been reported that hypoxia induced by hypoxia-mimetic agent cobalt chloride $(CoCl_2)$ modulates the invasive potential of primary and metastatic breast cancer cells (Fu et al., 2009). Hypoxiainducible factor (HIF-1 α), the main regulator of hypoxic conditions, as well as contributes to survival rather than cell death by inducing autophagy possibly mediated via HIF-dependent gene products BNIP3 (Bcl2/adenovirus EIB 19 kDa-interacting protein 3) or BNIP3L (Bcl-2/ adenovirus E1B19 kDa-interacting protein 3-like protein) proteins (Bellot et al., 2009).

Autophagy is a homeostatically controlled pathway by which autophagosomes fuse with lysosomes for subsequent degradation and recycling of macronutrients and impaired organelles by lysosomal enzymes (Klionsky and Emr, 2000). Autophagy is up-regulated in response to stress conditions such as nutrient deprivation, growth factor depletion, and hypoxia (Levine and Kroemer, 2008). Autophagy requires a regulator that contains lipid kinase activity named class III phosphoinositide 3-kinase (PI3KC3), for its activation under metabolic stress

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Suyanee Thongchot et al

(Byfield et al., 2005). It has been reported that autophagy was activated in CCA tissues of patients demonstrating by increasing the expression level of Beclin-1, a part of PI3KC3 complex (Hou et al., 2011). Interestingly, elevation of autophagy extends the survival in pancreatic cancer patients (Fujii et al., 2008) and inhibition of autophagy leads to apoptotic cell death in CCA cells (Hou et al., 2011).

In the present study, we aimed to investigate the role of hypoxia-induced autophagy on CCA survival and metastasis. Expressions of HIF-1 α , BNIP3 and PI3KC3 were determined in human CCA tissues and the correlation between those proteins and clinicopathological data of CCA patients was analyzed. In addition, we demonstrated that the hypoxic-mimicking condition activated the autophagy leading to the control over tumor cell migration.

Materials and Methods

Patients and samples

CCA tissue microarrays (CCA-TMAs) were constructed from archival paraffin embedded tissue of intrahepatic CCA specimens originated from primary tumors of patients. All patients underwent liver resection at Srinagarind Hospital, Khon Kaen University, Thailand during 1999-2010. Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki and its later revision. The Human Research Ethics Committee, Khon Kaen University (#HE43201 and #HE471214), approved the research protocol.

Cell lines, chemicals, and reagents

Two human CCA cell lines, M139 and M214, were cultured in Ham's F-12 medium supplemented with 44 mM NaHCO₃, penicillin (100 units/ml), streptomycin (100 mg/ml) and 10 % fetal bovine serum in a humidified atmosphere containing 5 % CO₂. CoCl₂ was purchased from Sigma, St. Louis, MO, United States. All other chemicals used were of analytical grade.

Immunohistochemical analysis

The CCA-TMAs of 183 cases were deparaffinized with xylene, dehydrated before exposure to 3% hydrogen peroxide for inactivating endogenous peroxidase activity and blocked with 10% skim milk for 1 h. Sections were incubated overnight with the primary antibodies against HIF-1α, BNIP3 and PI3KC3 (1:25, 1:50 and 1:100, respectively; Abcam; MA, USA) at 4°C, followed by peroxidase-conjugated Envision secondary antibodies (DAKO; Glostrup, Denmark) for an additional 1 h at room temperature. The presence of brown color corresponding to the peroxidase activity was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB) solution (Sigma-Aldrich; MO, USA), counterstained with Mayer's hematoxylin, dehydrated and mounted. The TMA sections were observed under a light microscope (Carl Zeiss, Germany) by using the high magnification power (X200); the 5 fields of each representative tumor section were evaluated.

Western blot analysis

Cells were washed with 1x PBS buffer and then lyzed with radioimmuno-precipitation assay (RIPA) buffer containing protease K inhibitor cocktail, 0.5M NaF, 0.2 M NaVO4, 1M Tris-HCl pH 7.5, 0.5M EDTA, 2.5M NaCl, 10% NP-40, 10% SDS, triton X-100 and deionized water. Ten µg of protein lysates were electrophoresed in 10% or 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Whatman, Dassel, Germany). Membranes were incubated with 10% skim milk to block the nonspecific binding. Then, membranes were incubated with primary antibodies against LC3, FAK and pFAK (1:1,000, 1:500 and 1:2,000, respectively; Abcam; MA, USA) overnight at 4°C, followed by secondary antibodies at room temperature for 1 h. Immunoreactive materials were developed by Enhanced Chemiluminescence Plus solution (GE Healthcare, Buckinghamshire, UK).

Wound-induced migration assay

CCA cell lines were cultured in medium containing 10%FBS at 37°C with 5% CO₂ in a 24-well plate until cells were confluent or nearly (>90%) confluent. Cell monolayers were scratched, then rinsed several times with 1x PBS to remove cell debris. Cells were incubated in medium containing CoCl₂ for 0, 8, 16, and 36 h. Cell migration in the wound area was monitored and visualized by microscopy and digitally photographed. The distance of the wound area was calculated by using the formula: Migration area=(Area of original wound - Area of wound during healing)/ Area of original wound.

Statistical analysis

Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL). The correlation of HIF-1 α , BNIP3 and PI3KC3 with clinicopathological parameters of CCA patients was analyzed by Fisher's exact test. The association among those proteins was performed by Pearson's correlation analysis. Patients' survival was calculated from the time of surgical resection to death and the survival curves were constructed according to Kaplan- Meier, with a Log-Rank test. A multivariate analysis was performed by the Cox proportional hazard regression model. The significance of different data was determined by the Student's t-test. A P value of less than 0.05 was considered statistically significant.

Results

Hypoxic- and autophagic-responsive proteins were expressed in human CCA tissues

Results of immunohistochemical staining in CCA-TMAs (n=183 cases) revealed that positive expressions of HIF-1 α , BNIP3 and PI3KC3 proteins (Figure 1) were 121 (66%), 81 (44%) and 85 (46%) of cases, respectively (Table 1). Among CCA-TMAs of 183 patients with intrahepatic CCA examined, 114 (62%) were male and 69 (38%) were female. The age of patients ranged from 26 to 89 years old (median age=58 years). In this study as shown in Table 2, the CCA histological types were classified as the papillary type of 62 (34%) cases and

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non-papillary type of 121 (66%) cases. Fisher's exact tests showed significant correlations between positive expressions of HIF-1 α (p=0.033), BNIP3 (p=0.040) and PI3KC3 (p=0.037) with lymph node metastasis. Age, gender and histological grade did not show any association with those proteins.

Cumulative survivals of CCA patients with positive expression of HIF-1 α (p=0.047) had significantly lower survival rates than those with negative expression (Figure 2A). The 5-year survival rates of patients who were positive with co-expressed HIF-1 α and BNIP3 (p=0.032)

 Table 1. Summary of Hypoxic-/Autophagic-Responsive

 Protein Expression in Tumor Tissues of CCA Patients

Proteins	Total cases	Results			
		Positives (%)	Negative (%)		
HIF-1α	183	121 (66%)	62 (34%)		
BNIP3	183	81 (44%)	102 (56%)		
PI3KC3	183	85 (46%)	98 (54%)		

or co-expressed HIF-1 α and PI3KC3 (p=0.043) were significantly greater than that of the negative groups (Figure 2B and Figure 2C respectively). Results in Table 3 showed that the expression of HIF-1 α was positively associated with BNIP3 (r= 0.3, p<0.01) and PI3KC3 (r= 0.2, p<0.01). Multivariate analysis was performed using the Cox proportional hazard model to investigate the independent value of each factor to predict overall survival. The results showed that HIF-1 α expression (HR=1.4, p=0.048), lymph nodes metastasis (HR=1.7, p=0.002) and non-papillary histological type (HR=1.4, p=0.038) were independent prognostic risk factors for overall survival (Table 4).

$CoCl_2$ stabilized HIF-1 α with induction of autophagicresponsive proteins in CCA cell lines

The CoCl₂ was added into cell culture with various concentrations and incubation times in order to determine the protein levels of HIF-1 α , BNIP3, PI3KC3 and LC3 (an autophagosomal marker) using western blotting. As shown

HIF-1αBNIP3PI3KC3Positive stainingImage: Comparison of the state of the s

Figure 1. HIF-1 α , **BNIP3 and PI3KC3 Expression in 183 Human Cholangiocarcinoma.** Representative HIF-1 α , BNIP3 and PI3KC3 immunohistochemistry images of tumor tissues in CCA-TMAs. Magnification was x200 for all figures



Figure 2. Kaplan-Meier Survival Analysis of CCA Patients. A) Cumulative survivals with HIF-1 α (+) and HIF-1 α (-) tumors (p=0.047; log-rank test); B) HIF-1 α (+) and BNIP3 (+) tumors compared to patients with HIF-1 α (-) and BNIP3 (-) (p=0.032; log-rank test); C) HIF-1 α (+) and PI3KC3 (+) tumors compared to patients with HIF-1 α (-) and PI3KC3 (-) (p=0.043; log-rank test)

 Table 2. Correlation between Expression of Hypoxic-/ Autophagic-Responsive Proteins in Tumor Tissues and

 Clinicopathology of CCA Patients Demonstrated by Immunohistochemical Staining

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Factors		n	HIF-1a		BNIP3			PI3KC3			
			-	+	Р	-	+	Р	-	+	Р
Age (Yr)	≤58	115	42	73	0.152	68	47	0.147	65	50	0.186
	>58	68	19	49	-	34	34	-	33	35	-
Gender	Female	69	17	52	0.055	34	35	0.112	36	33	0.445
	Male	114	44	70	-	68	64	-	62	52	-
Lymph nodes metastasis	No	98	39	39	0.033*	61	37	0.040*	59	39	0.037*
	Yes	85	22	59	-	41	44	-	39	46	-
Histological type	Non-papillary	121	41	80	0.48	70	51	0.259	69	52	0.123
	Papillary	62	20	42	-	32	30	-	29	33	-

*Fisher's exact probability was used for the comparison of variables that had two categories; **P value less than 0.05 was considered statistically significant

Suyanee Thongchot et al

in Figure 3A for M139 cells and 3B for M214 cells, HIF-1 α was markedly increased after 24-h incubation with 50, 100, 150 and 200 μ M of CoCl₂ in M139 cells and M214 cells compared with untreated cells. Treatment with 100 μ M CoCl₂ (12 to 48 h) increased the levels of HIF-1 α , BNIP3, PI3KC3 and LC3-II as a time-dependent manner (Figure 3C and 3D).

CoCl, induced migration ability of CCA cells

M139 and M214 cell lines were treated with 100 μ M CoCl₂ and the migration ability were assessed. As shown in Figure 4A and 4B, cells treated with 100 μ M CoCl₂ significantly migrated faster (1.7 times in M139 cells and 1.2 times in M214 cells) than untreated cells in which their wounds had closed at 36 h for M139 and 16 h for M214 cells. A metastasis marker, pFAK (phosphorylated focal adhesion kinase (Jiang et al., 2010)) and LC3-II were determined in CCA cells treated with CoCl₂ using western blotting. As depicted in Figure 4C and 4D, the 100 μ M CoCl₂ treated cells increased the levels of LC3-II

Table 3. Correlation Coefficients between Immunohistochemistry Scores of Hypoxic- (HIF-1α) and Autophagic-Responsive Proteins in CCA Tissues

Factors		HIF-1α	BNIP3	PI3KC3
HIF-1α	Pearson correlation	1	0.3*	0.2*
	Sig. (2-tailed)	-	0	0.001
	N	183	183	183
BNIP3	Pearson correlation	0.3*	1	0.9*
	Sig. (2-tailed)	0	-	0
	Ν	183	183	183
PI3KC3	Pearson correlation	0.2*	0.9*	1
	Sig. (2-tailed)	0.001	0	-
	N	183	183	183

*Correlation is Significant at the 0.01 level



Figure 3. CoCl₂ Stabilized HIF-1 α with Induction of Autophagic-Responsive Proteins in CCA Cell Lines. Western blot analysis of HIF-1 α in A) M139 and B) M214 cell lines treated with various concentrations of CoCl₂ (50, 100, 150 and 200 μ M) for 24 h. *p<0.05; Western blot analysis of HIF-1 α , BNIP3, PI3KC3 and LC3 in C) M139 and D) M214 cell lines treated with 100 μ M CoCl₂ for 0, 12, 24 and 48 h. Each culture was done in three independent experiments

and pFAK when compared with untreated cells.

Discussion

Previous studies have suggested that hypoxia activates autophagy may promote tumor progression in many cancers (Bellot et al., 2009; Mazure and Pouysségur, 2010). We demonstrated that a hypoxia-inducible factor HIF-1 α was positively expressed in CCA tissues in 66% of cases and was strongly correlated with shorter patients' survival. Our finding is consistent with previous studies revealing that HIF-1 α correlated with shorter survival of breast cancer (Deb et al., 2014), lung cancer (Li et al., 2012), pancreatic cancer (Hoffmann et al., 2008), colorectal cancer (Baba et al., 2010) and Japanese type of intrahepatic CCA (Morine et al., 2010). In addition, it has

Table 4. Multivariate Analysis by a Cox ProportionalHazard Regression Model

Variable		Adj. HR	95% CI	p value
HIF-1α	Positive	1.4	1.0-1.9	0.048*
	Negative	1		
HIF-1α and BNIP3	Positive	1	0.8-1.4	0.781
	Negative	1		
HIF-1α and PI3KC3	Positive	1.2	0.9-1.6	0.290
	Negative	1		
Age (Yr)	≤58	1.1	0.8-1.5	0.690
	>58	1		
Gender	Female	0.9	0.7-1.3	0.648
	Male	1		
Lymph nodes metastasis	Yes	1.7	1.2-2.3	0.002*
	No	1		
Histological type	Non-papillar	y 1.4	1.0-1.9	0.038*
	Papillary	1		

HR indicates hazard ratio; 95% CI, 95% confidence interval



Figure 4. CoCl₂ Induced Migration Ability in CCA Cells. CoCl₂ (100 μ M) were added in A) M139 and B) M214 cell lines and incubated for 36 h or 16 h, respectively, and the migration of the cells towards the wound was visualized. Images were taken at various time points. Data were presented as mean±SEM, *p<0.05. Each culture was done in three independent experiments. Western blotting of LC3, pFAK and total FAK in C) M139 and D) M214 cell lines treated with 100 μ M CoCl₂. β -actin was used as an internal control. Each culture was done in three independent experiments.

been revealed that HIF-1 α was presented in intrahepatic CCA tissues of Thai patients (Pinlaor et al., 2005), and in this study, we found that HIF-1 α had a high expression in CCA patients with a shorter survival. We then revealed that HIF-dependent gene product BNIP3 was positively expressed in CCA tissues with 44% of cases and the autophagy regulator PI3KC3 was positive in 46% of CCA cases. Patients who positively expressed two markers, HIF-1a plus BNIP3 or HIF-1a plus PI3KC3, showed the significant correlation with the shorter survivals. In addition, HIF-1a, BNIP3 or PI3KC3 were positively associated with lymph node metastasis. Furthermore, we also elucidated that HIF-1 α was positively correlated with BNIP3 and PI3KC3 by Pearson's correlation analysis. This implies that HIF-1 α might trigger autophagy in CCA tissues via its target gene BNIP3 cross-linking to autophagy via activation of PI3KC3 complex (Bellot et al., 2009), providing the role in tumor progression. In addition to HIF-1 α alone, combination between HIF-1 α and BNIP3 or HIF-1 α and PI3KC3 could predict a survival and metastasis of CCA.

We established the hypoxia-mimicking condition using CoCl₂ to mimic an oxygen-depleted atmosphere in two CCA cell lines to explore the cellular functions in CCA. Our results revealed that CoCl_2 generated a hypoxic condition and induced autophagy by increasing levels of HIF-1 α , BNIP3, PI3KC3 and autophagosomal marker LC3-II in CCA cell lines. A chemical mimetic of hypoxia CoCl, increased autophagy has been demonstrated in several models such as in the H9c2 rat cardiomyoblast cell culture (Gallo et al., 2014) and human periodontal ligament cells (Song et al., 2012) resulting in an increasing LC3-II level that is similar to O₂ depletion induced hypoxia (Hu et al., 2012). The hypoxia induced LC3-II/LC3-I ratio has been shown that it activates autophagosome maturation (Gallo et al., 2014). We demonstrated that treatment with $100 \,\mu M$ CoCl₂ accelerated CCA cell migration by increasing the level of pFAK, a metastasis marker. Our results imply that a synchronized function of HIF-1a, BNIP3, PI3KC3 and LC3-II promotes CCA cell migration. Likewise, hypoxiamimetic agent CoCl, has previously been demonstrated that it has potential in modulating the invasive ability of primary and metastatic breast cancer cells (Fu et al., 2009). Knockdown HIF-1 α by siRNA inhibiting cell migration and invasion under hypoxic environment was determined in malignant gliomas (Mendez et al., 2010). BNIP3 supports melanoma cell migration and vasculogenic mimicry by orchestrating the actin cytoskeleton (Agostinis et al., 2013). Furthermore, autophagy itself can promote tumor metastasis (Kenific et al., 2010).

In conclusion, our results with CCA tissues in patients and cell lines support that several molecular markers closely correlated with tumor hypoxic conditionassociated autophagy activated in CCA reflect severity of the disease, contribute to their survival and metastasis and provides as prognostic markers.

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Suyanee Thongchot et al

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