RESEARCH ARTICLE

Hypoxia Enhances Aggressiveness of Cholangiocarcinoma Cells

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Abstract

Hypoxia, a common feature of solid tumors, plays a significant role in determining tumor phenotype and tumor progression. In this study, using an in-house PCR-array, we investigated phenotypic changes and differentially expressed hypoxia related genes in the KKU-M213 CCA cell line, cultured under hypoxic (1% O_2) condition. Trefoil factor-1 (*TFF1*), a disintegrin, and metalloprotease 12 (*ADAM12*), integrin-alpha 5 (*ITGA5*) and baculoviral IAP repeat-containing 5 (*BIRC5*/*survivin*), proteins involved with cell proliferation, metastasis and apoptosis resistance, were up-regulated whereas uridine 5'-monophosphate synthase (*UMPS*) and S100 calcium binding protein P (*S100P*), involved with chemosensitivity and cell adhesion, were down-regulated. Growth arrest, apoptosis resistance to UV-irradiation and chemotherapeutic drugs (5- flourouracil, cisplatin, doxorubicin) as well as cell adhesion were thus significantly enhanced upon exposure to hypoxic condition. These findings emphasize the significance of a hypoxic state in the induction of an aggressive phenotype and suggest the potential of targeting hypoxia regulated genes to enhance the sensitivity of chemotherapeutic drug against CCA.

Keywords : Bile duct cancer - hypoxia - PCR-array-*TFF1* - *BIRC5*/survivin

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Introduction

Cholangiocarcinoma (CCA), an aggressive and lethal cancer, is now increasing worldwide. CCA arises from biliary epithelium within either the intrahepatic or the extrahepatic biliary tract. It is difficult to diagnose CCA at early stage and hence almost all patients present with advanced, incurable disease. The low 5-year survival rate (0% to 40%) was reported even in patients who had undergone complete surgical resection (Gores, 2003; Anderson et al., 2004). CCA is known as a chemo-resistant cancer with a high recurrence rate; therefore, a new therapeutic regimen using specific targeted molecule is deemed essential to improve the clinical outcomes.

Hypoxia can develop at distances (typically 100-150 μm) beyond the diffusion capacity of oxygen from blood vessels or in areas of a tumor with compromised blood flow due to aberrant vasculature formation and high interstitial pressure. Recent studies suggest that tumor hypoxia contributes to the progression of a more malignant phenotype by selecting for cells with a diminished apoptotic potential and a reversibly inhibition of cell-cycle progression (Green and Giaccia, 1998). This population of cells significantly impacts clinical response to anticancer therapies.

Hypoxia was correlated with a lower probability of disease-free survival in head and neck carcinoma patients (Nordsmark et al., 1996; Brizel et al., 1997) and with increased incidence of metastases in several cancers (Bennewith and Dedhar, 2011). Nonetheless, hypoxia predicted distant failure not only in patients treated with radiotherapy but also in those treated with surgery. Several studies suggest that hypoxia alters fundamental, physiologically important pathways that result in more aggressive tumor behaviors in a wide variety of tumors (Rockwell et al., 2009; DeClerck and Elble, 2010). However, the mechanisms of chemo/radio-resistance are remaining unclear, especially in CCA. For a better understanding and beneficial outcome in the treatment of CCA patients; therefore, it is valuable to characterize the hypoxia related genes in CCA.

The aim of this study was to characterize the transcriptome profile of CCA in hypoxic condition using in-house PCR array. In addition, the progression of a more malignant phenotype induced by hypoxia –namely growth, apoptosis and adhesion in CCA cell lines were explored.

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Materials and Methods

Cell lines

Human CCA cell lines, KKU-M139, KKU-M213, KKU-M214 and KKU100 were established from primary tumor of CCA patients with different histological types according to Sripa et al (2005). CCA cell lines and immortalized normal cholangiocyte, MMNK-1 were cultured in DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% penicillin-streptomycin at 37°C, 5% CO_2 and 21% O_2 for normoxia or at 37°C, 5% CO_2 and 1% O_2 for hypoxia condition.

RNA Extraction

Total RNA was isolated from CCA cell line using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quality of RNA was monitored by gel electrophoresis on 1.5% denaturing agarose gels. A NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) was used to quantify the amount of RNA.

In-house PCR array

In total, 176 CCA associated genes were included in this study based on 1) functions reported in CCA and 2) gene expression data deposited in the National Center for Biotechnology Information (NCBI) and Gene Expression Omnibus (GEO) database. There were 19 genes involved in apoptosis (e.g., *BIRC5*); 27 genes in metabolism (e.g., *TYMP*, *UMPS*); 26 genes in cell adhesion (e.g., *ADAM12*); 19 genes in cell migration and invasion (e.g., *ITGA5*, *S100P*); 30 genes in cell differentiation and cell proliferation (e.g., *PDGFA, PRKCA*); 21 genes in signal transduction (e.g., *ESR1*); 12 genes in transporters (e.g., *ABCC1*) and 22 genes in others mechanism. Primers were designed based on unique sequences and the BLAST and BLAT tools on the NCBI and United States Cancer Statistics website.

Quantitative real-time PCR analysis

RNA (2 μg) was reverse-transcribed to cDNA using oligo-dT primers and quantitative real-time PCR for Trefoil factor-1 (*TFF1*), a disintegrin and metalloprotease 12 (*ADAM12*), integrin-alpha 5 (*ITGA5*), baculoviral IAP repeat-containing 5 (*BIRC5*/*survivin*), uridine 5'-monophosphate synthase (*UMPS*) and S100 calcium binding protein P (*S100P*) were performed using a LightCycler 480 (Roche Applied Science, Indianapolis, IN). ACTB was used as a housekeeping gene. The differences of gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method for relative quantification and expressed as the fold change relative to normoxic or hypoxic groups.

In vitro growth assay

Numbers of CCA cells were analyzed by sulphorhodamine B (SRB) assay. Cells were fixed with cold 40% trichloroacetic acid (Sigma-Aldrich, St. Louis, Mo, USA) for 1 h, washed, and then stained with 0.4% (w/v) SRB (Sigma-Aldrich, St. Louis, Mo, USA) dissolved in 1% acetic acid for 30 min. Unbound dye was removed by four washes with 1% acetic acid. Protein bound dye was dissolved with 10 mM unbuffered Tris base. The optical density was measured at 540 nm using a microtiter plate reader (Tecan Austria GmbH, Salzburg, Austria).

Cell adhesion assay

CCA cells (2x104 cells) in serum free medium were plated onto a matrigel-coated 24 well plate (BD Bioscience, San Jose, CA, USA) and allowed to attach for 6 h. After incubation, the wells were washed three times with phosphate-buffered saline. The attached cells were then fixed and stained with SRB as described above. The optical density obtained was used as adhesion index. All assays were performed in triplicate in each of two independent experiments.

Statistical Analysis

The results were presented as the mean±S.D. of at least three separated experiments. Statistical significance was determined by Student's t-test. $P<0.05$ was considered to be significant.

Results

Profiling of hypoxia related genes in human CCA cell lines

To gain an insight into the expression profile of hypoxia regulated genes in human CCA cell lines, we performed an in-house PCR array containing 176 genes that associated with carcinogenesis and progression of CCA. CCA cells, KKU-M213, were subjected to normoxic or hypoxic condition for 72 h, before submitted to PCR array. The expression levels of genes with 2.5-fold difference were classified as up-regulated and those with <0.5-fold difference were classified as down-regulated. There were 31 genes up-regulated and 48 genes down-regulated when CCA cells were exposed to hypoxic condition for 72 h (Table 1). Majority of the identified genes are involved in cell cycle, apoptosis, cellular movement, free radical scavenging and DNA repair. Genes with highly differential expressed in hypoxic state, e.g., *TFF1*, *ADAM12*, *ITGA5*, *BIRC5*/*survivin*, *UMPS* and *S100P* were validated by real-time PCR in KKU-M213 (Table 2).

Hypoxia induces growth arrest in CCA cell lines

To analyze the influence of hypoxia on proliferation and viability, four CCA cell lines established from different histological types of primary tumors from CCA patients, KKU-M139 (adeno-squamous CCA), KKU-M213 (mixed papillary and non-papillary CCA), KKU-M214 (well-differentiated CCA) and KKU-100 (poorly differentiated CCA) and immortalized normal cholangiocyte, MMNK-1 were exposed to hypoxia for 72 h. Cell numbers were investigated every 24 h with SRB assay. A remarkable reduction of growth rates were found in four hypoxic CCA cells (Figure 1). MMNK-1 exhibited a dramatically growth rate reduction compared to CCA cell lines. As compared to the control cells in normoxia, hypoxia reduced cell numbers, 54.43%, 61.89%, 61.33% and 66.63% for KKU-M139, KKU-M213, KKU-M214 and KKU-100 ($P<0.001$), respectively. In contrast to CCA cells, 90.33% of MMNK-1 cells were inhibited ($P<0.001$).

It is important to note that significant amount of dead cells were observed in the MMNK-1 under hypoxic condition.

Hypoxia induces apoptosis-resistance in CCA cell lines

As UV-radiation is known to cause cell death via apoptosis, we examined further the effect of hypoxia induced apoptosis-resistance in CCA cells (KKU-M139, KKU-M213, KKU-M214 and KKU100) compared to normal cholangiocyte, MMNK1. Cells were exposed under 4 mJ UV for 4 sec before incubating in normoxic or hypoxic condition for 48 h. Cells without UV exposure and cultured in normoxia or hypoxia were used as control (100%) for each condition. UV-radiation significantly reduced cell numbers of CCA cell lines and MMNK-1 in normoxic condition to 60-70% of the control. However, CCA cells cultured in hypoxic condition were not much affected by UV-radiation, 90-100% of cells were remained. In contrast, similar method induced cell death in MMNK1 cells to the same extent as observed in untreated cells cultured in normoxic condition. The numbers of CCA cells remained in UV treated-normoxia were significantly reduced compared to those of UV treated-hypoxia (P<0.001; Figure. 2). Retardation of cell death was observed in all CCA cells cultured in hypoxic condition but not in those cultured in normoxic condition.

We next investigated the effect of hypoxia on chemotherapeutics-induced cell death. Cells were cultured in normoxia or hypoxia for 24 h prior treated with different concentrations of chemotherapeutic agents (5-FU, cisplatin and doxorubicin) and further cultured in normoxic or hypoxic condition for 48 h. Regardless to the culture condition, viability of three CCA cell lines was decreased with increasing dose of chemotherapeutic agents comparing to non-treated cells (P<0.001). At 0.01-2,000 μM 5-FU, numbers of hypoxic cells of all CCA cell lines were significant higher than those in normoxic cells (P<0.001; P<0.05). The similar results were also observed in M139 and M213 cells treated with cisplatin (0.1-10

Table 1. Differential Expression Profile of CCA Cell Line, KKU-M213, under Hypoxic vs. Normoxic Conditions (Ratio Hypoxia/Normoxia)

Up-regulation		Down-regulation			
Gene l Symbol	Fold Change	Gene Symbol	Fold Change	Gene Symbol	Fold Change
TFF1	2592.27	*UMPS*	0.01	*USF2*	0.3
IGFBP5	88.95	*CD14*	0.02	*MDK*	0.31
ADAM12	33.36	*S100P*	0.02	*PDGFA*	0.31
ANLN	23.59	*TYMP*	0.03	*PCDH1*	0.32
GC	21.19	*SDC1*	0.04	*MMP14*	0.33
INSIG1	18.44	*S100A11*	0.04	*ERBB2*	0.33
BIRC5	17.57	*SYNGR2*	0.04	*SLC12A2*	0.33
ITGA5	13.78	*POU1F1*	0.04	*BGN*	0.34
ESR1	11.84	*SERPINC1*	0.05	*RAB27B*	0.36
CKS2	8.49	*PRKAR1A*	0.06	*PMEPA1*	0.37
GPC3	7.7	*RECK*	0.06	*VCAN*	0.41
EMP1	6.75	*TACSTD2*	0.07	*MUC5AC*	0.42
HOXB7	5.98	*TOP2B*	0.07	*GSTP1*	0.43
EGR2	5.43	*MOBKL2B*	0.09	*ABCC1*	0.44
ENO1	4.87	*NCOR1*	0.1	*EBAG9*	0.48
CIT	4.07	*KRT7*	0.14	*SRI*	0.5
SCG2	3.84	*ABCA3*	0.15	*KNG1*	0.5
KRT19	3.66	*CKMT1A*	0.16		
BUB1B	3.34	*LMO4*	0.17		
PPIA	3.2	*TIMP3*	0.18		
CEACAM6	3.08	*TMEM63A*	0.19		
MUC1	3.06	*VDR*	0.19		
AKR1C4	2.98	*PRKCA*	0.2		
B2M	2.91	*SFN*	0.21		
HCN3	2.88	*METAP2*	0.21		
AXIN1	2.8	*YWHAZ*	0.23		
COL7A1	2.67	*ACTB*	0.24		
HPD	2.66	*JAG1*	0.25		
GAPDH	2.65	*REG1A*	0.26		
IQGAP1	2.62	*ACSS1*	0.3		
KIF2C	2.54	*RRM1*	0.3		

Table 2. Six Hypoxia Related Genes Obtained from PCR Array were Validated in KKU-M213 using Real Time-PCR

Genes	KKU-M213	
	PCR-array	Real-Time PCR
TFF1	2592.3	1917.48
ADAM12	33.36	1.5
BIRC5	17.57	3.19
ITGA5	13.78	24.5
UMPS	0.01	0.23
S100P	0.02	0.41

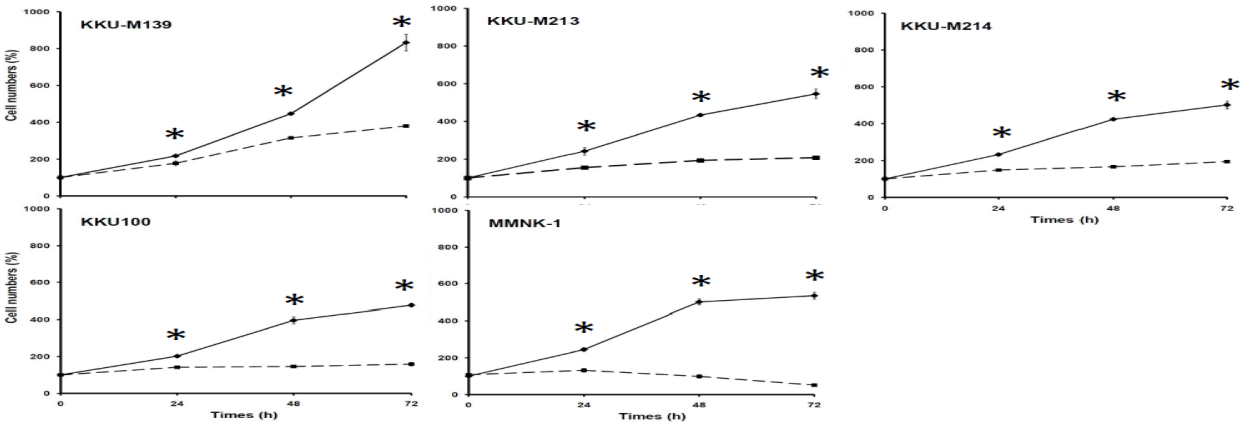

Figure 1. Growth Inhibition Effect of Hypoxia in CCA Cell Lines. Cells were cultured in normoxic (21% O_2) or hypoxic (1% O_2) conditions. Cell numbers were measured by SRB assay. Growth of CCA cell lines, KKU-M139, KKU-M213, KKU-M214 and KKU100 were retarded by hypoxic condition, whereas, apoptotic cells were observed in immortalized normal choalangiocyte, MMNK-1. Solid line represents normoxia; dotted line represents hypoxia

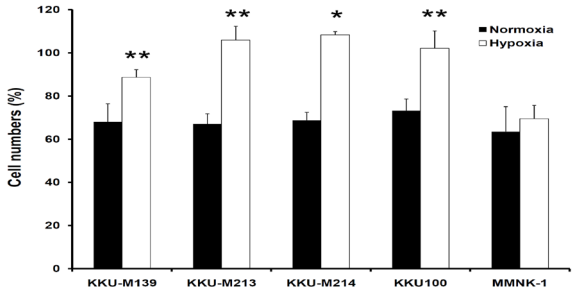

Figure 2. Hypoxia Induced Apoptosis-Resistance to UV-Irradiation in CCA Cell Lines. Cells were exposed to UV 4 mJ/cm^2 for 4 sec before incubating in a normoxic (21% O_2) or hypoxic (1% O_2) conditions for 48 h. Anti-apoptosis was found in all CCA cell lines but not normal cholangiocytes (MMNK1). Cells without UV exposure and cultured in normoxia or hypoxia were used as control (100%) ** P<0.05; * P<0.01

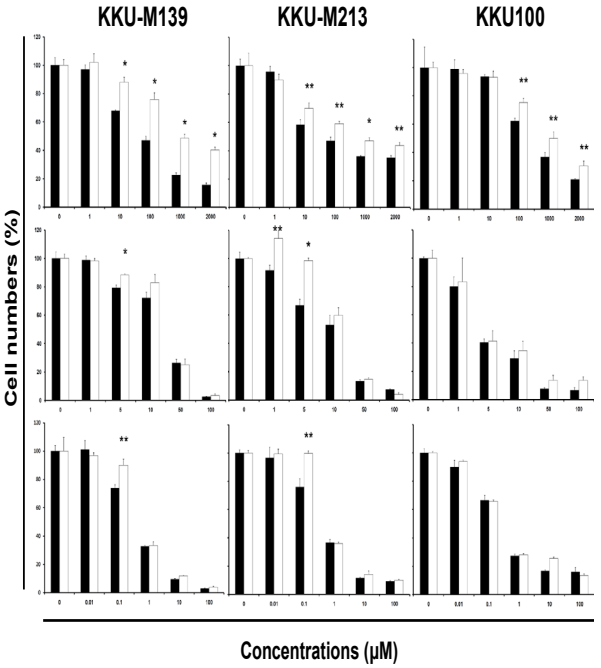

Figure 3. Hypoxia Promoted Chemotherapeutic Drug-Resistance in CCA Cell Lines. CCA cell lines (KKU-M139, KKU-M213 and KKU-100) were cultured in normoxia or hypoxia for 24 h prior treated with different concentrations of chemotherapeutic agents and further cultured in normoxic or hypoxic condition for 48 h. Cells without chemotherapeutic agent and cultured in normoxia or hypoxia were used as control (100%). Black bar and open bar represent cells cultured in normoxic and hypoxic conditions. **P<0.05; *P<0.01

μM) and doxorubicin (0.1 μM) (Figure 3). In contrast to M139 and M213, we did not observe the chemotherapeutic resistance in hypoxic condition in KKU-100.

Hypoxia enhances adhesion in CCA cell lines

To study the effect of hypoxia on cell adhesion, the matrigel adhesion test was performed. Cells cultured in normoxia or hypoxia for 24 h before subjecting to adhesion assay. All CCA cell lines at hypoxic condition had significantly higher numbers of adhered cells than the nomoxic cells (P<0.05). In contrast, hypoxic MMNK-1 cells had lower adhesion ability compared to nomoxic cells (P<0.05) (Figure. 4)

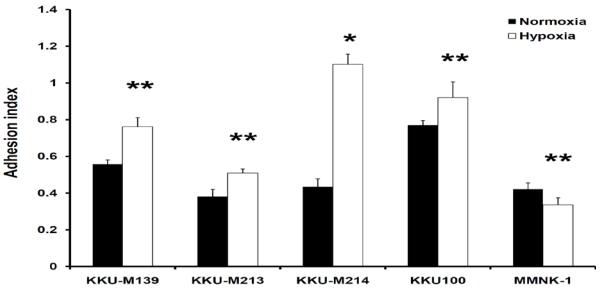

Figure 4. Hypoxia Promoted Cell Adhesion of CCA Cell Lines. Cells were allowed to adhere on a matrigel-coated well for 6 h and the adhered cells were determined using SRB assay. The obtained optical density was used as adhesion index. **P<0.05; *P<0.01

Discussion

Using an in-house PCR array, we have demonstrated the differential expression profile induced by hypoxia in CCA cell lines. Several genes played roles in cell cycle, cell movement, free radical scavenging and DNA repair were altered in hypoxic condition. Hypoxia contributes to the progression of a more malignant phenotype of CCA. CCA cells at hypoxic state significantly enhanced cell cycle arrest, apoptosis resistance to UV-irradiation and chemotherapeutic drugs, and increased cell adhesion to matrigel. These evidences raise the possibility of targeting hypoxia related genes to enhance the sensitivity of chemotherapeutic drugs for CCA.

Cellular adaptations to hypoxia are well documented in many cancer cells. We found that the differentially expressed genes were involved in the processes of adaptation and cell death, indicating a delicate balance between these processes under hypoxic stress. Up-regulated genes included *TFF1, ADAM12, ITGA5* and *BIRC5/survivin,* whereas down-regulation of *UMPS* and *S100P* were found in hypoxic CCA cell lines. These genes had been reported to involve with an aggressive phenotype of cancer cells including the inhibition of apoptosis and increasing metastasis.

All up-regulated genes had positive correlation with tumor progression and poor patients' outcomes. *TFF1* is a signaling protein that can activate epithelial cell invasion and has been considered as a metastasis stimulating agent. Over expression of *TFF1* has been reported in several cancers including CCA (Thuwajit et al., 2007; Hunsawong et al., 2012). Increasing of *TFF1* expression promoted tumor invasion in CCA cell lines. *ADAM12*, a disintegrin and metalloprotease, is a member of the ADAMs family which expressed at low levels in most normal adult tissues but at high levels in many human carcinomas, e.g., breast, gastric, colon carcinomas, and liver metastases (Iba et al., 1999; Le Pabic et al., 2003; Carl-McGrath et al., 2005). Over-expression of *ADAM12* was associated with increasing of tumor cell adhesion (Iba et al., 1999; Thodeti et al., 2005) and apoptosis resistance (Kveiborg et al., 2005). In CCA, the levels of serum *ADAM12* were inversely correlated with overall survival of CCA patients (Daduang et al., 2011).

Integrins are heterodimeric cell adhesion receptors that mediate intercellular communication through cell-

extracellular matrix interactions and cell-cell interactions. Integrins have been demonstrated to play a direct role in cancer progression, specifically in tumor cell survival, tumor angiogenesis, and metastasis. Over-expression of *ITGA5* has been demonstrated in many cancers with poor prognosis and insensitivity to treatment (Adachi et al., 2000). The negative correlation between *ITGA5* expression and cell growth has been shown in colon cancer and hepatocellular carcinoma both in vitro and in vivo (Varner et al., 1995; Zhou et al., 2000).

BIRC5/survivin is a protein in the intrinsic apoptotic pathway that interacts with XIAP and DIABLO leading to caspase-3 and caspase-9 inactivation, and finally inhibits apoptosis (Yamamoto et al., 2008). Up-regulation of *BIRC5* has been shown in almost all human malignancies including esophageal, breast, lung and CCA (Javle et al., 2004; Hinnis et al., 2007; Krepela et al., 2009; Zhu et al., 2011). A higher *BIRC5* expression has been correlated with an unfavorable survival or disease recurrence. Additionally, over-expression of *BIRC5* reduced the percentage of cell death induced by radiation in esophageal cancer cell lines (Zhu et al., 2011). Expression level of *BIRC5* was related to chemosensitivity. Patients with lower *BIRC5* expression were more responsive to preoperative chemotherapy with 5-flourouracil and cisplatin in esophageal cancer. In addition, rectal cancer patients with high *BIRC5* expression in pretreatment biopsies were more resistant to chemo-radiotherapy (Kim et al., 2011). Regarding to CCA, over-expression of *BIRC5* was reported (Chang et al., 2004) and associated with poor patients' outcomes (Javle et al., 2004).

UMPS and *S100P* were down-regulated in hypoxic CCA cells. *UMPS* catalyze the synthesis of UMP from orotate. Decreasing of *UMPS* expression is associated with resistant to 5-fluorouracil in colorectal cancer (Hidaka et al., 2003). *S100P* is a calcium-binding protein in the S100 family. Expression of *S100P* is related with reduction of cell adhesion (Du et al., 2012).

The profiles of genes expressed in hypoxic CCA cells (up-regulation of *TFF1*, *ADAM12*, *ITGA5* and *BIRC5/survivin*, and down-regulation of *UMPS* and *S100P*) indicated aggressive phenotypes of tumor cells. This led us to investigate cell growth, anti-apoptosis and adhesion of CCA cell lines under hypoxic condition. Growth retardation and resistance to apoptosis seem to be the adaptive characters of tumor cells but not normal cells in hypoxic condition. In our study, retardation of cell growth without apparent apoptosis was observed in hypoxic CCA cells. In contrast, hypoxia obviously induced cell death in normal cholangiocytes, MMNK1. The anti-apoptosis induced by hypoxia was more obvious when cells were induced to apoptosis by UV-irradiation. Hypoxic stage induces apoptosis resistance to UV-irradiation in CCA cells but not the MMNK1 cells.

Hypoxia induced anti-apoptosis in CCA cells was demonstrated in the present study. All three CCA cell lines were resisted to 5-FU treatment comparing to cisplatin and doxorubicin at the same concentration. Many studies demonstrated that hypoxia suppressed several genes involved in the metabolic activation pathway of 5-FU, e.g., Uridine 5'-monophosphate synthase (*UMPS*) (Hidaka et al., 2003) and Thymidine phosphorylase (*TYMP*) (Longley et al., 2003). As a result, hypoxia induced chemoresistance was more obvious in 5-FU treatment than in cisplatin or doxorubicin treatments.

In conclusion, hypoxia induced modification of gene expression that promoted aggressiveness of CCA cells. Under hypoxic state, CCA cells exhibited growth retardation, anti-apoptosis and adhesion. The present results suggest possible means for controlling growth and metastasis as well as enhancing chemosensitivity of CCA cells by suppressing hypoxia regulated genes. Future works are needed to understand the mechanism by which hypoxia mediated expression of *TFF1*, *ADAM12*, *BIRC5*, *ITGA5*, *UMPS* and *S100P*.

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