

## Prognostic Value of Eicosanoid Pathways in Extrahepatic Cholangiocarcinoma

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**Abstract.** *Background: Chronic inflammation of the bile duct is linked to an increased risk for the development of cholangiocarcinoma. Arachidonic acid and linoleic acid oxidation through cyclooxygenase and lipoxygenase – two major pro-inflammatory pathways – have rarely been investigated in extrahepatic cholangiocarcinoma. Materials and Methods: Paraffin-embedded specimens from 51 resected adenocarcinomas of the extrahepatic bile duct were immunostained for cyclooxygenase 2 (COX-2) and 5-lipoxygenase (5-LOX) to evaluate their intracellular distribution and prognostic value. Results: Cholangiocarcinoma had significantly higher levels of 5-LOX and COX-2 expression compared with normal tissue ( $p=0.015$ ). High expression of nucleus-located 5-LOX was significantly associated with intensive staining for COX-2, ( $p=0.023$ ). Median disease-free survival (DFS) in patients with low expression of 5-LOX was significantly better than in patients with high expression of 5-LOX (log rank  $p=0.046$ ). DFS in patients with low COX-2 expression was also significantly better than DFS in patients with high COX-2 expression (log rank  $p=0.0187$ ). Conclusion: The present study demonstrates that 5-LOX and COX-2 protein expression was increased in cholangiocarcinoma suggesting that these two enzymes might be of prognostic value and offer a potential additional adjuvant therapeutic approach to this disease.*

Considerable epidemiological and molecular evidence indicates that chronic diseases that are triggered by persistent inflammatory stimuli are associated with an

increased risk for cancer development (1). Although a number of exogenous and genetic factors probably influence the risk of an individual to develop cholangiocarcinoma and may determine its clinical course, it is clear that chronic inflammation is an important feature that links this cancer to many other types of malignancy. Patients with persistent hepatitis B or C, *Helicobacter pylori* gastritis, inflammatory bowel disease, or chronic pancreatitis, all carry an increased risk of cancer development in the respective chronically inflamed organ. Two known risk factors of chronic inflammation in cholangiocarcinoma are liver fluke infestation and hepatolithiasis (2).

Chronic inflammation is characterized by infiltration by various numbers of inflammatory cells, growth factors and cytokines. An important pathway in chronic inflammation, and as recently described in cancer development, is the increased rate of arachidonic acid and linoleic acid oxidation through both cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) (3). Cyclooxygenase is a key enzyme in the conversion of arachidonic acid to prostaglandins. Two isoforms of COX, namely COX-1 and COX-2 have been identified. COX-1 does play a role in the cytoprotection of gastric mucosa. COX-2 is an inducible intermediate early gene, and its role has been associated with inflammation and carcinogenesis (4). A recently published *in vitro* study showed that the inhibition of proliferation and induction of apoptosis in human cholangiocarcinoma cells by celecoxib, a rather cyclooxygenase-2 specific inhibitor, may involve COX-dependent mechanisms and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) pathways (5). Thus, the COX-2 inhibitors may have chemo preventive potential. Arachidonic acid is also metabolized by 5-lipoxygenase to hydroxyl derivatives of fatty acids and leukotrienes. The production of leukotrienes is associated with acute and chronic inflammation, but the role of LOX in carcinogenesis is thought to be more complex because at least 6 responsible genes have been identified. There seems to be a certain expression pattern that encodes for the course of cancer development. The major “procarcinogenic”

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isoforms appear to be the 5- and 12-LOX (3). 5-LOX is localized in the nucleus and cytosol but on cellular activation the enzyme undergoes  $\text{Ca}^{2+}$  dependent translocation to the nuclear envelope (6). Cellular overexpression has been shown for different types of cancer, such as prostate cancer, colon cancer and leukemia (7-9). In cholangiocarcinoma, LOX and its correlation to cyclooxygenase expression has not been investigated. The aim of the present study was to evaluate the coexpression of COX-2 and 5-LOX in cholangiocarcinoma and define their prognostic value.

## Materials and Methods

**Patient selection.** Fifty-one patients with histologically confirmed extrahepatic cholangiocarcinoma who underwent tumor resection between January 1997 until August 2005 were included into the study. Written informed consent was obtained. The protocol was approved by the Ethics committee at the University of Leipzig. In all patients, the presence of distant metastasis had been excluded by standard staging techniques such as abdominal ultrasound (US), computed tomography (CT scan), magnet resonance imaging (MRI), and positron emission tomography (PET) in recent cases. Resection specimens were assessed according to the tumor node metastasis (TNM) classification system (10). The follow-up was carried out on a regular basis of 3 months in the first year after the operation and then once a year. Physical examination, a CT scan of the thorax and the abdomen and tumor markers were included in follow-up.

**Tumor samples.** The 51 tumor specimens were sectioned according to a standard protocol. Based on initial review of the hematoxylin-eosin stained slides of all surgical specimen sections, two representative paraffin blocks from each case were selected. Invasive margin and viable tumor were present in all selected blocks. Three successive sections from one representative block were used for evaluation. Normal background tissue (n=23) which were from the same patients with cholangiocarcinoma and away from the tumor margin were also collected.

**Immunohistochemistry.** Paraffin-embedded tumor sections were stained for COX-2 (polyclonal antibody; Zymed, Wien, Austria) and 5-LOX (monoclonal antibody; BD Bioscience, San Jose, USA). COX-2 expression was analyzed using the standard avidin-biotin method. Sections (2  $\mu\text{m}$ ) were deparaffinized and pretreated with citrate buffer using a heat-induced epitope retrieval protocol (11). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 minutes at room temperature followed by incubation with COX-2 antibody (dilution 1:100) for 30 minutes. A biotinylated goat anti-mouse immunoglobulin G secondary antibody (Dako Cytomation, Hamburg, Germany) was then applied to each slide for 30 minutes. After washing in TRIS-hydrochloric acid buffer (TBS), the slides were incubated with peroxidase-conjugated-streptavidin complex reagent (Dako) and developed with 3,3'-diaminobenzidine for 5 minutes. The slides were counterstained and dehydrated. Positive controls consisted of lung sections that were stained *via* the same process as the tumor sections. The replacement of the primary antibodies by mouse immunoglobulin served as negative controls.

For 5-LOX staining sections were incubated with hydrogen peroxide to block endogenous peroxidase. After incubation with the primary monoclonal anti-5-LOX antibody overnight at a dilution of 1:1000, incubation with the secondary antibody (AK BA9200, Vector laboratories, goat anti-mouse, Burlingame, California) was carried out for 30 minutes. Further steps were performed with a commercially available detection kit (ABC, Vector Laboratories). Positive controls consisted of liver sections that were stained *via* the same process as the tumor sections. The replacement of the primary antibodies by mouse immunoglobulin served as negative controls.

**Analysis of COX-2 expression.** COX-2 expression was analyzed according to a previously published method (11). In brief, the slides were evaluated under a transmission light microscope by two separate investigators (M.C. and A.T.) in a blind way in terms of the patient's background. For COX-2 assessment, staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), or 3 (strong). Extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), or 4 (76-100%) according to the percentage of the positive staining area in relation to the whole carcinoma area. The product of the intensity and extent score was used as the final staining score (0-12) for COX-2.

**Analysis of 5-LOX expression.** The 5-LOX expression was evaluated by determining the percentage of stained nuclear envelopes of cancer cells at the invasive edge of the tumor, where three areas were selected containing the highest percentage of tumor cells. The assessment was performed on a 400x field (40x objective and 10x ocular, area 0.18  $\text{mm}^2$ ) and at least 200 cells were counted in each area. Patients with a score below or equal to the median were classified as belonging to a low 5-LOX group and above the median as to a high 5-LOX group.

**Statistical analysis.** Statistical calculations were performed using SPSS, version 10.0 (SPSS Inc., Chicago, IL, USA). Differences of distributions were tested by chi square analysis. Data are expressed as mean  $\pm$  standard deviation or median (range). Patient survival rates were estimated using the Kaplan-Meier method and compared by means of the log-rank test. *P*-values less than 0.05 were considered significant.

## Results

**Patient follow-up and survival.** We studied the resection specimens of 51 patients with histologically proven cholangiocarcinomas: 32 men and 19 women, with a median age of 60 years (range 2-78 years). None of the patients had any prior neoadjuvant treatment. The surgical procedures comprised right trisegmentectomy (n=21), right-sided hemihepatectomy (n=15), left hemihepatectomy (n=12) and hilar resection (n=3). In 39 of 51 patients, curative resection (R0) was achieved. Microscopic infiltration of the resection or dissection margins (R1 resection) was found in 10 patients. The remaining 2 patients had gross residual disease (R2 resection). Metastases in regional lymph nodes were observed in tumors of 18 patients (35%) and 42 patients (82%) had well- or moderately differentiated tumors. Postoperatively

UICC IA, IB, IIA, IIB, and III tumor stages were found 3, 13, 17, 15, and 3 patients, respectively. The median follow-up time was 21.3 months (range 1 to 66.3 months). The median disease-free survival time was 22.8 months (range 0.3-66.3 months) in the R0 group. Median time to progression was 12.2 months (4-37.8 months) in the R1 group and 5.2 months (2.8-6.4 months) in the R2 group. The R1 and R2 groups were excluded from further survival analysis.

**Immunohistochemical expression of 5-LOX.** Normal liver tissue showed only slight staining of the cytoplasm, without any nuclear staining. The cholangiocytes in normal liver showed some staining of the cytoplasm and in a few cells also nuclear staining, but the nuclear envelope was not stained. In cancer cells, the cytoplasm and the nucleolus did show a slight, but not regular staining similar to the cholangiocytes, unlike specific nuclear envelope staining (Figure 1a and 1b).

**Immunohistochemical expression of COX-2.** The cholangiocytes in normal liver tissue close to the tumor did not stain for COX-2. Slight staining was observed in hepatocytes. Weak staining was regularly seen in endothelial cells. In cancer cells, COX-2 expression was mainly localized at the invasive edge of the tumor and in the cytoplasm.

**Relationship between 5-LOX and COX-2 expression.** Cholangiocarcinoma had significantly higher levels of 5-LOX and COX-2 expression compared with normal tissue ( $p=0.015$ ). To investigate whether there was overexpression of 5-LOX and COX-2 in the same tumor, the COX-2 score in low expressing 5-LOX and high expressing 5-LOX tumors was evaluated. The mean COX-2 score in the low 5-LOX group was  $2.7 \pm 0.9$  while in the high 5-LOX group it was  $8.0 \pm 2.3$  ( $p=0.023$ ).

**5-LOX expression and patient survival.** 5-Lipoxygenase expression was evaluated by the percentage of stained nuclei. The median percentage was 82.5% (range 0-100%). Patients with a score below or equal to 82.5% were classified as belonging to a low 5-LOX group and those above 82.5% as to a high 5-LOX group. Low 5-LOX expression was seen in 23 patients while high 5-LOX expression was seen in 28 patients. There was no significant difference between the two groups as regards UICC stage distribution. DFS in patients with low expression of 5-LOX was also significantly longer than in patients with high expression of 5-LOX (33.7 months (range 0.3-66.3 months) vs. 19.1 months (range 0.62-63.1 months); log rank  $p=0.046$ ), (Figure 2).

**COX-2 expression and patient survival.** According to the modified Remmler score COX-2 expression of the tumors was evaluated by intensity and extent of staining (11). The

maximum score was 12 and the median score was 4 (range 0-12). Patients with a score below or equal to 4 were classified as belonging to a low COX-2 group and those above 4 as to a high COX-2 group. Low COX-2 expression was seen in 29 patients while high COX-2 expression was seen in 22 patients. There was no significant difference between the two groups as regards UICC stage distribution. DFS in patients without residual tumor disease after resection with low COX-2 expression was significantly longer as compared to patients with high COX-2 expression (31.7 months (range 0.3-66.3 months) vs. 21.1 months (range 0.62-63.1 months); log rank  $p=0.0187$ ), (Figure 3).

## Discussion

Several risk factors have been associated with the development of cholangiocarcinoma. The association between cholangiocarcinoma and chronic biliary-tract inflammation in patients with conditions such as primary sclerosing cholangitis, liver-fluke infestation or hepatolithiasis is well recognized. Bile stasis and recurrent biliary-tract inflammation might predispose individuals with these conditions to cancer (12). In the present study, 5-LOX was overexpressed in 28 patients and was associated with elevated COX-2 expression and reduced survival. Numerous *in vitro* and *in vivo* studies indicate that downstream targets such as  $\text{PGE}_2$ , prostaglandin  $\text{F}_{2\alpha}$  ( $\text{PGF}_{2\alpha}$ ) and leukotriene B4 ( $\text{LTB}_4$ ) are involved in the stimulation of proliferation and inhibition of apoptosis in tumor cells, promotion of angiogenesis, stimulation of invasion and motility and immune modulation (3). 5-LOX is present in the soluble compartment of the cytoplasm and the nucleus before activation. Factors stimulating 5-LOX activity include phospholipids, lipid hydroperoxides and the elevation of intracellular  $\text{Ca}^{2+}$ . After stimulation, cytoplasmatic 5-LOX moves to the nuclear envelope and attaches to the nuclear membrane located 5-lipoxygenase-activating protein (FLAP). Following this docking process, the synthesis of leukotrienes is increased (13). Because of this underlying mechanism, only tumor cells with stained nuclear envelope were counted as positive in the present study. The precise mechanism by which 5-LOX impacts on the prognosis and survival of the patients is not clear. In cholangiocarcinoma, interleukin 6 ( $\text{IL-6}$ ) appears to be a pivotal cytokine for cholangiocarcinogenesis (14).  $\text{IL-6}$  appears to promote the growth of cholangiocarcinoma cells by stimulating a mitogen-activated protein kinase signaling pathway and has been shown to monitor response in photodynamic therapy (15, 16). Studies on nonredox-type 5-LOX inhibitor have shown that the inhibition especially modulates the  $\text{IL-6}$  productions in chronic liver inflammation (17). In cholangiocarcinoma, this possible link has not yet been investigated.

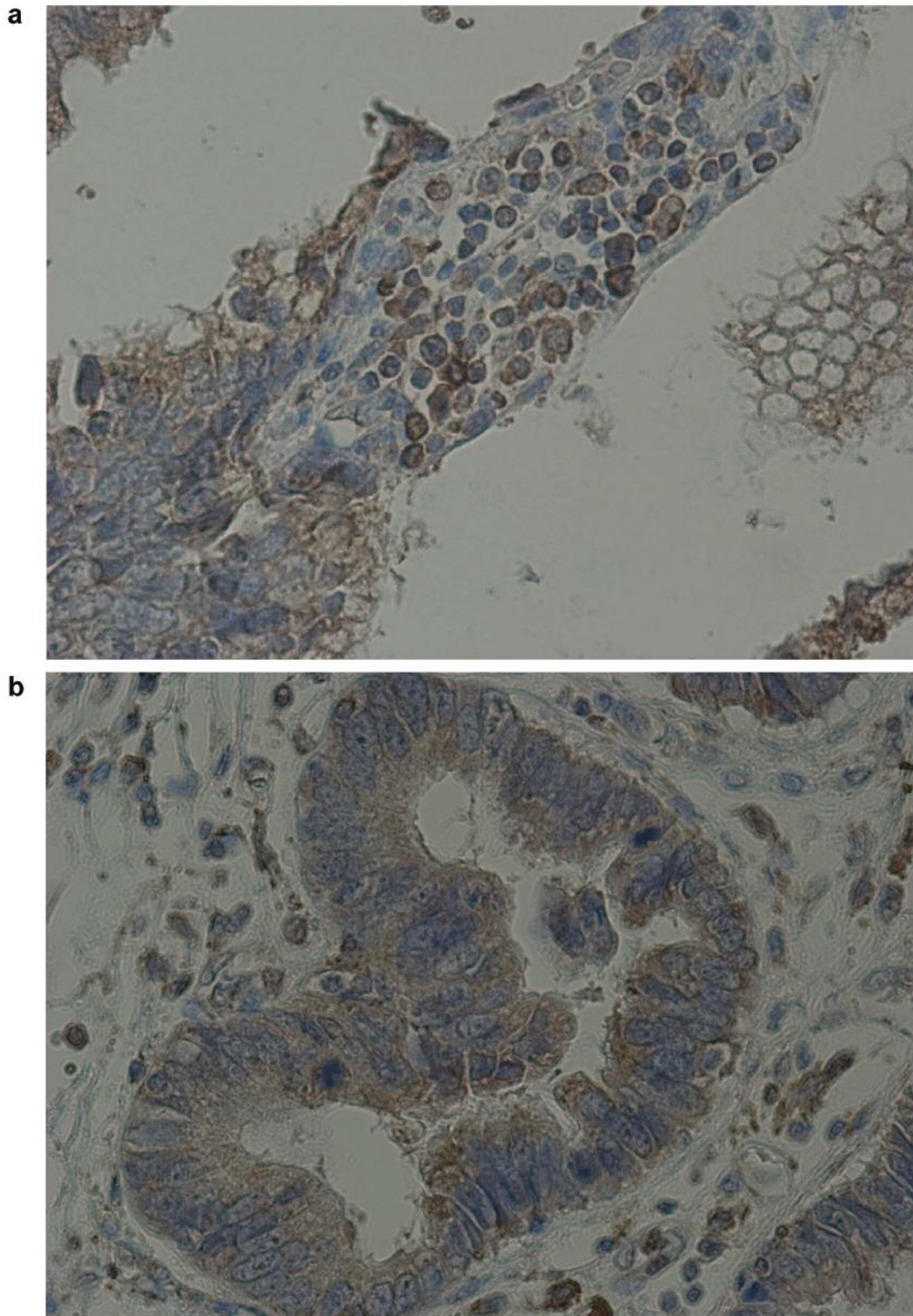


Figure 1. a) Representative example of 5-lipoxygenase immunohistochemistry with intensive staining of the cytoplasm of cancer cells and typical round staining of the nuclear envelope symbolizing activated 5-LOX; highly expressing 5-LOX tumor. b) Representative example of 5-lipoxygenase immunohistochemistry with intensive staining of the cytoplasm of cancer cells, but without typical staining of the nuclear envelope; low expressing 5-LOX tumor.

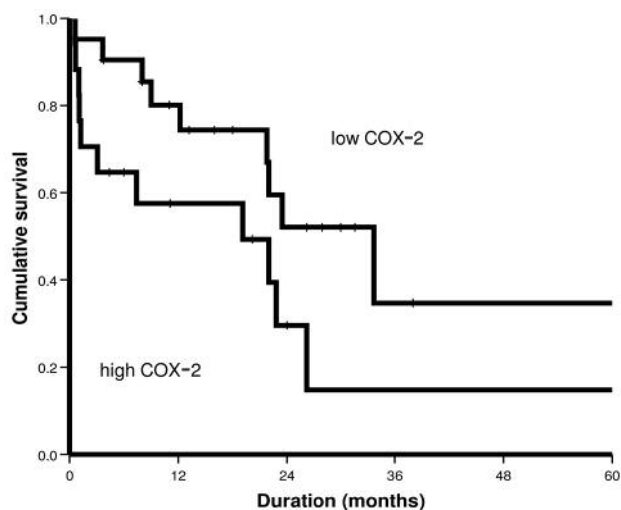


Figure 2. Disease-free survival (DFS) of patients with extrahepatic cholangiocarcinoma is shown as a function of 5-lipoxygenase (5-LOX) expression. High 5-LOX expression: 19.1 months (range 0.62-63.1 months) versus low 5-LOX expression: 31.7 months (range 0.3-66.3 months);  $p=0.046$ .

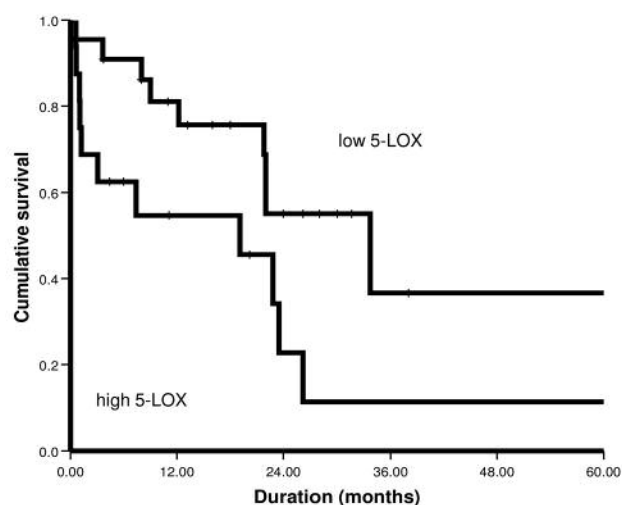


Figure 3. Disease-free survival (DFS) of patients with extrahepatic cholangiocarcinoma is shown as a function of cyclooxygenase-2 (COX-2) expression. High COX-2 expression: 21.1 months (range 0.62-63.1 months) versus low COX-2 expression: 31.7 months (range 0.3-66.3 months);  $p=0.0187$ .

Besides 5-LOX, COX-2 was also overexpressed in extrahepatic cholangiocarcinoma and associated with reduced survival. Recent *in vitro* and chimeric mice studies investigated the role of COX inhibitors on cholangiocarcinoma cell lines (18-20). Our findings are in accordance with these results. The COX inhibitor investigations indicate that selective COX inhibitors preferentially induce apoptosis in cholangiocarcinoma cells through a mechanism involving Akt inactivation, Bax translocation and cytochrome c release. The *in vivo* results suggest that COX inhibitors may have a potential therapeutic or chemopreventive value against cholangiocarcinoma. The results of the various studies provide the rationale for clinical trials with COX-2 inhibitors for cancer prevention especially in the situation with chronic inflammation or cancer treatment. Another preventive or therapeutic approach could be the use of licoferone, a dual anti-inflammatory drug that inhibits both 5-LOX and COX-2 (21).

In summary, to the authors' knowledge, this is the first study that showed a possible association of the two main metabolic routes of arachidonic acid oxidation with prognosis in extrahepatic cholangiocarcinoma. The present data indicate, that COX-2 and 5-LOX immunoreactivity might become a new useful tool to predict tumor prognosis and to select patients for adjuvant, or preventive tumor therapies.

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