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Original Article

Carbonic anhydrase 2 overexpression is associated with poor prognosis and improved by metformin treatment in intrahepatic cholangiocarcinoma

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Abstract: Background: Previous studies have indicated dysregulation of carbonic anhydrase 2 (CA2) in several types of cancer. However, its role in cancer progression remains a subject of controversy, particularly in relation to different tumor types. Intrahepatic cholangiocarcinoma (CCA) is a rare and highly aggressive tumor, with limited understanding of the involvement of CA2 in its development. Aim: This study aims to explore the possible association between CA2 and the prognosis and treatment of intrahepatic cholangiocarcinoma. Methods: We performed RT-PCR and IHC staining to observe the expression of CA2 in both human and rat models of thioacetamide (TAA)-induced CCA. The association between CA2 expression and clinical outcomes was assessed using univariate and multivariate analyses. Additionally, we quantified changes in CA2 expression both in vivo and in vitro following treatment with Metformin, a commonly used inhibitor of CCA development. Results: The results showed that patients with high CA2 expression had a significantly poorer prognosis compared to those without CA2 expression. In the TAA-induced CCA model, CA2 expression was found to be significantly correlated with disease progression. Treatment with Metformin resulted in a reduction of CCA lesions induced by TAA, accompanied by a significant decrease in CA2 expression. Conclusions: These findings suggest that CA2 has potential as a prognostic factor in intrahepatic CCA, highlighting its potential as a novel therapeutic target for future CCA treatment.

Keywords: Cholangiocarcinoma, carbonic anhydrase 2, thioacetamide, metformin

Introduction

Intrahepatic cholangiocarcinoma (CCA) is considered to be one of the most aggressive types of liver cancer globally. It originates from the epithelial lining of the secondary biliary tree [1]. Currently, clinical diagnosis primarily relies on imaging findings of masses within the liver, while postoperative pathological examination remains the gold standard for confirming CCA diagnosis. The widely used serum biomarker for CCA is carbonic anhydrases 19-9, but its diagnostic performance is limited, as elevated levels can also indicate bacterial cholangitis, cholestasis, and other malignancies. Unfortunately, only approximately one-third of CCA patients are suitable for radical excision and liver transplantation upon diagnosis. Due to its anatomical location, the excision rate is low, and CCA also exhibits resistance to conventional chemotherapies, resulting in a bleak prognosis for patients [2]. The incidence and mortality rates of CCA have increased worldwide over the past decades. In the United States, about 7500 new CCA cases are diagnosed per year, and the 5-year survival rate is less than 30% [3].

Carbonic anhydrases (CAs), which are zinc-containing enzymes, belong to the category of metalloenzymes. Currently, there are 16 known isozymes in this enzyme family, with 15 of them identified in humans [4]. These isozymes play a vital role in catalyzing the reversible hydration process of carbon dioxide to bicarbonate, thereby regulating acid-base homeostasis. As a result, they are involved in various physiological

and biological processes. In recent decades, the roles of CAs have been extensively studied, particularly their potential as diagnostic markers for different types of cancers. For instance, CA9 has been identified as a biomarker in primary cervix cancer, renal cell carcinoma, and prostate cancer [5]. Similarly, CA12 has shown potential as a diagnostic marker in early breast cancer and cervical cancer [6].

Carbonic anhydrase 2 (CA2) is the most widely expressed isoform in normal tissues. However, its role in human cancers remains controversial. While it is weakly expressed in non-small cell lung cancer (NSCLC), hepatocellular carcinoma [7], colorectal cancer [8], and gastric cancer [9], it is highly expressed in nasopharyngeal cancer [10] and urinary bladder cancer [11]. For instance, a study conducted by Chenyue Zhang *et al*. discovered that CA2 inhibits epithelial-mesenchymal transition and metastasis in hepatocellular carcinoma [12]. On the other hand, Hirokazu Tachibana *et al*. reported that it acts as a novel invasion-associated factor in urinary bladder cancers [11]. Therefore, the expression pattern and effects of CA2 in CCA remain poorly understood and require further investigation.

Metformin (Met) is a commonly prescribed oral medication for type 2 diabetes [13]. Growing evidence suggests that Met also possesses anticancer properties in various tumors. Son Xuan Trinh *et al*. found that Metformin can inhibit the migration and invasion of CCA cells. It was observed that exposure to Metformin led to decreased expression of anti-apoptotic proteins Bcl-2 and Mcl-1, while upregulating the expression of E-cadherin. Additionally, the expression of N-cadherin, Snail, and MMP-2 was downregulated [14].

In this study, we have made a groundbreaking discovery by identifying a significant increase in CA2 expression in both rat and human CCA. Importantly, we have also established a strong correlation between elevated CA2 expression and poor outcomes in patients with CCA. Additionally, our findings demonstrate that administration of Metformin can effectively decrease the level of CA2 expression, subsequently reducing the incidence of CCA in rats. These findings underscore the potential of CA2 as a promising biomarker for prognostic evaluation in CCA patients. Moreover, our results suggest that targeting CA2 may hold promise as a therapeutic strategy for the treatment of CCA.

Material and methods

Tissue specimens and patient information

Forty-nine human CCA and ten paired adjacent noncancerous bile duct tissues were obtained from 49 CCA patients enrolled in Eastern Hepatobiliary Hospital in Shanghai, China from 2015 to 2017 in this retrospective study. Detailed clinical and pathological information of these patients was listed in **Table 1**, including age at diagnosis, gender, tumor size, the depth of invasion, nodal metastases, and cancer stages according to American Joint Committee on Cancer stage (AJCC) manual. The mean age of patients at tumor resection was 55 years; 35 (71.4%) were male and 14 (28.6%) were female. None of these patients received preoperative treatment. Clinical follow-ups were available for all patients (median, 16 months [range, 1-59 months]). All tissue specimens were obtained together with written informed consent, the study was approved by an ethical review committee of the Eastern Hepatobiliary Hospital Institutional Review Board (EHBHKY201803006). The study followed principles and guidelines for reporting this preclinical research.

Inclusion criteria: 1. Patients with locally or metastatic CCA who have been histologically or cytologically verified. 2. Age 18 years or older. 3. Patients who have not received any chemotherapy prior to surgery.

Exclusion criteria: 1. Patients diagnosed with hepatic cellular cancer (HCC) through pathological examination. 2. Patients with extrahepatic CCA. 3. Patients with a current clinically significant primary malignancy other than CCA.

Cell lines and culture conditions

The human Cholangiocarcinoma cell lines (CCA cells) QBC939 were purchased from the Cell Center of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FBS and 100 μg/mL penicillin/streptomycin. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Table 1. CA2 expression was highly associated with CCA tumor development

Parameters	N	CA2 positive	CA2 negative	*P*
		N (%)	N (%)	
Gender				
Male	35	23 (65.7)	12 (34.3)	0.3078
Female	14	7 (50.0)	7 (50.0)	
Tumor size				
≤3 cm	18	8 (44.4)	10 (55.6)	0.0662
>3 cm	31	22 (71.0)	9 (29.0)	
T stage				
T1-3	7	2 (28.6)	5 (71.4)	0.0555
T4	42	28 (66.7)	14 (33.3)	
N stage				
No	16	5 (31.3)	11 (68.7)	0.0027
Yes	33	25 (75.8)	8 (24.2)	
Differentiation				
High/moderate	37	22 (59.5)	15 (40.5)	0.6561
Poor/undifferentiated	12	8 (66.7)	4 (33.3)	
Disease stage				
I/II	20	9 (45.0)	11 (55)	0.0529
III/IV	29	21 (72.4)	8 (27.6)	

CA2: Carbonic anhydrase 2; *CCA*: Cholangiocarcinoma.

Animal experiments

Male SD rats weighing between 250-350 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The rats were randomized into two groups (n=10/group), administrated with PBS water (control group), thioacetamide (TAA group, Changzhou Sea Billiton Experiment Instrument Co., Ltd., Jiangsu, China) alone or TAA+Metformin (TAA+Met group, Sangon Biotechnology, Shanghai, China). TAA was supplied through TAA water (300 mg/L) daily; Met (134 mg/kg) was intragastric 5 times/week. Two rats per group were harvested at the following time points: 8 weeks, 12 weeks, and 16 weeks. The remaining rats were harvested at 20 weeks. All animal experiments were approved by the Animal Ethics Committee of Tongji University School of Medicine.

Colony formation assay and RT-PCR assay

CCA cells were seeded in 6-well plates in triplicate at the density of 400 cells per well. After 24 h, cells were treated with Met at doses of 0 mM, 10 mM, 20 mM, and 50 mM. Foruteen days later, colonies were fixed with methanol/acetone (1:1), then stained with crystal violet and counted. Total RNA was extracted from tissues with TRIzol. The retroviral reverse transcriptase kit (Takara, Tokyo, Japan) was used to synthesize cDNA with the reaction conditions of 37°C for 60 min and 95°C for 3 min. CA2 gene primers: sense: 5-GTGACCTGGATTGTGCTCAAGG-3, antisense: 5-GTTGTCCACCATCAGTTCTTCGG-3, GAPDH gene primers: sense: 5-GAAGGTGAAGGTCGGAGTC-3, antisense: 5-GAAGATGGTGATGGGATTTC-3. Real-time PCR was performed by the 7500 realtime quantitative PCR instrument (Applied Biosystems, CA, United States) with the following conditions: 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s for 40 cycles. Data were normalized using the GAPDH gene and expressed as $2^{-\Delta Ct}$. Each experiment was repeated three times.

Cell treatment and western blotting

CCA cells were seeded in a 6-well plate at a density of 1.2×10^6 cells/well. After growing to 80% of confluence, cells were treated either with Met (0, 10, 20, and 50 mM) for 24 h, or 50 mM Met for 0 h, 48 h, and 72 h. Total proteins from CCA cells were extracted in RIPA buffer. BCA protein assay kit (Takara Bio Inc., Otsu, Shiga, Japan) was used to measure the protein concentrations. Cell lysates were electrophoresed by SDS-PAGE, and the protein was then transferred onto polyvinylidene difluoride membranes (EMD Millipore Corp., Kenilworth, NJ, USA). The membranes were blocked by 5% nonfat milk for 1 h at RT, followed by incubation with primary antibody anti-CA2 (1:1000, Cat. PB1045, Boster Biological Technology, Wuhan, China) overnight at 4°C. After 3 washes with PBST for 5 minutes each, membranes were probed with HRP conjugated secondary antibody (1:2000, SC-2004, Santa Cruz Biotechnology, Dallas, TX, USA), then hybridization bands were visualized in the Odyssey Infrared Imaging System (Li-COR, USA). β-actin (Santa Cruz Biotechnology, USA) served as the internal control. Each experiment was repeated 3 times.

Immunofluorescence assay

CCA cells were seeded and incubated on chamber slides overnight, then treated with Met at doses of 0 mM and 50 mM. At 48 h, cells were fixed with freshly-made 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 in PBS for 10 min, blocked by 3% BSA for 30 minutes, and hybridized with primary antibody anti-CA2 overnight at 4°C. After 3 washes, cells were probed with appropriate secondary antibodies and counterstained with DAPI. Images were taken under a fluorescence inverted microscope (Olympus IX73). Each experiment was repeated 3 times.

Immunohistochemistry (IHC)

Paraffin-embedded mice liver tissue and human CCA tissue microarray slides were subjected to immunohistochemically (IHC) staining. Briefly, 4 μm paraffin sections were dewaxed in xylene 3 times, followed by a gradient concentration of ethanol hydrate. After antigen retrieval in 0.01 M citric acid buffer solution preparation, sections were blocked endogenous peroxidase by 3% H_2O_2, and probed with CA2 antibody (Cat. PB1045, Boster Biological Technology, Wuhan, China) at 1:100 dilutions overnight at 4°C. Before and after incubation with secondary antibody for 20 minutes at room temperature, sections were washed 3 times in PBS. Colored products were developed by SP immunohistochemical kit and DAB chromogenic reagent kit (Fuzhou Maixin Biotech, Fuzhou, China). After re-dyeing with hematoxylin, dehydrating, and transparent, slides were finally mounted using coverslips. PBS, instead of primary antibody, was used as the negative control. The mean area of immunostaining with tumor cells was calculated in 5 areas of a given sample at a magnification of ×400 by Image Pro Plus software. The intensity of immunostaining was scored as 0 for negative, 1 for weak, 2 for moderate, and 3 for strong. Finally, a weighted score was generated (Area * Intensity) for each case. We defined the score <75 as a low expression and ≥75 as high expression. The score ≥75 was defined as positive expression, while score <75 was defined as negative expression.

Statistical methods

The Chi-squared (χ^2) test was used to analyze categorical data. Survival rates were estimated using the Kaplan-Meier method while survival differences between groups were assessed by log-rank test. Two-tailed Mann-Whitney U test and two-tailed Student's *t*-test were used to determine the significance between different groups. Multiple groups were compared by ANOVA test. SPSS v13.0 statistical software was used for the analyses. $P<0.05$ was defined as statistically significant.

Results

CA2 is highly expressed in tumors compared with that in normal tissues and correlated with poor outcomes in CCA patients

Generally, CA2 is overexpressed in 61.2% (30/49) cases with cholangiocarcinoma, while no CA2 over-expression (score >1) is observed in the normal bile duct epithelium by the IHC assay. The immunoreactivity of CA2 is mainly localized in the cytoplasm of CCA cells without obvious stromal staining (**Figure 1A**). Furthermore, we grouped CCA patients into different sets, based on gender, tumor size, T stage, N stage, differentiation, and disease stage. The results show that over-expression of CA2 is correlated significantly with regional lymph node metastasis (75.8% *vs* 31.3%, $P=0.003$). In addition, although the difference is not statistically significant, there is a trend showing that high CA2 expression is more often observed in larger tumor size ($P=0.063$), invasive tumors ($P=0.055$), and higher cancer stage ($P=0.051$) (**Table 1**).

T stage, regional lymph node metastasis, and positive margin are significant prognostic factors of tumor recurrence. We also evaluated the prognostic value of CA2 in CCA. Patients with CA2-positive tumors had a significantly shorter time of tumor recurrence compared with those CA2 negative tumors (11 months *vs* 41 months, $P<0.001$) (**Figure 1B**). After adjustment for confounding variables in a Cox model, T stage and CA2 expression are identified as the independent prognostic factors (**Table 2**). Similarly, patients with CA2-positive tumors have a significantly shorter overall survival period than CA2 negative patients (13 months *vs* 50 months, $P<0.001$) (**Figure 1C**). When adjusting with a multivariate Cox model, only CA2 expression was found as the independent prognostic factor (**Table 3**).

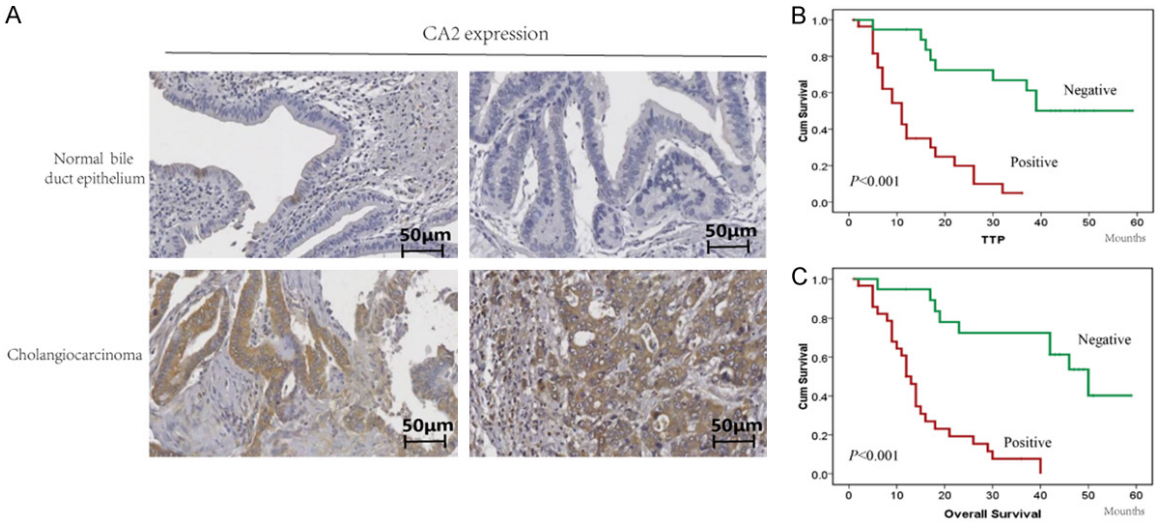

Figure 1. CA2 is over-expressed in human CCA samples and highly correlated with poor outcomes in CCA patients. A. CA2 expression in cancerous and paired noncancerous bile duct epithelium. Magnification: IHC ×200. B, C. Patients with CA2 positive expression had a higher chance to recur (time to progression, TTP) and a decreased survival duration (overall survival) than that with negative expression.

Table 2. CA2 expression and several clinicopathological factors were evaluated their relationships with time to progression in 49 CCA patients by univariate and multivariate analysis

Clinicopathological factor	Case (n)	TTP (mo)	Univariate		Multivariate		*HR (95% CI)*
			X^2	*P*	X^2	*P*	
T stage							
T1-3	7	>52	9.437	0.002	4.780	0.029	0.101 (0.013-0.789)
T4	42	16					
N stage							
No	16	39	4.759	0.029	0.124	0.724	0.833 (0.301-2.303)
Yes	33	15					
Differentiation							
High/moderate	37	26	3.450	0.063	2.151	0.142	0.547 (0.244-1.225)
Poor/undifferentiated	12	11					
Positive margin							
No	23	37	3.983	0.046	0.252	0.616	1.235 (0.541-2.819)
Yes	26	17					
CA2							
Negative	19	41	19.698	0.001	8.737	0.003	0.191 (0.064-0.573)
Positive	30	11					

CA2: Carbonic anhydrase 2; *CCA*: Cholangiocarcinoma; *TTP*: time to tumor progression; *HR*: hazard ratio; *CI*: confidence interval.

CA2 expression is significantly increased in TAA-induced CCA

To verify the change of CA2 expression in different stages of cholangiocarcinoma, we successfully duplicated an oral TAA-induced rat CCA model as previously described [15]. Thioacetamide is a well-established carcinogenic compound known to induce bile duct epithelial carcinomatosis in rats, making it a reliable and established animal model for studying spontaneous CCA. During the 5-month study period of administering TAA, no obvious changes were observed at 8 weeks; rats began to develop

Table 3. CA2 expression and several clinicopathological factors were evaluated their relationships with overall survival in 49 CCA patients by univariate and multivariate analysis

Clinicopathological factor	Case (n)	OS (mo)	Univariate analysis		Multivariate analysis		*HR (95% CI)*
			χ^2	*P*	χ^2	*P*	
T stage							
T1-3	7	50	7.559	0.006	3.707	0.054	0.225 (0.049-1.027)
T4	42	16					
N stage							
No	16	42	5.602	0.018	0.015	0.904	0.940 (0.344-2.566)
Yes	33	14					
Differentiation							
High/moderate	37	23	3.640	0.056	2.851	0.091	0.515 (0.238-1.113)
Poor/un-differentiated	12	16					
Positive margin							
No	23	46	4.096	0.043	0.088	0.767	1.130 (0.504-2.536)
Yes	26	16					
CA2							
Negative	19	50	24.923	<0.001	12.081	0.001	0.136 (0.044-0.419)
Positive	30	13					

CA2: Carbonic anhydrase 2; *CCA*: Cholangiocarcinoma; OS: overall survival; *HR*: hazard ratio; *CI:* confidence interval.

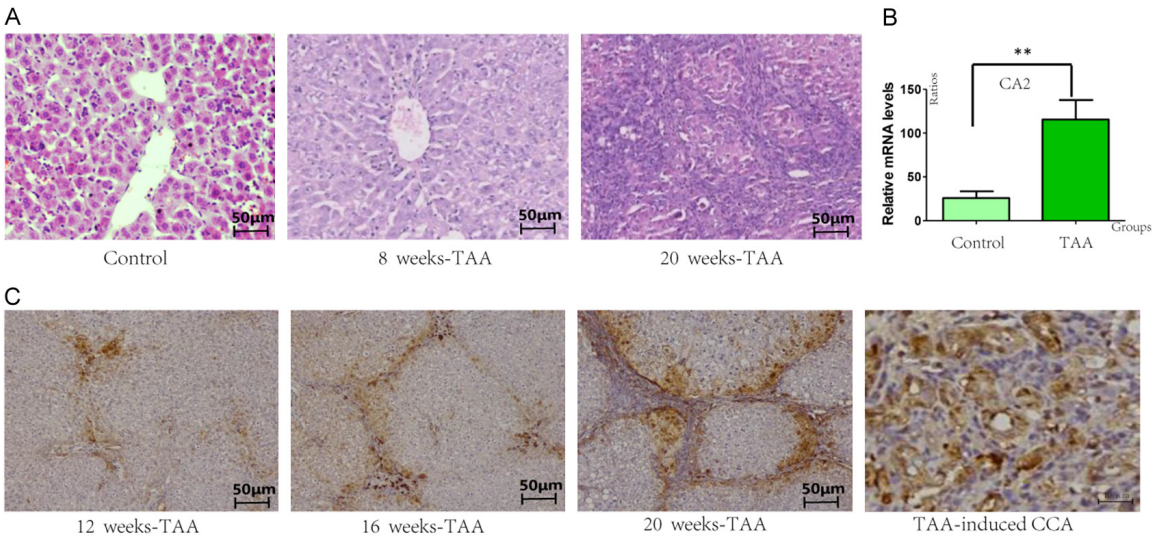

Figure 2. Expression of CA2 is significantly induced in TAA-induced CCA. A. HE staining of normal livers and TAA treated livers with 8 weeks and 20 weeks. B. Relative mRNA expression of CA2 in liver tissues between control group and TAA group (after 20 weeks of treatment). C. IHC staining of CA2 in TAA-induced liver lesions at different time periods. ***P*<0.01 (Unpaired Student's t-test). Magnification: HE ×200; IHC ×200.

multifocal bile ductular proliferation and biliary dysplasia at 12 weeks, and all rats developed invasive intestinal-type CCA at 20 weeks (**Figure 2A**). The RT-PCR revealed that the mRNA level of CA2 was remarkably increased in CCA compared with noncancerous liver (*P*<0.01) after 20 weeks of treatment (**Figure 2B**). IHC analysis proved that the protein level of CA2 in the liver was gradually increased along with the continuous administration of TAA and was highest when developing CCA (**Figure 2C**).

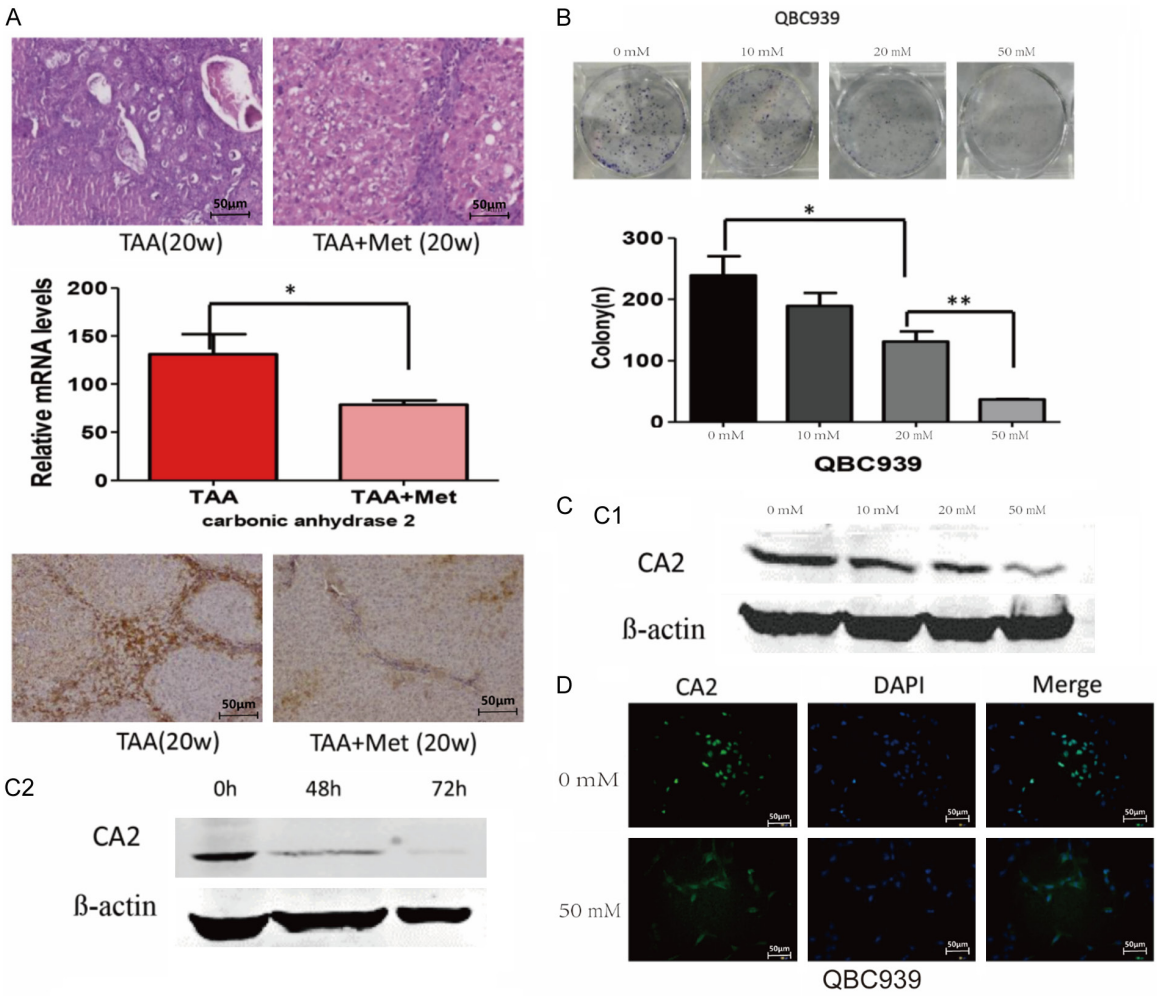

Figure 3. Metformin inhibits CCA development and down-regulates CA2 expression. A. Metformin (Met) alleviates TAA induced liver lesions (Upper) and down-regulates CA2 expression both in mRNA level and protein level (Lower); *P<0.05 (Unpaired Student's t-test). B. The colony formation of QBC939 cells is significantly inhibited by Met with a dose-dependent manner. *P<0.05; **P<0.01 (ANOVA test). C, D. The CA2 expression of CCA cells are significantly decreased by Met with a dose and time-dependent manner (QBC939 cells are tested by Western blotting, QBC939 cells are tested by immunofluorescence).

Metformin reduces the TAA-induced CCA development accompanied by the decreasing CA2 expression of tumors and inhibits the colony formation of human CCA cells

Metformin is an effective agent for inhibiting CCA development. We then administrated TAA-feeding to rats with and without Met to explore the expression of CA2 changes. Long-term use (20 weeks) of Met led to decreased incidence of biliary dysplasia and CCA (**Figure 3A**). RT-PCR and IHC revealed that CA2 expression was significantly reduced in the TAA+Met group compared with TAA alone group both in mRNA and protein levels (**Figure 3A**). Colony formation assay in QBC939 cells showed a strongly reduced number of colonies when cells were treated with a high dose of Met (**Figure 3B**). Western blot assay showed CA2 protein level was significantly decreased by Met treatment in a dose-dependent (**Figure 3C1**) and time-dependent (**Figure 3C2**) manner. This result was further confirmed by an immunofluorescence test in QBC939 cells (**Figure 3D**).

Discussion

Previous studies have indicated that the expression of CA2 is generally low in hepatocellular cancer (HCC) and higher expression levels are usually associated with favorable outcomes for patients. On the other hand, cholangiocarcino-

ma, which is also a primary malignant liver tumor, exhibits an opposite pattern of CA2 expression and functionality compared to HCC. In our study, we reported for the first time that CA2 is overexpressed in human CCA tissues compared to paired normal tissues. Furthermore, through univariate and multivariate analyses, we demonstrated that CA2 serves as an independent risk factor for tumor recurrence and poor outcomes in patients.

To evaluate potential chemopreventive and therapeutic approaches for human CCA, we utilized a highly effective pre-clinical model in rats. We successfully replicated a 20-week invasive intestinal-type CCA rat model by administering TAA. This model allowed us to mimic the progression from normal cholangioles to biliary dysplasia and ultimately invasive CCA [15]. Comparing the TAA-induced CCA group with the control group, we observed a significant increase in the levels of CA2 expression throughout the entire development of CCA.

Acetazolamide, the most widely used inhibitor of CA, has been shown to enhance the anti-CCA effects of bevacizumab [16]. However, clinical treatment with acetazolamide has been associated with side effects. In our study, we investigated Met as a potential non-specific CA2 inhibitor and its effectiveness in preventing CCA. Previous research has demonstrated the strong antineoplastic properties of Met and has explored its mechanisms of action, including its targeting of mTOR/AMPK, Akt, and ERK signaling pathways [17, 18]. In our study, we observed that Met significantly inhibited the development of CCA in a rat model. Additionally, we found that Met treatment dose-dependently and time-dependently suppressed the expression of CA2, which is known to be upregulated in CCA. These findings suggest that Met has the potential to effectively inhibit CCA development and regulate the expression of CA2.

However, it is important to note that our study has some limitations. Firstly, the small sample size is due to the low incidence of CCA. This may limit the generalizability of our findings. Secondly, we did not assess the impact of overexpression or knockdown of CA2 in CCA cell lines on the biological behavior of CCA in vivo and in vitro. This is an area that requires further investigation in future studies. Nonetheless, our findings suggest that CA2 may hold promise as a potential therapeutic target for anti-CCA treatment, and we intend to explore this in future research.

Conclusion

In this study, we made significant discoveries regarding the role of CA2 in both rat and human CCA. Our findings indicate that CA2 expression was notably elevated in both species and was strongly associated with unfavorable outcomes in CCA patients. Furthermore, we demonstrated that the administration of Met effectively decreased CA2 expression, leading to a reduction in the incidence of rat CCA. These findings collectively propose CA2 as a promising biomarker for CCA with potential implications in developing targeted therapeutic interventions. Overall, our study provides valuable insights into the diagnostic and therapeutic potential of CA2 in CCA.

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Disclosure of conflict of interest

None.

Abbreviations

CA2, carbonic anhydrase 2; CCA, cholangiocarcinoma; Cas, carbonic anhydrases; AJCC, American Joint Committee on Cancer stage; HCC, hepatic cellular cancer; DMEM, Dulbecco's Modified Eagle Medium.

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References

[1] Bergquist A and von Seth E. Epidemiology of cholangiocarcinoma. Best Pract Res Clin Gastroenterol 2015; 29: 221-32.

[2] Plentz RR and Malek NP. Clinical presentation, risk factors and staging systems of cholangiocarcinoma. Best Pract Res Clin Gastroenterol 2015; 29: 245-52.

[3] Shaib Y and El-Serag HB. The epidemiology of cholangiocarcinoma. Semin Liver Dis 2004; 24: 115-25.

[4] Nishimori I and Onishi S. Carbonic anhydrase isozymes in the human pancreas. Dig Liver Dis 2001; 33: 68-74.

[5] Kalavska K, Cierna Z, Chovanec M, Takacova M, Svetlovska D, Miskovska V, Obertova J, Palacka P, Rajec J, Sycova-Mila Z, Machalekova K, Kajo K, Spanik S, Mardiak J, Babal P, Pastorekova S and Mego M. Prognostic value of intratumoral carbonic anhydrase IX expression in testicular germ cell tumors. Oncol Lett 2017; 13: 2177-2185.

[6] Kato Y, Ozawa S, Miyamoto C, Maehata Y, Suzuki A, Maeda T and Baba Y. Acidic extracellular microenvironment and cancer. Cancer Cell Int 2013; 13: 89.

[7] Kuo WH, Chiang WL, Yang SF, Yeh KT, Yeh CM, Hsieh YS and Chu SC. The differential expression of cytosolic carbonic anhydrase in human hepatocellular carcinoma. Life Sci 2003; 73: 2211-23.

[8] Viikilä P, Kivelä AJ, Mustonen H, Koskensalo S, Waheed A, Sly WS, Pastorek J, Pastorekova S, Parkkila S and Haglund C. Carbonic anhydrase enzymes II, VII, IX and XII in colorectal carcinomas. World J Gastroenterol 2016; 22: 8168-77.

[9] Hu X, Huang Z, Liao Z, He C and Fang X. Low CA II expression is associated with tumor aggressiveness and poor prognosis in gastric cancer patients. Int J Clin Exp Pathol 2014; 7: 6716-24.

[10] Luo Y, Mok TS, Lin X, Zhang W, Cui Y, Guo J, Chen X, Zhang T and Wang T. SWATH-based proteomics identified carbonic anhydrase 2 as a potential diagnosis biomarker for nasopharyngeal carcinoma. Sci Rep 2017; 7: 41191.

[11] Tachibana H, Gi M, Kato M, Yamano S, Fujioka M, Kakehashi A, Hirayama Y, Koyama Y, Tamada S, Nakatani T and Wanibuchi H. Carbonic anhydrase 2 is a novel invasion-associated factor in urinary bladder cancers. Cancer Sci 2017; 108: 331-337.

[12] Zhang C, Wang H, Chen Z, Zhuang L, Xu L, Ning Z, Zhu Z, Wang P and Meng Z. Carbonic anhydrase 2 inhibits epithelial-mesenchymal transition and metastasis in hepatocellular carcinoma. Carcinogenesis 2018; 39: 562-570.

[13] Maruthur NM, Tseng E, Hutfless S, Wilson LM, Suarez-Cuervo C, Berger Z, Chu Y, Iyoha E, Segal JB and Bolen S. Diabetes medications as monotherapy or metformin-based combination therapy for type 2 diabetes: a systematic review and meta-analysis. Ann Intern Med 2016; 164: 740-51.

[14] Trinh SX, Nguyen HT, Saimuang K, Prachayasittikul V and Chan On W. Metformin inhibits migration and invasion of cholangiocarcinoma cells. Asian Pac J Cancer Prev 2017; 18: 473-477.

[15] Yeh CN, Maitra A, Lee KF, Jan YY and Chen MF. Thioacetamide-induced intestinal-type cholangiocarcinoma in rat: an animal model recapitulating the multi-stage progression of human cholangiocarcinoma. Carcinogenesis 2004; 25: 631-6.

[16] Vaeteewoottacharn K, Kariya R, Dana P, Fujikawa S, Matsuda K, Ohkuma K, Kudo E, Kraiklang R, Wongkham C, Wongkham S and Okada S. Inhibition of carbonic anhydrase potentiates bevacizumab treatment in cholangiocarcinoma. Tumour Biol 2016; 37: 9023-35.

[17] Cauchy F, Mebarki M, Leporq B, Laouirem S, Albuquerque M, Lambert S, Bourgoin P, Soubrane O, Van Beers BE, Faivre S, Bedossa P and Paradis V. Strong antineoplastic effects of metformin in preclinical models of liver carcinogenesis. Clin Sci (Lond) 2017; 131: 27-36.

[18] Yu G, Fang W, Xia T, Chen Y, Gao Y, Jiao X, Huang S, Wang J, Li Z and Xie K. Metformin potentiates rapamycin and cisplatin in gastric cancer in mice. Oncotarget 2015; 6: 12748-62.