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## Expression and Significance of COX-2 and Ki-67 in Hepatolithiasis with Bile Duct Carcinoma

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**Background:** As an induced enzyme, COX-2 expression is elevated under stimuli from inflammatory mediator or growth factor product. Ki-67, a cell cycle-related proliferative antigen, reflects the tissue proliferative activity. This study analyzed the expressional profile of cyclooxygenase-2 (COX-2) and Ki-67 in hepatolithiasis and bile duct carcinoma tissues, in an attempt to provide evidence for diagnosis and prognosis prediction of disease.





**Material/Methods:** A cohort of tissue samples from hepatolithiasis with bile duct carcinoma (N=47) patients were analyzed using immunohistochemical (IHC) staining method for the expression of COX-2 and Ki-67, in parallel with hepatolithiasis (N=44) and normal bile duct tissues (N=30). The relationship between expression pattern of COX-2 and Ki-67 and pathological conditions was also analyzed, in addition to the correlation with positive expression in hepatolithiasis samples.

**Results:** The positive expression rate of COX-2 and Ki-67 in bile duct carcinoma was 76.6% and 80.9%, respectively, and was significantly higher than those in the hepatolithiasis group, which was also higher than the control group. Expression of both COX-2 and Ki-67 is closely related to TNM staging, lymph node metastasis, and differentiation stage. They were also correlated with the mortality rate of patients.

**Conclusions:** Both COX-2 and Ki-67 are abundantly expressed in hepatolithiasis and bile duct carcinoma tissues and may play an important role in the disease occurrence, progression, and metastasis.

**MeSH Keywords:** **Adenoma, Bile Duct • Bile Duct Neoplasms • Cyclooxygenase 2 Inhibitors**

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## Background

Although hepatolithiasis is a common disease, the combination of hepatolithiasis and bile duct carcinoma only accounts for 1~3% of total cases [1]. Hepatolithiasis patients typically manifest with jaundice, fever, and upper abdominal pains. But once being complicated with bile duct cancer, patients often present typical symptoms similar to those of hepatolithiasis with cholangitis. Imaging examination, on the other hand, may not clearly visualize the tumor due to the coverage by the lithiasis. Serum tumor markers such as CA199 have inherent high sensitivity but low specificity. Due to such diagnostic difficulty and its insidious onset, hepatolithiasis with bile duct carcinoma patients often receive confirmed diagnosis only at a late stage, thus severely compromising patient prognosis.

It is well known that angiogenesis is closely related with the invasion and metastasis of malignant tumors [2]. Cyclooxygenase (COX) is a critical enzyme converting arachidonic acid into prostaglandin. There are 2 isozymes of COX: COX-1 potentiates the formation of new vessels in tumors and COX-2 has high expression under the stimuli of inflammatory mediator or growth factors [3]. Ki-67, on the other hand, is a proliferative antigen closely related with cell cycle regulation, as supported by its pleiotropic functions in cytokinetics and oncogenesis [4]. Studies have shown that the co-assay of COX-2 and Ki-67 expression had significantly higher sensitivity or specificity in breast cancer invasive ductal carcinoma when compared to either single test [5]. The present study therefore investigated the co-expression of COX-2 and Ki-67 in hepatolithiasis with bile duct carcinoma patients, in an attempt to find the significance of gene expression in disease occurrence and progression.

## Material and Methods

### Patient information

A total of 44 patients diagnosed with hepatolithiasis with bile duct carcinoma in our hospital between April 2012 and January 2014 were recruited in this study, along with 47 hepatolithiasis patients. Inclusion criteria: (1) had not received surgical resection or radio-/chemo-/hormonal treatment before tissue collection; (2) with full information of clinical/TNM staging; (3) with confirmed diagnosis of hepatolithiasis with bile duct carcinoma by post-operative pathological examination. Exclusion criteria: (1) complicated with other malignant tumors; (2) incomplete medical history; (3) accompanied with acute or chronic infection. There were 27 males and 20 females in the cancer group, ages 37~73 years old (average age=62.5±3.9 years). A further TNM staging following the American Joint Committee on Cancer (AJCC) standard [4] identified 6 stage I patients, 11

stage II patients, 12 stage III patients, and 18 stage V patients. Lymph node metastasis were discovered in 13 patients. A further differentiation score based on Gleason system [6] showed 7 low, 21 moderate, and 19 highly differentiated tumors. The invasive condition of tumors was: 11 cases of deep muscular layer invasion; 26 cases of shallow muscular layer invasion, and 10 cases without any invasion. Among 47 hepatolithiasis patients, 26 were males and 21 were females, ages 36~77 years old (average age=61.8±4.5 years). Thirty samples of normal bile duct tissues from hemangioma resection were recruited in the control group. There were 17 males and 13 females, ages 35~79 years old (average age=62.1±4.5 years).

The study protocol was approved by the Research Ethics Committee of our hospital, and all patients gave their informed consent before study commencement.

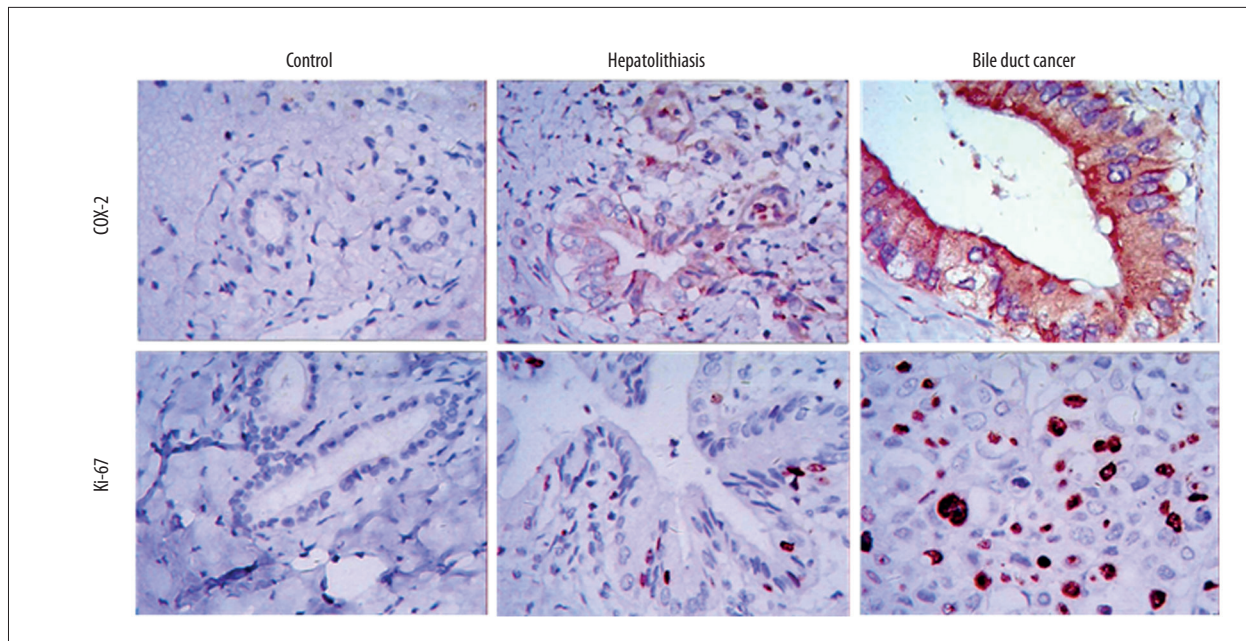
### Histological staining and scoring

All tissue samples were frozen and sectioned for further hematoxylin-eosin staining and immunohistochemical (IHC) staining following standard protocols as stipulated in the IHC kit (GIBCO, US). Mouse anti-human COX-2 and rabbit anti-human Ki-67 antibody were purchased from Baijing Biotech (China). Parallel negative controls were performed using PBS to replace the primary antibody. Five high-magnification fields were randomly selected from each slide.

The staining score was calculated as the product of: (1) percentage of positive cells and (2) staining intensity. In brief, the positive cell% was deduced as 0, 1, 2, and 3 scores when positive cells accounts for less than 5%, between 5% and 25%, between 26% and 50%, and more than 50% of total cells, respectively. The staining intensity score was 0 for no color, 1 for moderate staining, and 2 for intensive staining. The total scores (cell percentage score X staining intensity score) thus range between 0 and 6. Negative (-), weak positive (+), moderate positive (++) , and strong positive (+++) were defined for scores of 0~1, 2~3, 3~4, and 5~6, respectively.

### Statistical analysis

The SPSS 20.0 software package was used to process all collected data, among which analysis of variance (ANOVA) was used for multiple group comparisons. Further between-group analysis was performed by Bonferroni post-hoc test. Enumeration data, on the other hand, was presented as percentage (%) and was tested by chi-square method. Cox regression method was used to analyze the factors of prognosis. A statistical significance was defined when  $p < 0.05$ .



**Figure 1.** COX-2 and Ki-67 protein expression. IHC staining was used to visualize the distribution of COX-2 (upper panels) and Ki-67 (lower panels) in control (left panels), hepatolithiasis (middle panels), and bile duct cancer (right panels) tissues. Brown dots indicate the positive signal. Magnification:  $\times 400$ .

**Table 1.** Positive rate of COX-2 and Ki-67 proteins.

Group	N	COX-2				Positive rate [n (%)]	Ki-67				Positive rate [n (%)]
		-	+	++	+++		-	+	++	+++	
Control	30	29	1	0	0	1 (3.33)	30	0	0	0	0
Hepatolithiasis	47	33	13	1	0	14 (29.79)	35	12	0	0	12 (25.53)
Bile duct cancer	47	11	3	12	21	36 (76.60)	9	10	13	25	38 (80.85)

## Results

### Staining of COX-2 and Ki-67

As shown in Figure 1, COX-2 mainly existed in the cytoplasm in tumor cells, while Ki-67 protein was concentrated in the nucleus of tumor cells.

### Positive rate of COX-2 and Ki-67

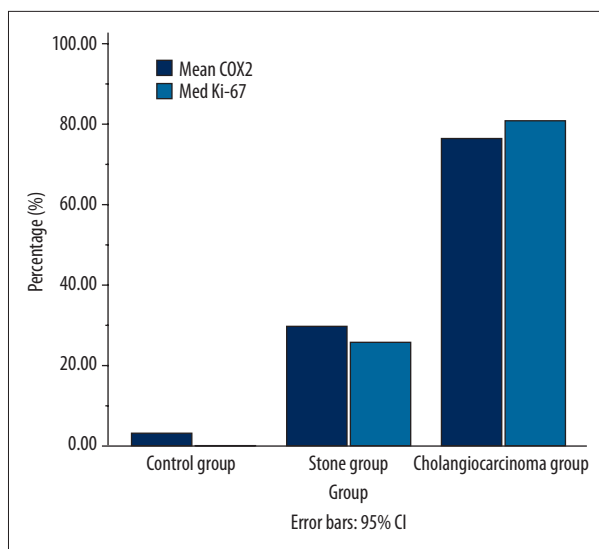
Further analysis showed that the positive rate of COX-2 and Ki-67 in bile duct carcinoma group was 76.60% and 80.85%, respectively, with significant elevation compared to those in control or hepatolithiasis group ( $p < 0.05$ ). Those rates in the hepatolithiasis tissues were also significantly higher than control group ( $p < 0.05$ ), as shown in Table 1 and Figure 2.

### COX-2 and Ki-67 expression and clinical features

As shown in Tables 2 and 3, positive expression rate of both COX-2 and Ki-67 were significantly correlated with various clinical parameters including TNM staging, lymph node metastasis and differentiation grade ( $p < 0.05$ ). Other general parameters such as patient age, sex, pathological type and size of tumors were not related to protein expression ( $p > 0.05$ ).

### Regression analysis of survival rates

Within 12-month follow-up, 43 patients survived within a total of 47 bile duct cancer patients, making the 1-year survival rate 91.59%. We further performed a COX regression survival model, considering patient age, sex, disease period, lymph node metastasis, TNM staging, differentiation grade, tumor



**Figure 2.** COX-2 and Ki-67 positive rates. Averaged positive rates (in%) were plotted across control, hepatolithiasis, and bile duct carcinoma groups.

size, tumor type, and expression of COX-2 and Ki-67. Results (Table 4) identified 4 factors significantly related to the survival rate: Ki-67 positive rate, COX-2 positive rate, tumor differentiation grade, and TNM staging.

## Discussion

The combined treatment plan involving surgical resection and chemo-/radio-therapy has obtained satisfactory results in bile duct carcinoma, especially for those early-stage tumors. Postoperative follow-ups, however, showed that about 17~51% of patients develop distal metastasis, in addition to the 6~21% rate of local recurrence within 3~5 years after surgery [7]. Such recurrent tumors are more difficult to treat and usually lead to death within 1 year. Past studies have identified the history of hepatolithiasis as a risk factor of bile duct carcinoma, mainly due to the persistent mechanical stimuli on the bile duct wall [8]. Currently, there are many biomarkers for evaluating the prognosis of bile duct carcinoma patients, such as growth factor, vesicular endothelial growth factor (VEGF), and transforming growth factor (TGF)-β1 [9].

As shown in this study (Figure 1), Ki-67 mainly locates within the nucleus, consistent with its major functions in mitosis. Previous studies have found an important role of Ki-67 in cell proliferation, as it is both a structural protein and scaffold of chromosomes [10]. Recent studies further identified the relationship between Ki-67 and cell cycle. Because an important feature of tumor cells is the loss of cell cycle control and hyper-proliferation, Ki-67 has been widely used as an index reflecting the proliferative activity and recurrence possibility of malignant tumors, including urinary cancer, breast cancer, gastrointestinal cancer, and lung cancer [11]. For example, Ki-67

**Table 2.** COX-2 expression and clinical indexes.

Clinical parameter	N	Positive cases	Percentage	χ <sup>2</sup> value	P value	
Age (yrs)	<60	31	22	70.97	1.609	0.205
	≥60	16	14	87.50		
Sex	Male	27	21	77.78	0.049	0.824
	Female	20	15	75.00		
Disease history (yr)	<3	15	10	66.67	1.212	0.271
	≥3	32	26	81.25		
Tumor size (cm)	<2	19	13	68.42	1.189	0.275
	≥2	28	23	82.14		
Type	Squamous	4	3	75.00	0.006	0.937
	Adenoma	43	33	76.74		
Differentiation	Low	10	10	100.00	5.987	0.017
	Moderate	21	17	76.19		
	High	16	9	68.75		
Lymph node metastasis	Yes	24	22	91.67	6.214	0.013
	No	23	14	60.87		
TNM staging	I+II	18	10	55.56	7.204	0.007
	III+IV	29	26	89.66		

**Table 3.** Ki-67 expression and clinical indexes.

Clinical parameter		N	Positive cases	Percentage	$\chi^2$ value	P value
Age (yrs)	<60	31	24	77.42	0.693	0.405
	≥60	16	14	87.50		
Sex	Male	27	22	81.48	0.016	0.898
	Female	20	16	80.00		
Disease history (yr)	<3	15	11	73.33	0.804	0.370
	≥3	32	27	84.38		
Tumor size (cm)	<2	19	14	73.68	1.058	0.304
	≥2	28	24	85.71		
Type	Squamous	4	3	75.00	0.097	0.759
	Adenoma	43	35	81.40		
Differentiation	Low	10	10	100.00	4.875	0.027
	Moderate	21	18	85.71		
	High	16	10	62.50		
Lymph node metastasis	Yes	24	23	95.83	7.111	0.008
	No	23	15	65.22		
TNM staging	I+II	18	11	61.11	7.342	0.007
	III+IV	29	27	93.10		

**Table 4.** COX regression survival analysis within 47 cases of hepatolithiasis with bile duct carcinoma.

Parameter	B value	Standard error	Wald $\chi^2$ value	P value	RR value	95%CI
Age	0.02	0.04	0.23	0.65	1.02	0.97~1.08
Sex	0.37	0.31	1.40	0.25	0.81	0.80~2.62
Tumor size	–	–	0.07	0.98	1.00	–
Pathological type	–0.04	0.31	0.02	0.93	0.74	0.55~1.76
Disease period	–0.07	0.24	0.07	0.81	0.99	0.61~1.49
Lymph node metastasis	0.33	0.30	1.19	0.29	0.89	0.78~2.46
Ki-67 positive	0.75	0.24	10.68	0.00	1.54	1.35~3.27
COX-2 positive	1.14	0.26	20.23	0.00	1.59	1.39~3.07
High differentiation	–	–	14.92	0.00	1.88	–
Moderate differentiation	0.73	0.32	5.40	0.03	2.05	1.13~3.75
Low differentiation	0.87	0.30	8.54	0.01	2.37	1.34~4.26
TNM stage III and IV	1.08	0.21	17.47	0.00	2.68	1.76~4.81

level was significantly elevated in primary stomach cancer patients, especially those patients with lymph node metastasis, distal recurrence, and lower-differentiation tumors [12]. The lung cancer also had higher Ki-67 antigen levels compared to normal ones [13].

COX-2 belongs to the immediate early gene family and has trace amounts in placenta, kidney, and brain tissues. The expression of COX-2 is only elevated when cells receive inflammatory stimuli and growth factor-like molecules, with its start codon playing an important role in transcriptional modulation.

Recently, COX-2 has been suggested to be involved in the pathogenesis and progression of malignant tumors, as it has higher expression in most malignant tumor tissues [14], and may participate in the differentiation, proliferation, metastasis, neoangiogenesis, and anti-apoptosis, thereby lowering the cell-to-cell adhesion and facilitating disease progression [15]. Within the occurrence and progression of malignant tumors, angiogenesis plays an important role. High-level expression of COX-2 may inhibit the immune response and escape from immune surveillance, thus facilitating formation of tumor vessels. For example, COX-2 has been shown to be highly expressed in upper urinary epithelioma by cDNA microarray analysis [16,17]. Other studies also reported the correlation between COX-2 expression level and the invasion, metastasis, and prognosis of bladder papillary tumors [18].

In this study, we analyzed the expression pattern of COX-2 and Ki-67 in hepatolithiasis with bile duct carcinoma tissues and its clinical significance. Table 1 shows that only 1 out of 30 normal bile duct tissue had COX-2 expression, significantly lower than that in the hepatolithiasis (29.79%) or bile duct carcinoma group (76.60%). These results suggest the possible involvement of COX-2 in the pathogenesis of hepatolithiasis with bile duct carcinoma, consistent with results of clinical studies [19]. Other studies found that the inflammation of gland and bile duct epithelial and secondary hyperplasia are indicators of bile duct cancer [20], which is preceded by hepatolithiasis, cholangitis, and bile duct epithelial hyperplasia. In this study, we also discovered higher expression level of COX-2 in hepatolithiasis compared to normal bile duct, suggesting that the abnormal COX-2 expression may be an early indicator of bile duct cancer. Further correlation analysis found elevated COX-2 expression in bile duct cancer patients with advanced TNM stage, higher malignancy, and lymph

node metastasis (Table 2). Therefore, COX-2 expression may be closely related to the occurrence and metastasis of bile duct cancer. The assay of COX-2 expression in patients who are susceptible to hepatolithiasis with bile duct cancer may help to make early diagnosis.

No Ki-67 protein has been found in normal bile duct tissues (Table 1, Figure 1), suggesting its lower expression under normal circumstances. However, hepatolithiasis and bile duct cancer tissues had 25.35% and 80.85% Ki-67 expression, respectively, significantly higher than in normal tissues. Our results are consistent with previous reports [21,22], suggesting the elevated Ki-67 expression in hepatolithiasis and bile duct carcinoma. Correlation analysis showed a strong relationship between Ki-67 and TNM stage, lymph node metastasis, and differentiation degree (Table 3). Further analysis showed that bile duct cancer with positive Ki-67 or COX-2 expression had 1.54- and 1.59-fold mortality rates, respectively, compared to those with negative expression. Those 2 proteins, although they had minimal expression in normal cells, can be activated by tumor-related pathways such as growth factor, oncogene, and cytokines. Ki-67 and COX-2 are closely related with short-term prognosis of bile duct patients complicated with hepatolithiasis.

## Conclusions

Our results collectively showed that COX-2 and Ki-67 were highly expressed in hepatolithiasis with bile duct carcinoma tissues, and might promote the pathogenesis, progression, and metastasis of bile duct tumors. These 2 factors are correlated with patient prognosis and should receive further research attention.

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