High Cyclooxygenase-2 Expression Is Associated with Advanced Stages in Colorectal Cancer

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Abstract. *Background: Despite compelling evidence from the genetic background and clinical studies indicating that cyclooxygenase-2 (COX2) up-regulation is a key step in carcinogenesis of colorectal carcinoma (CRC), controversy regarding its role as a prognostic factor exists. However, all evidence indicates that increased COX2 activity promotes progression of CRC. This study, aimed to evaluate the expression of COX2 in CRC, and correlate it with different patient clinicopathological data, emphasizing on the role of COX2 as a prognostic factor for CRC. Materials and Methods: In the present study, archival samples from 145 patients with stage I, II, III, or IV CRC treated during 1981-1990 at the Turku University Hospital (Finland) were used (as microarray blocks) to analyze COX2 expression by immunohistochemistry (IHC). Results: Higher levels of COX2 expression were associated with higher TNM class ($p<0.06$), and higher Dukes' stage ($p<0.045$). In contrast, there was no significant correlation with age, gender, tumor grade or lymph node status. However, univariate survival analysis of metastases showed borderline association with COX2 expression in that patients with metastases with COX2-positive tumors were alive for shorter periods of time compared with patients whose tumors had no COX2 expression ($p<0.023$, log-rank). Conclusion: COX-2 expression has shown a significant correlation with tumor stage and hence is assumed to be a prognostic factor in our cohort of colorectal cancer patients.*

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Invasive colorectal carcinoma (CRC) is potentially a largely preventable disease if diagnosed early (1-3). Early detection through widely applied screening programs in asymptomatic populations is the most important factor in the recent decline of CRC in developed countries. In the USA, little improvement in the 5-year relative survival rates of patients with colonic cancer has been achieved over the past 30 years, with survival reaching 65% (4). New and more comprehensive screening strategies are thus needed.

However, fundamental advances in our understanding of the biology and genetics of CRC are also being made (5-7). This knowledge is slowly making its way into the clinic and is being employed to better-stratify individual risks of developing CRC, discover better screening methodologies, allow for better prognostication, and improve the ability to predict benefit from new anticancer therapies.

In spite of the tremendous efforts made to achieve personalized prediction in molecular-targeted therapeutic models, tumor node metastasis (TNM) stage and residual disease after initial surgery are still considered the most significant and independent prognostic factors for CRC (8, 9). Furthermore, many of the published data on molecular markers are contradictory; hence, the American Society of Clinical Oncology Tumor Markers Expert Panel does not currently recommend their use in routine practice. The current reality is that no molecular marker, other than the *KRAS* gene in the case of epidermal growth factor receptor (*EGFR*)-targeted therapy for metastatic disease, has been implemented in clinical practice (10, 11).

Cyclooxygenase-2 (COX2) was discovered in 1991 by the Daniel Simmons Laboratory at the Brigham Young University (12). It is an enzyme encoded by the (*PTGS2*) gene and converts arachidonic acid to prostaglandin endoperoxide H2 (PGH2), which is sequentially metabolized to five active, structurally related prostanoids, including PGE_2 and PGD_2, the

targets for non-steroidal anti-inflammatory drugs (NSAIDs) and COX2-specific inhibitors (13).

It seems that the pro-inflammatory PGE_2 plays a predominant role in promoting colorectal tumor growth (14). The genetic deletion of *mPGE* (S-1) suppressed intestinal tumorigenesis in experimental models (15), and many studies showed that PGE_2 promotes colorectal tumor growth by stimulating angiogenesis, cell invasion, cell growth and survival (16). COX2 may also induce resistance to apoptosis, changes in adhesion of the extracellular matrix and enhance metastatic potential (17). Furthermore, PGH2 is converted by PGE_2 synthase into PGE_2, which in turn can stimulate cancer progression; thus inhibiting COX2 may have benefit in the prevention and treatment of these types of cancer (18). This evidence is also supported by the observation of elevated COX2 expression found in nearly 50% of colonic adenomas and 85% of adenocarcinomas (19). It has also been shown that the prevention of COX2 pathway by regular use of NSAIDs, including aspirin for over a 10 to 15 year period reduces the relative risk of developing CRC by 40-50% (20). Regular intake of NSAIDs was found to significantly reduce the risk for CRC, breast, lung, and prostate cancer (21), but to be associated with worse survival among patients with CRC (22). In the present study, we examined the expression of COX2 in 145 CRCs using (IHC), and correlated the results with the established clinicopathological factors of the disease.

Materials and Methods

The present series consisted of tissue samples obtained from 145 patients with stage I-IV CRC who had undergone bowel resection during 1981-1990 at Turku University Hospital (TUH), available for study at the archives of the Department of Pathology. IHC staining was carried out at the Department of Pathology, Benghazi University, Benghazi, Libya. All pertinent clinical and histopathological data of the patients were collected from the patients' case records and are summarized in Table I. All patients were prospectively followed-up until death or when last seen alive at their clinical visit (March 2007), with median follow-up time of 77.0 months (range=2.0-263 months). The study was approved by the TUH Ethics Committee and was conducted in accordance with the endorsement of the National Authority for Medico-legal Affairs.

Table I. *Clinicopathological characteristics of 145 patients with colorectal cancer.*

Characteristic	No. of patients (%)
Gender	
Male	64 (44%)
Female	81 (56%)
Age	
<65 years	72 (49.7%)
≥65 years	73 (50.3%)
Primary tumor status	
T1	3 (2%)
T2	16 (11%)
T3	82 (56.5%)
T4	44 (30.5%)
LN involvement	
No	99 (68.2%)
Yes	46 (31.8%)
Metastasis	
No	123 (84.8%)
Yes	22 (15.2%)
Stage	
I	18 (12.4%)
II	81 (55.8%)
III	24 (16.5%)
IV	22 (15.3%)
Histological grade	
I	14 (9.6%)
II	112 (77.2%)
III	19 (13.2%)
Localization	
Rt colon	50 (34.5%)
Lt colon	47 (32.4%)
Rectum	48 (33.1%)
Recurrence during follow-up	
Yes	53 (36.5%)
No	70 (48%)
Unknown	22 (15.5%)
Status at the end of follow-up	
Alive	49 (33.8%)
Dead as result of disease	60 (41.4%)
Dead from other cause(s)	36 (24.8%)

Tissue microarrays (TMA). Archival paraffin-embedded CRC samples were used to build TMA blocks for IHC staining. Areas of invasive tumor with the lowest degree of differentiation, abundant in cells with the highest number of mitoses, were chosen from the original blocks. Necrotic and autolytic areas and areas containing predominantly stromal tissue were avoided. For tumors producing abundant intra- or extra-cellular mucin, invasive areas with the highest number of epithelial cells were chosen. These representative areas were marked by an experienced pathologist on hematoxylin and eosin (H&E)-stained slides from selected paraffin blocks, and a cylinder of tissue 1 mm in diameter was cut with a TMA instrument (Beecher Instruments, Sun Prairie, WI, USA) into a new paraffin block. This size of tissue section (1-mm wide) was equal to the often used three cores, 0.6-mm wide (23). As the core was larger than usual, sampling differences were less than that of 0.6 mm cores. Serial 4-μm sections were then cut from the TMA paraffin blocks. The sections were mounted on ChemMate TM Capillary Gap plus Slides (Grey) (Dako, Glostrup, Denmark). Normal colorectal mucosa was selected adjacent to, but at least 2 mm from the malignant tissues of the section. If available, another normal sample was obtained from normal colorectal mucosa or the resection margins in the surgical specimens. Consequently, two normal controls were usually available. Lymphatic follicles, hyperplastic and inflamed areas were avoided. To obtain enough mucosa for tissue arrays, tangentially-cut areas were avoided.

COX2 immunostaining. Formalin-fixed, paraffin-embedded primary colorectal tumor tissue was obtained from 145 patients. Sections

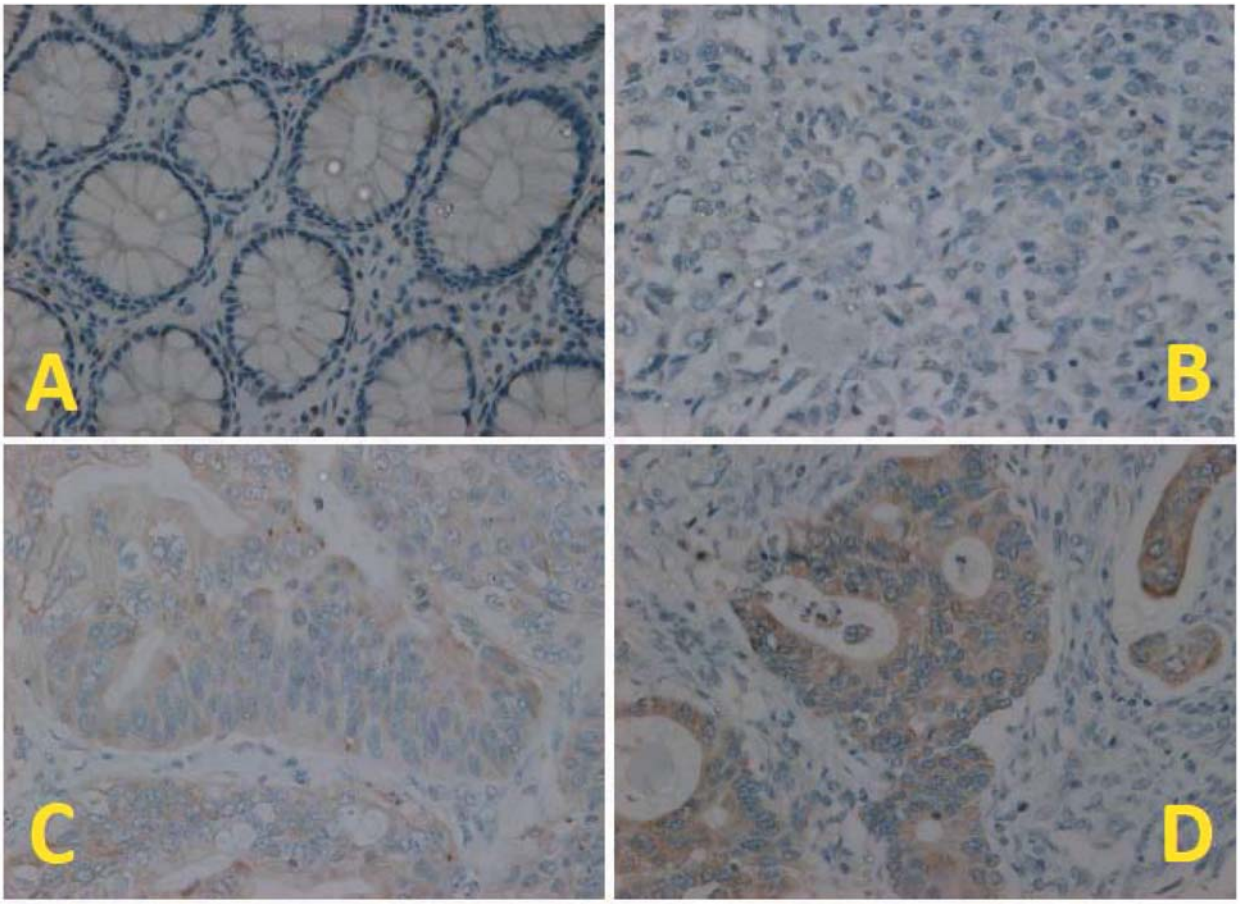

Figure 1. *Normal colonic mucosa showed no expression of COX2 (A). Adenocarcinoma of colon also showed no expression of COX2 (B). Diffuse weak cytoplasmic expression (C) and diffuse moderate cytoplasmic expression (D), magnification (×40).*

were cut serially at 5 μm for routine H&E staining and for IHC analysis. IHC analysis was carried out using an automatic system (Bench-Mark XT; Ventana Medical Systems, Inc., Tucson, AZ, USA). This fully automated processing of bar code-labeled slides included baking of the slides, solvent-free deparaffinization, antigen retrieval in a cell conditioning buffer CC1 (mild: 36 min conditioning, and standard: 60 min conditioning), incubation with the monoclonal antibody to COX2 (clone SP21; Ventana Medical Systems Inc.), for 32 min, at 37˚C. Application of Ultra View™ Universal DAB Inhibitor, Ultra View Universal DAB Chromogen, Ultra View Universal DAB H_2O_2, and Ultra View Universal DAB Copper UltraView Universal HRP Multimer. Counterstaining with hematoxylin (II) took 4 min, and post-counterstaining with bluing reagent also took 4 min. After staining, the sections were dehydrated in ethanol, cleared in xylene, and covered with Mountex and cover slips.

Evaluation of COX_2 staining. The evaluation of staining of all TMAs was performed with a light microscope at a magnification of x40, blinded to information on tumor grade, stage and clinical outcome. The typical expression patterns of COX2 are illustrated in Figure 1.

Three different grading (A, B, and C) systems were applied to assess the pattern of COX2 expression in tumor cells. In system A, the cytoplasmic staining was graded into four categories: 0, no expression (no detectable staining); 1+, weak staining; 2++, moderate staining intensity; and 3+++, strong staining intensity. In system B, cytoplasmic staining was graded into two categories: i, no/weak expression and ii, moderate/strong expression. Finally, the C system categorized COX2 expression as negative 0, or positive +, ++, +++.

In calculating the staining index: cytoplasmic index, the intensity of staining and the fraction of positively stained cells were taken into account, using the following formula:

I=0 x f0+1 x f1 +2 x f2 +3 xf3

Where I is the staining index, f0–f3 are the fractions of the cells showing a defined level of staining intensity (from 0 to 3). Theoretically, the index could vary between 0 and 3 (24).

All three systems A, B, C, were statistically tested, and the negative/positive system (C) seemed to provide the most meaningful correlates of COX2 with the clinically relevant data.

Negative *vs*. positive is the grading system proved to be most useful and was adopted for all statistical calculations.

Table II. *Correlation between COX2 expression and clinicopathological features of colorectal cancer.*

Features	Number of cases (%)	COX2 Expression		*p*-Value
		Negative (0)	Positive (+1,++2,+++3)	
Gender				0.19
Male	64 (44%)	20 (31.7%)	43 (68.3%)	
Female	81 (56%)	18 (22.0%)	64 (78%)	
Age group (years)				0.70
<60	72 (49.7%)	20 (27.8%)	52 (72.2%)	
≥60	73 (50.3%)	18 (24.7%)	55 (75.3%)	
Lymph node involvement				0.31
Yes	46 (31.8%)	15 (32.6%)	31 (67.4%)	
No	99 (68.2%)	23 (23.2%)	76 (76.8%)	
Distant metastasis				0.18
Yes	22 (15.2%)	8 (38.1%)	13 (61.9%)	
No	123 (84.8%)	30 (24.2%)	94 (75.8%)	
Tumor stage				0.04
I	18 (12.4%)	8 (44.4%)	10 (55.6%)	
II	81 (55.8%)	15 (18.5%)	66(81.5%)	
III	24 (16.5%)	6 (25.0%)	18 (75.0%)	
IV	22 (15.2%)	9 (40.9%)	13 (59.1%)	
Tumor grade				0.17
Well	14 (9.6%)	5 (35.7%)	9 (64.3%)	
Moderate	112 (77.2%)	31 (27.7%)	81 (72.3%)	
Poor	19 (13.2%)	2 (10.5%)	17 (89.5%)	
Tumor location				0.68
Colon	97 (66.9%)	24 (24.7%)	73 (75.3%)	
Rectum	48 (33.1%)	14 (29.2%)	34 (70.8%)	
Recurrence				0.52
Yes	53 (36.5%)	14 (26.4%)	39 (73.6%)	
No	70 (48%)	15 (21.4%)	55 (78.6%)	
Unknown	22 (15.5%)	No data		
TNM class				0.06
1	18 (12%)	8 (44.4%)	10 (55.6%)	
2	87 (60%)	18 (20.7%)	69 (79.3%)	
3	2 (1%)	0 (0%)	2 (100%)	
4	14 (9%)	6 (42.9%)	8 (57.1%)	

Statistical analyses. Statistical analyses were performed using the IBM SPSS® Statistics (IBM Company, New York, NY, USA) and STATA (StataCorp, TX, USA) software packages (IBM PASW Statistics for Windows, version 18.0.3 and STATA/SE 11.1). Frequency tables were analyzed using the Chi-squared test, with likelihood ratio or two-sided Fisher's exact test being used to assess the significance of the correlation between the categorical variables. Odds ratio and their 95% confidence intervals (95% CI) were calculated where appropriate, using the exact method. Differences in the means of continuous variables were analyzed using non-parametric tests (Mann-Whitney or Kruskal Wallis) for two and multiple independent samples, respectively. Analysis of variance (ANOVA) was only used for the mean values (and their 95% CI) of each individual stratum. Univariate survival analysis for the outcome measure [disease-specific survival (DSS), disease free-survival (DFS)] was based on the Kaplan-Meier method, with log-rank (Mantel–Cox) comparison test. To assess the value of COX2 as an independent predictor, multivariate survival analysis was performed, using the Cox proportional hazards regression model controlling for confounding by the following variables: age, sex, tumor, localization, T stage, grade, (for DFS) , and recurrence as an additional variable (for DSS). In all tests, values of $p \leq 0.05$ were regarded as being statistically significant.

Results

Patients' characteristics. The 145 cases included in this study were nearly equally grouped into patiemts below and those above 65 years of age. Most of the cases were females (56%). Seventy-seven percent of the cases were grade 2 and 55.8% were tumor stage B. Lymph node metastatic cases were 68%; while 36.5% had recurrence, only 41.5% died from their disease during the first five years. All clinical and histological characteristics of the cases are summarized in Table I.

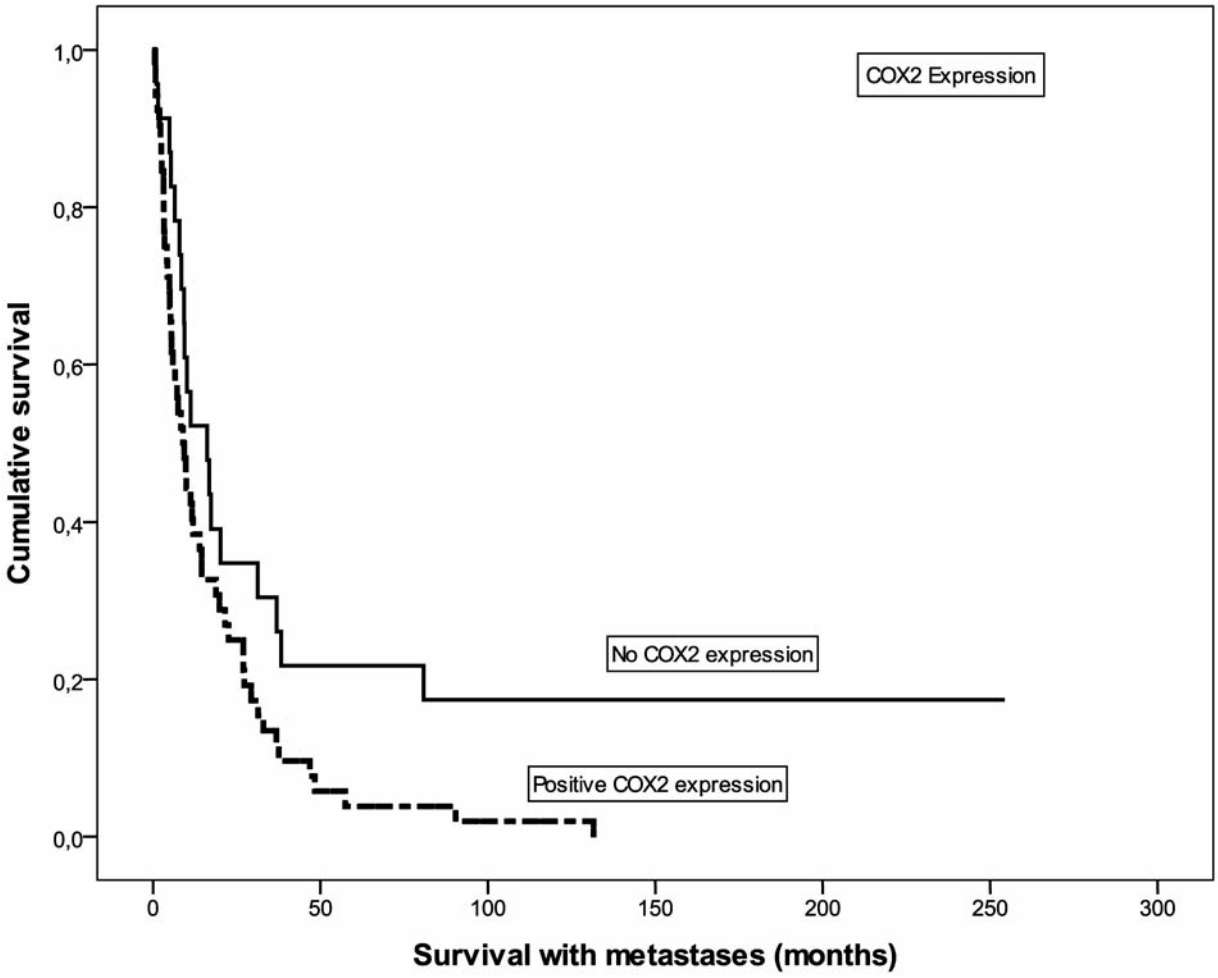

Figure 2. *COX-2 expression (negative/positive) as determinant of survival with metastases in univariate (Kaplan-Meier) analysis ($p<0.023$, log rank).*

Correlation of COX2 expression with clinicopathological features. The associations between COX2 expression and clinicopathological features are presented in Table II. COX2 expression was mainly cytoplasmic in the tumor cases, and about 74% of the cases (n=107) exhibited positive COX2 expression (staining intensity >1) (Figure 1 C and D). While 26% (n=38) were negative (staining intensity=0) (Figure 1B). Normal colonic epithelium exhibited no expression of COX2 (Figure 1A).

Age, gender, tumor location, lymph node status and the presence of distance metastases had no significant relationship with the expression of COX2. However, the tumor stage was significantly associated with COX2 expression, with higher expression being more common in advanced tumors ($p<0.045$) and COX2 expression had a borderline association ($p<0.068$) with TNM classification.

Surprisingly, COX2 expression seems to correlate with less differentiated tumors, as 89.5% of grade 3 tumors exhibited positive COX2 expression, compared to 64.3% grade 1. However, this was statistical significant. The respective data are shown in Table II.

Survival analysis. At 3-years follow-up, 3-year survival of patients with metastases was significantly associated with COX2 expression ($p<0.023$): 15% of the patients with COX2-positive tumors were alive with metastases compared with 30% of patients with metastases whose tumors had no COX2 expression (Figure 2).

Discussion

The aims of the present study were to cast further light on the issues related to prognostication of CRC, and to assess the value of COX2 expression profiles as predictive and prognostic markers. We focused on stage I-IV disease, in which molecular and other markers may help to pinpoint a subgroup of patients who would eventually benefit from the use of adjuvant therapy.

This important therapeutic decision involves a careful weighing of the risks of drug toxicity and complications against the potential curability of the disease (25).

Increased prostanoid activity is a well-recognized characteristic of CRC (26). However, it is controversial whether COX2 expression itself is a prognostic factor for local recurrence and/or survival of patients with CRC (17). Although COX2 has a well-known role in tumorigenesis, studies are conflicting regarding the prognostic significance of COX2 in CRC, with some supporting and others refusing an independent adverse effect of COX2 overexpression (22).

The expression of COX2 is up-regulated in many types of cancers; in additions the product of COX2, PGH2, is converted by PGE_2 synthase into PGE_2, which in turn can stimulate cancer progression. Consequently, inhibiting COX2 may have benefit in the prevention and treatment of these types of cancer (18).

However, compelling evidence from genetic and clinical studies indicates that COX2 up-regulation is a key step in carcinogenesis. Overexpression of COX2 is sufficient to cause tumorigenesis in animal models and inhibition of the COX2 pathway results in reduction in tumor incidence and progression (27).

In our series, 74% of the primary CRCs expressed COX2, whereas results in previous studies have shown that COX2 was expressed in 85%-95% of CRCs (28, 29). Al-Maghrabi *et al*. found that only 56% of primary tumors expressed COX2 (30). This implies its important role in cancer progression. The discrepancies between the different studies may be explained by the variability in the size of the cohorts and their characteristics, use of antibodies and differences in the cut-off levels. Other reasons for discrepancy could also be differences of interpretation of expression profiling. The COX2 immunostaining pattern was predominantly cytoplasmic within the tumors. Normal colonic epithelium adjacent to the tumor showed no staining for COX2, which is in alignment with previous studies (30-32).

Positive expression of COX2 was more common among advanced-stage tumors than in early-stage tumors. This is in agreement with a study by Lim *et al*. (33), who showed that COX2 expression was correlated with the depth of invasion and advanced tumor stage. This observation also substantiates the data of Al-Maghrabi *et al*. (30). Interestingly, Peng *et al*. in their meta-analysis concluded that COX2 expression detected by IHC was associated with poor overall survival in CRC, but not DFS (34); other studies failed to prove a prognostic relevance of COX2 expression in CRC (35). Our study shows a borderline association between survival of patients with metastases and COX2 expression.

COX2 expression was found to have a significant correlation with tumor stage and is assumed to be a prognostic factor in our cohort of patients with CRC. These findings support the view that COX2 plays an important role in advanced CRC.

Conflicts of Interest

The Authors declare that they have no competing interests.

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