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RESEARCH

Overexpressions of CK2β and XIAP are Associated with Poor Prognosis of Patients with Cholangiocarcinoma

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Abstract To investigate the expressions of casein kinase II β(CK2β) and X-Linked inhibitor of apoptosis protein (XIAP) in cholangiocarcinoma (CCA) and evaluated their correlations with major clinicopathologic features and patients' survival. Fifty CCA specimens and 20 normal liver tissues were included in the study. Immunohistochemical staining was used to determine the expression levels of CK2β, XIAP in normal and CCA tissues. The relationships of CK2β and XIAP expressions with clinicopathologic parameters and clinical outcome were evaluated. High immunostaining of CK2β and XIAP were observed in 66 % (33/50) and 68 % (34/50) of CCA tissues, which were significantly higher than that of normal liver tissues 0 % (0/20) and 25 % (5/20). The high expression of CK2β was significantly associated with TNM stage (P=0.036), histological grade (P=0.020) and high serum CEA level(P=0.010), while high expression of XIAP was only associated with TNM stage(P=0.014) and high serum CEA level(P=0.001). By univariant analysis, patients with high expression of CK2β and XIAP demonstrate significantly poorer overall survival (P=0.003 vs P=0.018). Cox regression model showed that positive expression of CK2βis an independent factor of prognosis (P=0.004). The expressions of CK2β and XIAP in CCA tissues showed strong correlations with the tumor progression, CK2β may be applied as a potential prognostic marker for CCA.

Keywords Protein kinase CK2 · Casein kinase 2 · X-Linked inhibitor of apoptosis protein · Cholangiocarcinoma · Prognosis

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Introduction

Cholangiocarcinoma (CCA) has been recongnized as the second most common primary malignancy of the liver [1]. Its slow-growing but highly metastatic characteristics lead to the high mortality, and recently, the morbidity and mortality of CCA are on the rise globally [2–4]. Despite improvements in surgical techniques and the advent of more targeted therapeutic agents, the 5-year recurrence rate is still high and the long-term survival of CCA patient remains poor. Therefore, it is urgent to improve on current understanding of the exact molecular mechanisms underlying cholangiocarcinogenesis and identify important prognostic factors and novel molecular targets of CCA, so as to develop better prognostic and predictive assays.

Protein kinase CK2 (previously known as casein kinase II) is a serine/threonine kinase that ubiquitously expressed in eukaryotic cells. It is involved in multiple cell processes such as proliferation, apoptosis and embryonic development. It comprises two catalytic subunits (42 kDa α and 38 kDa α') and two regulatory subunits (28 kDa β). The catalytic α subunits are linked via the regulatory β subunits to form configurations such as α2β2, α'2β2, αα'β2 in different cells. In the β subunit, certain cysteine residues may play a role in anchoring the kinase to nuclear structures. CK2 plays key roles in diverse biological processes by transferring signals to key sites in nuclear matrix and chromatin structures [5]. Thus, the β subunits that form a linkage with the nuclear matrix is a key locus for CK2 signaling in the nucleus [6]. Several growth stimuli can activate CK2 signals so that higher nuclear localization can be observed in tumor cells than that in normal cells [7, 8]. Abnormal expression of CK2 in the nucleus of tumor cell can influence cell apoptotic activity and enhance cell survival [9, 10]. Inhibition of CK2 induced prostate cancer cells apoptosis mainly through tumor necrosis factor–related ligand (TRAIL/Apo2-L)–mediated apoptosis [11]; Interfering CK2 expression using CK2

inhibitior or specific siRNA led to a reduction of pancreatic cancer cell viability through caspase-dependent apoptosis [12]. Wang reported that CK2-mediated suppression of apoptosis impacts on several downstream targets in the apoptotic machinery including Bax, Bcl-2, Bcl-xL, NF-kB, cytochrome c, and caspase [13, 14]. The inhibitors of apoptosis proteins (IAPs) which are over expressed in cancer cells and block caspase were also modulated by CK2 [15].

To date, less is known about the status of CK2 expression in cholangiocarcinoma and its clinical/prognostic relevance. In this study, we sought to investigate the expression of CK2β and its downstream molecule (X-Linked inhibitor of apoptosis protein, XIAP) in CCA by immunohistochemical staining. We then correlated the expression of CK2β and XIAP with the clinicopathological features and prognosis of cholangiocarcinoma.

Materials and Methods

Patients

The research ethics committee of our institution approved the present study. Fifty paraffin-embedded specimens of consecutive 50 CCA patients who underwent surgical treatments in Second Affiliated Hospital, School of Medicine, Zhejiang University from 2004 to 2009 were enrolled in this study. The control specimens were collected from normal liver tissues beside liver lesions in 20 patients with hepatic hemangioma. The histopathologic diagnosis was based on the criteria of World Health Organization [16]. The clinical classification of tumor was assigned according to the seventh edition of TNM staging system published by the Union for International Cancer Control (UICC). The histologic grade of tumor differentiation was determined according to the classification proposed by Edmondson and Steiner [17]. The main clinical and pathologic variables of the patients are shown in Table 1. Follow-up data including overall survival time were available for all of the cases. The median overall survival, which was defined as the time between the date of surgery and the date of either death or last contact, was 12.5 months (range, 4–72 months).

Immunohistochemistry

Formalin-fixed, paraffin embedded tissues were freshly cut (5 um) and mounted onto slides. Sections were then deparaffinized by sequential washing with xylene, 100 % ethanol, 95 % ethanol, 80 % ethanol, and PBS. For antigen retrieval, slides incubating in 0.1 mol/L of sodium citrate in dH_2O (pH 6.0) were heated in a steam cooker for 2.5 min. After washed with PBS, slides were incubated with a rabbit polyclonal antibodies (Santa Cruz Biotechnology Inc Santa Cruz, CA, USA) to total CK2β (1: 300 dilution) for 2 h at room temperature. Then slides were washed for three times with PBS and incubated with a biotinylated anti-rabbit secondary antibody conjugated streptavidin/horseradish peroxidase (60 min; Dako), then followed by diaminobenzidine (Phoenix Biotechnologies) substrate for 5 min, washed with water, and counterstained with hematoxylin (Sigma) for 20 s.

Evaluation of the Immunohistochemical Stainings

The scoring procedure was carried out twice by two independent pathologists without any knowledge of the clinical data. We evaluated the cytoplasmic and the nuclear staining intensity of CK2β separately. CK2β expression was determined semiquantitatively by assessing the percentage of stained tumor cells and the staining intensity. The percentage of positive cells was rated as follows: 0 points: <10 %; 1 points: 10 % to 25 %; 2 points: 26 % to 50 %; 3 points: 50 % to 75 %; 4 points: >75 %. The staining intensity was rated as follows: 0 points: no staining;1 point: weak intensity (equivalent to normal epithelium); 2 points: moderate intensity; 3 points: strong intensity. Points for expression and percentage of positive cells were added (total score of 0–7), and negative (total 0–1), + (total 2–3), ++ (total 4–5), or +++ (total 6–7) was assigned. For all cases, the scores (−) and (+) were defined as low expression of CK2β, (++) and (+++) as high expression [18].

Statistical Analysis

The data were compiled with the software SPSS (version 16.0). Fisher's exact and $\chi 2$ tests were used to assess the associations between CK2β, XIAP expression and clinicopathological parameters. Spearman correlation was used to analysis the correlation between CK2β and XIAP. Univariate survival analysis was performed according to Kaplan-Meier, and differences in survival curves were assessed with the log rank test. Multivariate survival analysis was performed on all of the parameters that were found to be significant on univariate analysis using the Cox regression model. P Value<0.05 was considered as statistically significance.

Results

Expression of CK2β, XIAP in Cholangiocarcinoma and Normal Liver Tissues

By the immunohistochemical stain, high expressions of CK2β were detected in tumor tissues of CCA, mainly localised in the nuclei of tumor cells (Fig. 1d). Negative or weak expressions were observed in normal liver tissue (Fig. 1a), normal intrahepatic bile duct (Fig. 1b) and normal bile duct (Fig. 1c). High expression of XIAP was mainly localised in cytoplasm of

Table 1 CK2β and XIAP expressions in normal live tissue and cholangiocarcinoma and their correlations with clinicopathological parameters

Parameters	CK2β expression		*P*-value	XIAP expression		*P* value
	Low(17)	High(33)		Low(16)	High(34)	
Normal or malignant			<0.001			<0.001
Normal (*n*=20)	20	0		15	5	
CCA (*n*=50)	17	33		16	34	
Age (years)			0.914			0.067
≤60 (*n*=27)	9	18		12	15	
>60 (*n*=23)	8	15		4	19	
Gender			0.765			0.544
Male(*n*=25)	9	16		9	16	
Female(*n*=25)	8	17		7	18	
Jaundice			0.740			0.500
Yes(*n*=31)	10	21		5	14	
No(*n*=19)	7	12		11	20	
TNM stage			0.036			0.014
I+II (*n*=27)	13	14		13	14	
III +IV (*n*=23)	4	19		3	20	
Tumor size (cm)			1.000			0.508
≤5 (*n*=37)	13	24		13	24	
>5 (*n*=13)	4	9		3	10	
Histological grade			0.020			0.161
Grade 1 (*n*=21)	11	10		9	12	
Grade 2+3 (*n*=29)	6	23		7	22	
Resection margin			0.181			1.000
R0(*n*=38)	15	23		12	26	
R1/2(*n*=12)	2	10		4	8	
Serum CEA(ng/ml)			0.010			0.001
≤5(*n*=20)	11	9		12	8	
>5(*n*=30)	6	24		4	26	
AST(IU/L)			0.318			0.222
≤34 (*n*=16)	7	9		7	9	
>34 (*n*=34)	10	24		9	25	
ALT(U/L)			0.890			0.778
≤36 (*n*=17)	6	11		5	12	
>36 (*n*=33)	11	22		11	22	
ALP(U/L)			0.744			0.666
≤94 (*n*=7)	2	5		3	4	
>94 (*n*=43)	15	28		13	30	
Total bilirubin(mg/dl)			0.558			0.427
≤1.3 (*n*=15)	6	9		6	9	
>1.3 (*n*=35)	11	24		10	25	
Albumin(ng/dl)			0.754			0.525
≤3.5 (*n*=22)	8	14		6	16	
>3.5(*n*=28)	9	19		10	18	
Postoperative complications			0.677			0.406
Yes(*n*=7)	3	4		1	6	
No(*n*=43)	14	29		15	28	
Adjuvant therapy			0.650			0.650
Yes (*n*=5)	1	4		2	3	
No (*n*=45)	16	29		14	31	

the tumor cell (Fig. 2d), whereas weak expression was observed in normal liver tissue (Fig. 2a), normal intrahepatic bile duct (Fig. 2b) and normal bile duct (Fig. 2c). The expression levels of CK2β and XIAP in cholangiocarcinoma were significantly

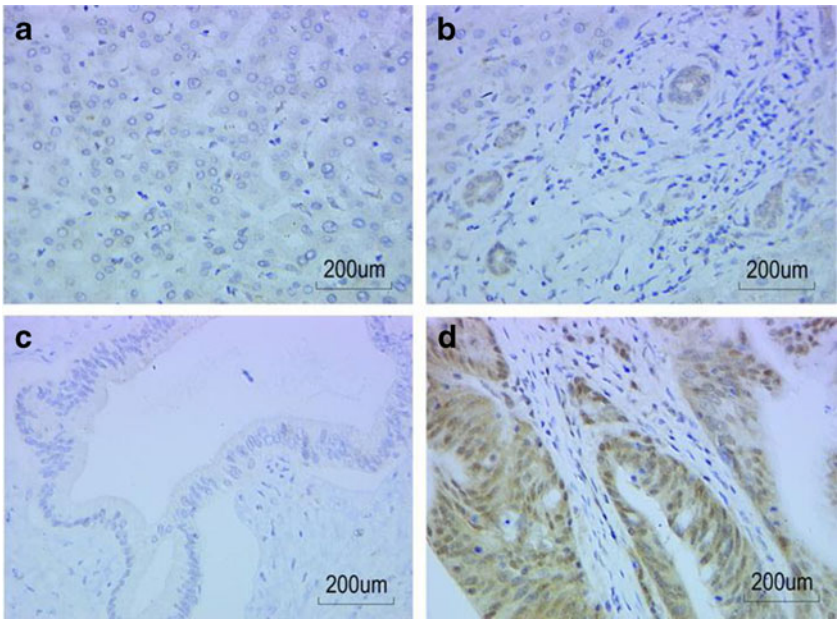

Fig. 1 Expression of CK2β by immunohistochemical stain in the tissue of normal liver (**a**), normal intrahepatic bile duct (**b**), normal bile duct (**c**) and CCA (**d**). No or weak expression was observed in normal liver tissue (**a**), normal intrahepatic bile duct (**b**) and normal bile duct (**c**). High expressions of CK2β were detected in tumor tissues of CCA, mainly localised in the nuclei of tumor cells (**d**)

higher than those in normal liver tissues ($P<0.001$, Table 1), and there was significant correlation between CK2β expression and XIAP expression($r=0.641$, $P<0.001$, Table 2).

Clinicopathological Significance of CK2β, XIAP Expression in Cholangiocarcinoma

To elucidate the significance of CK2β and XIAP in CCA, we correlated their expression with major clinicopathological features. As shown in Table 1, high expression of CK2β tended to occur in CCA with high TNM stage ($P=0.036$), high histological grade ($P=0.020$) and high CEA level($P=0.010$), whereas high expression of XIAP tended to occur in CCA with high TNM stage($P=0.014$) and high CEA level($P=0.001$). No associations were observed between CK2β and XIAP expression with age, gender and tumor size.

CK2β Expression is an Important Predictive Marker for Poor Prognosis of CCA

We next tended to analysis the prognostic effects of CK2β and XIAP expression on CCA patients using Kaplan-Meier method and log-rank test. CCA with high CK2β and XIAP expressions were correlated to shorter overall survival ($P=0.003$, $P=0.018$, Fig. 3). Besides, as shown in Tables 3 and 4, the survival benefits were also found in those with

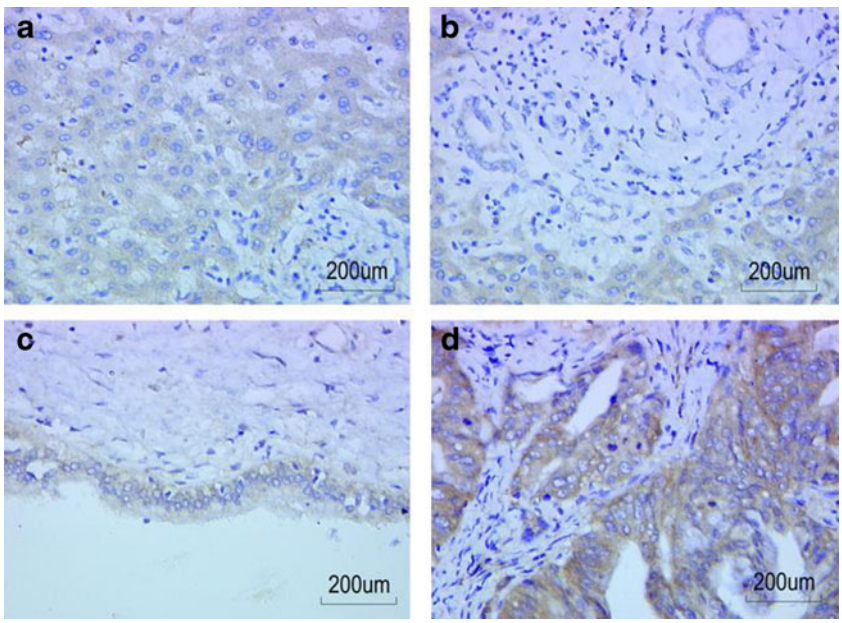

Fig. 2 Expression of XIAP by immunohistochemical stain in the tissue of normal liver (**a**), normal intrahepatic bile duct (**b**), normal bile duct (**c**) and CCA (**d**). High expression of XIAP was mainly localised in cytoplasm of the tumor cell (**d**), whereas weak expression was observed in normal liver tissue (**a**), normal intrahepatic bile duct (**b**) and normal bile duct (**c**)

Table 2 Correlation between CK2β expression and XIAP expression

Intensity of CK2β	Intensity of XIAP expression				Total	
Expression	−	+	++	+++		*P*-value
−	2	2	0	1	5	
+	3	4	3	2	12	
++	0	3	8	2	13	
+++	1	1	0	18	20	
Total	6	10	11	23	50	<0.001

earlier TNM stage (P=0.026), resection margin R_0 (p=0.047), low serum CEA (P=0.006), low bilirubin (P=0.019) and without complication (P=0.005). Multivariate Cox regression analysis enrolling above-mentioned significant parameters revealed that no complication (P<0.001), TNM stage (P=0.004), high CK2β expression (P=0.004) and low bilirubin (P=0.004) were independent prognostic markers for overall survival (Table 3).

Discussion

In this study, we analyzed the expressions of CK2β and XIAP in 50 CCA surgical specimens by immunohistochemistry. We found that high expression of CK2β was associated with high expression of XIAP. Moreover, high expressions of CK2β and XIAP were correlated with unfavorable clinicopathological parameters and poor survival, and CK2β was an independent prognositic factor for CCA. To the best of our knowledge, this is the first report to demonstrate the clinical implication of CK2β and its downstream molecule XIAP expression in CCA.

We detected that the expression of CK2β in CCA tissue was significantly higher than that in normal liver tissue. High CK2β expression was significantly correlated with TNM stage, histological grade and CEA level of CCA, which may suggest that CK2β may contribute to invasive growth and progression of CCA. Interestingly, the survival analysis revealed that CCA patients with high CK2β expression had significantly poorer overall survival when compared with CK2β (−/low) patients. Multivariate analysis demonstrated that among the factors analyzed, CK2β expression was an independent prognostic factor for overall survival in patients with CCA, which was consistent with findings in many other malignant tumors, including gastric cancer and prostate cancer [19, 20].

The close relationships between CK2β expression and clinicopathological features predicted that CK2β may play an important role in carcinogenesis and tumor progression. Currently, a number of studies have reported that CK2 participates in neoplastic transformation and tumor development involving a variety of mechanisms. Of these, apoptosis (or programmed cell death) has gained considerable attention in regard to its potential significance in cancer cell death[10]. There have been some clues that are able to help to explain its mechanisms. As described by some authors, the over expression of CK2 could activate some anti-apoptotic gene, such as Bcl-2, Bcl-xL, Mcl-1, survivin and XIAP, and thus inhibit apoptosis of multiple myeloma cells[21]. Inhibition of CK2 also induced cancer cells apoptosis through tumor necrosis factor-related ligand (TRAIL/Apo2-L)- mediated apoptosis, caspase-dependent apoptosis[11, 12]. Hence, CK2 may function as a potent suppressor of apoptosis.

Based on the mechanism findings of CK2 above, we choose one of its downstream molecule, XIAP and analysis its expression in CCA tissue. We found that there was close correlation between CK2β and XIAP expressions in CCA, which comfirmed previous studies that CK2 modulate cell apoptotic activity via its impact on cellular IAPs[15]. CK2β may interact with XIAP to the contribution of development of CCA. In addition, we also found that high expression of XIAP was significantly correlated with TNM stage and CEA level of CCA, which may suggest that XIAP, together with CK2β, synergistically contribute to invasive growth and

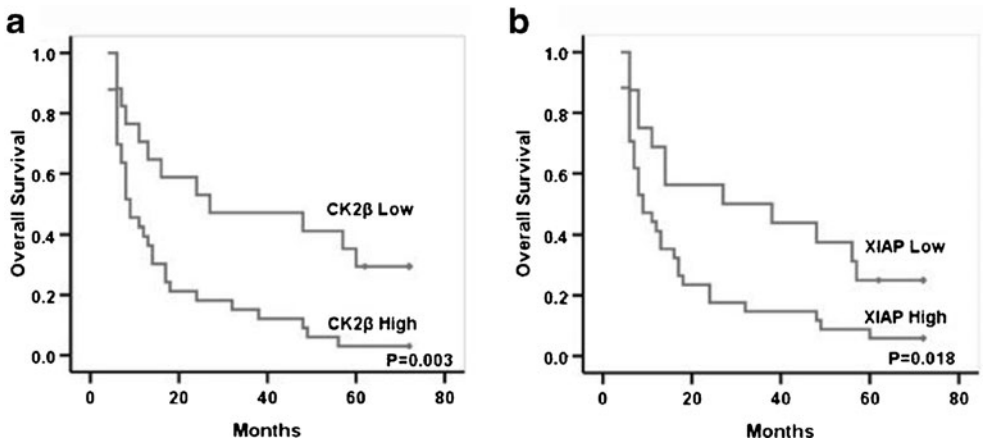

Fig. 3 CCA with high CK2β (**a**) and XIAP (**b**) expression were correlated to shorter overall survival

Table 3 Univariate analysis for overall survival in 50 patients with cholangiocarcinoma

Factors	3-year rate (%)	5-year rate (%)	*P*-value
All (*n*=50)	26.0	12.0	
Age (yr)			0.182
≤60 (*n*=25)	28.0	16.0	
>60 (*n*=25)	20.0	8.0	
Gender			0.526
Male(*n*=25)	24.0	8.0	
Female(*n*=25)	24.0	16.0	
Jaundice			0.153
Yes(*n*=31)	19.4	9.0	
No(*n*=19)	31.6	15.8	
TNM stage			0.026
I+II (*n*=27)	37.0	18.5	
III +IV (*n*=23)	13.0	4.3	
Tumor size (cm)			0.326
≤ 5 (*n*=37)	24.3	10.8	
>5 (*n*=12)	25.0	8.3	
Histological grade of CCA			0.297
Grade 1 (*n*=21)	33.3	14.3	
Grade 2+3 (*n*=29)	20.7	10.3	
Resection margin			0.047
R0 (*n*=38)	31.6	15.8	
R1/2 (*n*=12)	8.3	0.0	
Serum CEA(ng/ml)			0.006
≤5 (*n*=20)	45.0	25.0	
>5 (*n*=30)	13.3	3.3	
AST(IU/L)			0.382
≤34 (*n*=16)	37.5	18.8	
>34 (*n*=34)	20.6	8.8	
ALT(U/L)			0.274
≤36 (*n*=17)	35.3	17.6	
>36 (*n*=33)	21.2	9.1	
ALP(U/L)			0.617
≤94 (*n*=7)	28.6	14.3	
>94 (*n*=43)	23.3	11.6	
Total bilirubin(mg/dl)			0.019
≤1.3 (*n*=15)	33.3	26.7	
>1.3 (*n*=35)	17.1	5.7	
Albumin(ng/dl)			0.936
≤3.5 (*n*=22)	22.7	13.6	
>3.5(*n*=28)	28.6	14.3	
Postoperative complications			0.005
Yes (*n*=7)	30.2	14.0	
No (*n*=43)	0.0	0.0	
Post-op adjuvant therapy			0.498
Yes (*n*=5)	20.0	0.0	
No (*n*=45)	24.4	13.3	
CK2β expression			0.003
Low (*n*=17)	41.2	29.4	
High (*n*=33)	15.2	3.0	
XIAP expression			0.018
Low (*n*=16)	43.8	25.0	
High (*n*=34)	11.8	5.9	

progression of CCA. In survival analysis, CCA patients with high XIAP also had significantly poorer overall survival when compared with patients with low expression of XIAP. However, multivariate analysis revealed that XIAP expression was not an independent prognostic factor for overall survival in patients with CCA, whereas CK2β provided independent prognostic factor. This raises the possibility that CK2β may be a prognostic parameter for CCA which is as or more reliable than the clinicopathological factors currently in use, and suggests the possibility to use CK2β as a target in individualization of adjuvant therapy. It should be noted that several other molecular markers, such as CD151, HSP70, p53 and β-catenin have been identified which show association with prognosis of CCA [22–24]. However, it is not yet clear whether such markers are effective for clinical application as replacements for, or in addition to, the prognostic factors currently in use. As such, further investigation is called for to determine whether combined detection of CK2β together with some of these molecules would be valuable in enhancing prognostic effectiveness.

Table 4 Multivariate analysis for overall survival in 50 patients with cholangiocarcinoma

Factors	Category	*P*-value	HR	95%CI
Stage of CCA	FIGO I+II			
	FIGO III +IV	0.004	2.583	1.346–4.956
Resection margin	R0			
	R1/2	0.289	1.545	0.691–3.452
Serum CEA(ng/ml)	≤5			
	>5	0.791	1.122	0.479–2.630
Bilirubin(total)(mg/dl)	≤1.3			
	>1.3	0.004	2.929	1.418–6.049
With or without complication	Yes			
	No	0.000	5.647	2.221–14.359
CK2β expression	Low			
	High	0.004	2.926	1.415–6.049
XIAP expression	Low			
	High	0.998	1.001	0.416–2.408

In conclusion, we demonstrated, here, that CK2β and XIAP were over expressed in a large proportion of CCA tissues. In addition, high expressions of CK2β and XIAP were correlated with the disease progression and poor clinical outcome of CCA. Furthermore, CK2β was proved to be an independent prognostic factor for overall survival in patients with CCA. Therefore, targeted inhibition of CK2β might be a new idea for therapy of CCA. Certainly, further strong supports from basic investigations are still needed.

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