Carbonic anhydrase 2 is a promising predictive factor in cholangiocarcinoma and potential mediator in Metformin treatment

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Abstract

Background

Cholangiocarcinoma (CCA) is a rare tumor with an aggressive behavior, early diagnosis is impossible as symptoms emerge only in advanced stages. The discovery of a promising biomarker is urgently needed. Carbonic anhydrase 2 (CA2) was found to be dysregulated in diverse cancers, however limited knowledge of CA2 in CCA development was known.

Materials and methods

CA2 expression was detected both in human and rat thioacetamide (TAA)-induced CCA model by RT-PCR and IHC staining. The relationship of CA2 expression with clinical outcomes was evaluated by univariate and multivariate analyses. CA2 expression changes were also detected both in vivo and in vitro by a potential CA2 inhibitor, Metformin (Met), which was used to inhibit CCA development.

Results

The level of CA2 expression was increased in biliary lesions with continuous administration of TAA. CA2 was overexpressed in CCA compared with normal tissue samples in rat model and human samples, and correlated significantly with disease progression. Patients with high CA2 expression had a poorer outcome than those without CA2 expression. Met alleviated TAA-induced CCA lesions, and significantly decreased CA2 expression. Colony formation assay showed Met inhibition in CCA cell abilities to form colonies and CA2 expression. CA2 expression was downregulated by Met in a dose- and time-dependent manner in vitro.

Conclusions

These findings indicate that CA2 is a promising predictive and prognostic factor, and might serve as a potentially novel therapeutic target for human CCA.

1. Introduction

Cholangiocarcinoma (CCA), one of the most aggressive malignant liver tumors worldwide, originates in the biliary tree following hepatocellular carcinoma [1].

Due to its local invasiveness and high metastasis rate, CCA is considered as a lethal cancer. CCA symptoms typically emerge very late, so early diagnosis of CCA is impossible. Given the fact that its anatomic position results in a low radical excision rate and CCA is resistant to common chemotherapies, CCA patients always have a very poor prognosis [15]. The incidence and mortality rates of CCA have
increased worldwide over 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% [16].

Carbonic anhydrases (CAs), a family of enzymes containing a zinc ion, are categorized as metalloenzymes. So far, 16 isozymes are described in this family, with 15 found in humans [10]. These isozymes efficiently catalyze the reversible hydration process from carbon dioxide to bicarbonate and regulate acid-base homeostasis, therefore play important roles in diverse physiological and biological processes. During recent decades, the roles of CAs have been extensively studied and CAs are deemed to be potential diagnosis markers for various cancers—e.g., CA9 was found to be a biomarker in primary cervix cancer[5], renal cell carcinoma[6], and prostate cancer[7] while CA12 in early breast cancer[8] and cervical cancer[9].

CA2 is the most widely expressed isoform in normal tissues. Studies of CA2 in human cancers are controversial. CA2 was weakly expressed in NSCLC, hepatocellular [7], colorectal [20], and gastric cancer [3], but highly expressed in nasopharyngeal [8] and urinary bladder cancers [17]. To date, knowledge of the CA2 expression pattern in CCA and its effects is limited and needs to be explored. Metformin (Met) is a first-line oral medicine for type 2 diabetes[9], however its anti-cancer properties was uncovered in CCA in inhibiting CCA cells migration and invasion [18], making it as a useful agent for CCA treatment.

In this study, we found that CA2 was remarkably increased in rat and human CCA, and correlated significantly with poor outcomes in CCA patients. Moreover, the level of CA2 could be decreased by administration of Met, which could reduce the incidence of rat intestinal CCA. Therefore, CA2 might serve as a promising biomarker in predicting the prognosis for CCA patients, and be a potential anticancer therapeutic target.

2. Material And Methods

2.1 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 2005 to 2007. 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. Mean age of patients at tumor resection was 55y; 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 informed consents, the study was approved by an ethical review committee of Eastern Hepatobiliary Hospital Institutional Review Board.
Table 1
CA2 expression was highly associated with CCA tumor development

| Parameters        | CA2 positive |
|-------------------|--------------|
|                  | N   | N (%) | P     |
| Median age        | 55 y (31–79 y) |
| Gender            |     |       |       |
| Male              | 35  | 23 (65.7) | 0.242 |
| Female            | 14  | 7 (50.0)  |
| Tumor size        |     |       |       |
| ≤ 3 cm            | 18  | 8 (44.4) | 0.063 |
| > 3 cm            | 31  | 22 (71.0) |
| T stage           |     |       |       |
| T1–3              | 7   | 2 (28.6) | 0.055 |
| T4                | 42  | 28 (66.7) |
| N stage           |     |       |       |
| No                | 16  | 5 (31.3) | 0.003 |
| Yes               | 33  | 25 (75.8) |
| Differentiation   |     |       |       |
| High/moderate     | 37  | 22 (59.5) | 0.656 |
| poor/undifferentiated | 12 | 8 (66.7)  |
| Disease stage     |     |       |       |
| I/II              | 20  | 9 (45.0) | 0.051 |
| III/IV            | 29  | 21 (72.4) |

CA2: Carbonic anhydrase 2; CCA: Cholangiocarcinoma.

2.2 Cell lines and culture conditions

The human Cholangiocarcinoma cell lines (CCA cells) QBC939 and RBE 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% CO₂.
2.3 Animal model

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 either thioacetamide (TAA, Changzhou Sea Billiton Experiment Instrument Co., Ltd., Jiangsu, China) alone or TAA + Metformin (Met, Sangon Biotechnology, Shanghai, China). TAA was supplied through TAA water (300 mg/L) daily; meanwhile intragastric administration with Met (134 mg/kg) was performed in TAA + Met group 5 times/week. Two rats per group were harvested at the following time points: 8 weeks, 12 weeks, and 16 weeks. Remaining rats were harvested at 20 weeks.

All animal experiments were approved by the Animal Ethics Committee of Tongji University School of Medicine.

2.4 Colony formation assay

QBC939 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. 14 days later, colonies were fixed with methanol/acetone (1:1), then stained with crystal violet and counted.

2.5 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% non-fat 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 three 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.

2.6 Immunofluorescence assay

CCA cells (RBE) 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 three washes, cells were probed with appropriate secondary antibody and counterstained with DAPI. Images were taken under a fluorescence inverted microscope (Olympus IX73).

2.7 Immunohistochemistry
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 for three times, followed by gradient concentration of ethanol hydrate. After antigen retrieval in 0.01 M citric acid buffer solution preparation, sections were blocked endogenous peroxidase by 3% \( \text{H}_2\text{O}_2 \), 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 three 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 transparenting, slides were finally mounted using coverslips. PBS, instead of primary antibody, was used as negative control. The mean percentage of tumor cells was calculated in five areas of a given sample at a magnification of ×400 and scored from 0 to 3. 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 for each case, ranging from 0 to 3. We defined the score < 75 as low expression and ≥ 75 as high expression [24].

2.8 Statistical methods

The Chi-squared (\( \chi^2 \)) 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. SPSS v13.0 statistical software was used for the analyses. \( P < 0.05 \) was defined as statistically significant.

3. Results

3.1 CA2 expression was significantly increased in TAA-induced CCA

We successfully duplicated an oral TAA-induced rat CCA model as previously described [21]. During 5-month period of administering TAA, no obvious changes were observed at 8 weeks; rats began to develop multifocal bile ductular proliferation and biliary dysplasia at 12 weeks, and all rats developed invasive intestinal CCA at 20 weeks (Fig. 1A). The difference of mRNA profiles between adjacent intestinal tissues and liver lesions was analyzed by RNA-sequencing (data not shown). RNA-seq revealed that the level of CA2 mRNA was remarkably increased in livers from TAA-induced CCA rats compared with that in paired noncancerous tissues (\( P < 0.01 \)) (Fig. 1B).

IHC analysis revealed that the protein level of CA2 was gradually increased along with the continuously administration of TAA (Fig. 1C). CA2 was extremely highly expressed in rat CCA (Fig. 1D).

3.2 CA2 was expressed highly in CCA compared with that in non-cancerous tissues
Immunoreactivity for CA2 was mainly localized in the cytoplasm of CCA cells without obvious stromal staining (Fig. 2). Similarly to the findings in animal model, CA2 was highly expressed in human CCA (Fig. 2E and F) (score: 4). Generally, CA2 was overexpressed in 61.2% (30/49) cases with cholangiocarcinoma, while no or weak (score: 0–1) CA2 immunostaining was observed in the normal bile duct epithelium.

3.3 CA2 expression is highly associated with CCA tumor development

Furthermore, we grouped CCA patients into different sets, based on gender, tumor size, T stage, N stage, differentiation, and disease stage (Table 1). Overexpression of CA2 was found in CCA tumors compared with paired noncancerous tissue (Fig. 2), and was correlated significantly with regional lymph node metastasis (75.8% vs 31.3%, \( P = 0.003 \)). In addition, there was a trend showing that high CA2 expression was more often observed in larger tumor size (\( P = 0.063 \)), invasive tumor (\( P = 0.055 \)), and higher disease stage (\( P = 0.051 \)).

3.4 CA2 positive is highly correlated with poor outcomes in CCA patients

T stage, regional lymph node metastasis, and positive margin were significant predictive factors of tumor recurrence. We also evaluated the predictive and prognostic value of CA2 in CCA (Table 2). Patients with CA2-positive tumors had a significantly shorter time of tumor recurrence compared with those without CA2 positive tumors (11 months vs 41 months, \( P < 0.001 \)) (Fig. 3A). After adjustment for confounding variables in a Cox model, T stage and Ca2 expression were identified as independent predictive factors (Table 2).
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 |
|----------------------------|----------|----------|------------|--------------|
|                            |          |          | χ²         | P            | χ² | P | HR (95%CI) |
| **T stage**                |          |          |            |              |    |    |            |
| T1-3                       | 7        | >52      | 9.437      | 0.002        | 4.780 | 0.029 | 0.101 (0.013–0.789) |
| T4                         | 41       | 16       |            |              |    |    |            |
| **N stage**                |          |          |            |              |    |    |            |
| No                         | 16       | 39       | 4.759      | 0.029        | 0.124 | 0.724 | 0.833 (0.301–2.303) |
| Yes                        | 32       | 15       |            |              |    |    |            |
| **Differentiation**        |          |          |            |              |    |    |            |
| High/moderate              | 36       | 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                        | 25       | 17       |            |              |    |    |            |
| **CA2**                    |          |          |            |              |    |    |            |
| Negative                   | 19       | 41       | 19.698     | 0.001        | 8.737 | 0.003 | 0.191 (0.064–0.573) |
| Positive                   | 29       | 11       |            |              |    |    |            |

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

Similarly, patients with CA2-positive tumors had a significantly shorter overall survival periods than CA2 negative patients (13 months vs 50 months, $P<0.001$) (Fig. 3B). After adjustment by a multivariate Cox model, CA2 expression was an independent prognostic factor (Table 3).
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 |
|----------------------------|----------|---------|---------------------|----------------------|
|                            |          |         | χ²   | P    | χ²  | P    | HR (95% CI) |
| 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       | 26      | 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.

3.5 Met inhibits proliferation and colony formation of human CCA cells by targeting CA2

Met is an effective agent for inhibiting CCA development, we then administrated TAA feeding rats with and without Met to explore the role of CA2. Long-term use (20 weeks) of Met led to decreased incidence of biliary dysplasia and CCA (Fig. 4A). RNA-seq and IHC revealed that CA2 expression was significant reduced in TAA + Met group compared with TAA alone group (Fig. 4A), indicating that the inhibited effects
of Met in CCA was mediated by CA2. Colony formation assay in QBC939 cells showed strongly reduced number of colonies when cells were treated with high dose of Met (Fig. 4B). Western blot assay showed CA2 protein level was significantly decreased by Met treatment in a dose-dependent (Figure C1) and time-dependent (Figure C2) manner. This result was further confirmed by immunofluorescence staining using CA2 antibody (Fig. 4D).

4. Discussion

Rat TAA-induced CCA model, which closely resembles the pathologic characteristics of its human counterpart, is a powerful pre-clinical platform for discovering and evaluating promising chemopreventive and therapeutic strategies for human CCA. In this study, we duplicated a 20-week invasive intestinal-CCA rat model induced by TAA administration to mimic the progression from normal cholangioles to biliary dysplasia to invasive CCA [21]. By comparing with noncancerous tissues, we disclosed the potentially positive correlation between dysregulated CA2 expression and TAA induced CCA. Similar results found in human samples showed that CA2 was overexpressed in human CCA compared with paired normal tissues. Moreover, by univariate and multivariate analyses we firstly reported that CA2 was an independent risk factor in predicting tumor recurrence and patients’ poor outcomes. Therefore, CA2 is more than a potential diagnostic marker, it is a promising biomarker in monitoring the progression of CCA.

Uncontrolled tumor growth led to insufficient delivery of oxygen and accumulation of acidic products of glycolytic metabolism. The microenvironment stresses hypoxia-inducible factor (HIF) and HIF-mediated pathway, which leads to increased aggressiveness of tumors and poor response to therapy [14]. Certain CAs, such as CA IX, mediated pH regulation in hypoxia condition and served as vital targets of HIF [13]. CA2 has been found to be overexpressed in astrocytomas, oligodendrogliomas, medulloblastomas [12], and bladder cancers [17]. However few studies demonstrated the potential role of CA2 in promoting CCA carcinogenesis and its therapeutic value in treating human solid cancer.

In the present study, CA2 was found to be significantly increased in rat CCA model. In human CCA samples, patients with CA2 positive tumors were highly associated with poor outcomes, suggesting that CA2 plays an important role in CCA development and progression and has the potential to be a therapeutic target.

Acetazolamide, a most common CA inhibitor, is able to inhibit intestinal carcinogenesis [11]. Acetazolamide can also enhance the anti-CCA effects of bevacizumab [19]. Despite its effective inhibition in CCA, acetazolamide was found to have side effects in clinic treatment. Met, which is a potential non-specific CA2 inhibitor, shows effective functions in preventing CCA in another ongoing study.

Previous studies demonstrated the strong antineoplastic effects of Met and explored its underlying mechanisms through targeting mTOR/AMPK, Akt, and ERK signaling pathways [2, 23]. Met treatment led to increased carbonic anhydrase-9 (CA-9), while CA IX is upregulated by hypoxia in several cancer types and correlated with a poor prognosis [5]. In our study, we found Met significantly inhibited the
development of CCA, meanwhile the expression of CA2 (increased in CCA) was dramatically inhibited by Met treatment in a dose-dependent and time-dependent manner, indicating Met inhibited CCA development through downregulating CA2 expression, thus suggesting CA2 is a potential target for CCA patients.

RNA-sequencing analysis of TAA-induced CCA and normal liver tissues helped in disclosing key molecules driving carcinogenesis and invasion of CCA in this model. Actually, a comprehensive profile of proteins including CA2 were involved in the development of intestinal CCA (data not shown). The interaction among these molecules and CA2 will facilitate the understanding of the mechanism of CCA carcinogenesis and highlight the role of CA2 in human cancers. A previous study revealed increased expression of CA2 in invasive rat urinary bladder cancers compared to the non-invasive UC and normal urothelium [17]. CA2 is also a potentially new diagnostic biomarker for nasopharyngeal carcinoma [8]. Our results revealed that CA2 expression correlated with lymph node metastasis and disease progression, thus confirming CA2 as an invasive-associated factor in human solid cancers.

CA enzymes play a key role in maintaining pH homeostasis, dysregulation of CAs leads to unbalanced extracellular pH of tumor tissues [4]. This acidic tumor microenvironment results in more aggressive behavior of tumor cells and facilitates invasion and migration [6, 22]. Although no direct evidence showed that the TAA-induced CCA had an acidic microenvironment, higher expression level of CA2 suggested a possible link between them. Further study is necessary to determine the relationships among CA2, tumor microenvironment, and CCA progression. Undoubtedly, the microenvironment was improved when Met reduced the incidence of CCA, thus leading to decreased expression of CA2.

5. Conclusions

In conclusion, our findings revealed that CA2 was increased during the development and progression of CCA both in rat model and human samples, indicating CA2 as an important candidate oncogene. The fact that CA2 can be targeted by Met elucidated one of the mechanisms of Met inhibited CCA development.

Declarations

Acknowledgment

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Author contributions

All authors conceived and designed the experiments; Chaofu Li, Li Qin, Jian Huang and Yu Zhan performed the experiments; Dongning Huang, Chaofu Li, Guanzhen Yu and Haixin Huang analyzed the
data; and Chaofu Li, Dongning Huang, and Guanzhen Yu wrote the manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

This study was conducted with the approval of the Ethics Committee of Eastern Hepatobiliary Hospital.

**Consent for publication**

Not applicable.

**Competing interests**

Authors have no conflict of interest.

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Tables

Table 1. CA2 expression was highly associated with CCA tumor development
| Parameters             | N (N (%)) | P  |
|-----------------------|-----------|----|
| Median age            | 55 y (31–79 y) |    |
| Gender                |           |    |
| Male                  | 35 (65.7) | 0.242 |
| Female                | 14 (50.0) |    |
| Tumor size            |           |    |
| ≤3 cm                 | 18 (44.4) | 0.063 |
| >3 cm                 | 31 (71.0) |    |
| T stage               |           |    |
| T1–3                  | 7 (28.6)  | 0.055 |
| T4                    | 42 (66.7) |    |
| N stage               |           |    |
| No                    | 16 (31.3) | 0.003 |
| Yes                   | 33 (75.8) |    |
| Differentiation       |           |    |
| High/moderate         | 37 (59.5) | 0.656 |
| poor/undifferentiated | 12 (66.7) |    |
| Disease stage         |           |    |
| I/II                  | 20 (45.0) | 0.051 |
| III/IV                | 29 (72.4) |    |

CA2: Carbonic anhydrase 2; CCA: Cholangiocarcinoma.

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) |
|----------------------------|----------|----------|------------|--------------|-------------|
|                           |          |          | χ² | P    | χ² | P    |          |
| T stage                   |          |          |    |      |    |      |          |
| T1–3                      | 7        | >52      | 9.437 | 0.002 | 4.780 | 0.029 | 0.101 (0.013–0.789) |
| T4                        | 41       | 16       |      |    |     |      |          |
| N stage                   |          |          |    |      |    |      |          |
| No                        | 16       | 39       | 4.759 | 0.029 | 0.124 | 0.724 | 0.833 (0.301–2.303) |
| Yes                       | 32       | 15       |      |    |     |      |          |
| Differentiation           |          |          |    |      |    |      |          |
| High/moderate             | 36       | 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                       | 25       | 17       |      |    |     |      |          |
| CA2                       |          |          |    |      |    |      |          |
| Negative                  | 19       | 41       | 19.698 | 0.001 | 8.737 | 0.003 | 0.191 (0.064–0.573) |
| Positive                  | 29       | 11       |      |    |     |      |          |

CA2: Carbonic anhydrase 2; CCA: Cholangiocarcinoma; TTP: time to tumor progression; HR: hazard ratio; CI: confidence interval.
CA2 expression and several clinicopathological factors were evaluated their onships with overall survival in 49 CCA patients by univariate and multivariate analysis.

| clinicopathological factor | Case (n) | OS (mo) | Univariate analysis | Multivariate analysis |
|----------------------------|----------|---------|---------------------|-----------------------|
|                            |          |         | $\chi^2$   | $P$       | $\chi^2$   | $P$   | HR (95%CI)   |
| age                        | 7        | 50      | 7.559         | 0.006     | 3.707      | 0.054 | 0.225 (0.049–1.027) |
|                            | 42       | 16      |               |           |            |       |               |
| age                        | 16       | 42      | 5.602         | 0.018     | 0.015      | 0.904 | 0.940 (0.344–2.566) |
|                            | 33       | 14      |               |           |            |       |               |
| differentiation/low grade  | 37       | 23      | 3.640         | 0.056     | 2.851      | 0.091 | 0.515 (0.238–1.113) |
| differentiation/moderate   | 12       | 16      |               |           |            |       |               |
| negative margin            | 23       | 26      | 4.096         | 0.043     | 0.088      | 0.767 | 1.130 (0.504–2.536) |
|                            | 26       | 16      |               |           |            |       |               |
| positive                   | 19       | 50      | 24.923        | <0.001    | 12.081     | 0.001 | 0.136 (0.044–0.419) |
| negative                   | 30       | 13      |               |           |            |       |               |

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