Original Article

Impact of cyclooxygenase-2 and prostaglandin-E2 expression on clinical outcome after pulmonary metastasectomy

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Background: Pulmonary metastasectomy (PM) is a standard procedure in the treatment of stage IV colorectal cancer (CRC). In most centers the indication for PM is solely based on clinical factors without taking the tumor biology into account. This results in diverse outcomes ranging from long-term remission to early recurrence. Inflammation is considered a hallmark of cancer development and progression. On the other hand the accessibility of CRC cells to the immune system reflects the grade of tumor aggressiveness. We sought to investigate the impact of cyclooxygenase-2 (COX-2) and prostaglandin-E2 (PGE2) expression in pulmonary metastases on different outcome parameters following PM.

Methods: From 04/2009 to 11/2013 53 patients with complete PM for CRC were included in this single-center study. Tissue samples of resected pulmonary metastases and available corresponding primaries were collected and assessed by immunohistochemistry for COX-2 and PGE2 expression of the tumor tissue and the peritumoral stroma. Results were correlated with clinical outcome parameters.

Results: COX-2 and PGE2 were detected in nearly every pulmonary CRC metastasis. Staining intensities of pulmonary metastases correlated only weakly with intensities found in primary tumors. When dividing metastases in high expressing and low expressing tumors, a trend towards longer recurrence free survival and improved survival was found in tumors with strong COX-2 and PGE2 staining.

Conclusions: In conclusion, this pilot study shows that COX-2 and PGE2 are uniformly overexpressed in pulmonary metastases from CRC. High expression of COX-2 and PGE2 seems to reflect a beneficial tumor biology with late tumor recurrence and prolonged overall survival after PM.

Keywords: Pulmonary metastasectomy (PM); prognosis; inflammation; selection criteria

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Introduction

Nearly 1.4 million people are annually diagnosed with colorectal cancer (CRC), making it the third most common type of cancer worldwide (1). Metastases are considered the major contributor of colorectal cancer-related morbidity and mortality. Although the liver is the primary site of metastasis, pulmonary spreading occurs in approximately 10% of patients (2,3). Despite ongoing efforts to improve patient care and recent advances in chemotherapy the overall 5-year survival rate of patients with stage IV CRC remains low at only around 10–15% (4-6). Surgical resection of oligometastatic lesions represents the only curative option for patients with lung metastases. In

carefully selected patients 5-year survival rates of 40–68% are reported (7). Commonly accepted inclusion criteria for pulmonary metastasectomy (PM) are: (I) controlled primary tumor; (II) complete resection of all metastatic lesions; (III) exclusion of disseminated, extrathoracic disease; and (IV) an adequate performance status (8).

In addition to these "traditional" criteria, different biological behaviours of CRC subtypes were proposed to be important when selecting patients for PM. Therefore, various biological markers, which could distinguish between aggressive and more benign phenotypes of pulmonary spreading are currently in the spotlight of research (9,10).

In 2011 Hanahan and Weinberg updated their model of tumor pathogenesis by adding the concept of continuous inflammation as a main component of malignant transformation and tumor growth (11). This "hallmark of cancer" includes inflammatory mediators such as cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE-2). On the other hand the immune system and its soluble factors are an important part of the body's endogenous antitumor defence. Cells of the immune system recognize tumorous tissue and consequently eliminate tumor cells.

The physiological function of COX-2 is to mediate inflammation by converting arachidonic acid to prostaglandin H2, which is subsequently transformed to PGE2. An overexpression of COX-2 has been previously reported in primary CRC and was associated with tumor progression in various studies (12-14). Nevertheless, the expression pattern of COX-2 has not yet been addressed in the setting of PM.

Prostaglandin-E2 is an essential inflammatory mediator, but also regulates other processes such as vasodilatation or muscle relaxation. PGE2 is overexpressed in various malignancies (15), whereas its prognostic value remains elusive. Furthermore, the expression of PGE2 in pulmonary metastases of CRC and its prognostic impact after PM is currently unknown.

Methods

Study population

This study was designed as a retrospective analysis and is based on a prospectively documented and actively followed patient cohort. Data on the primary tumor, number and distribution of metastases, previous treatments, etc were collected at the time of PM and documented in our institutional PM database. A total of 53 CRC patients with pulmonary metastases undergoing curative metastasectomy from April 2009 to November 2013 were included in this study. In case of patients who had undergone a PM before the inclusion period, the specimen of the first PM was also assessed. For 26 patients paraffin embedded specimens from the primary tumor were obtained and stained. Tumor staging prior to metastasectomy was performed by abdominal and thoracic computed tomography (CT) scan. In case of inconclusive CT scans, positron emission tomography (PET) was used to exclude extrathoracic spreading. All patients were accessed through a muscle-sparing anterolateral or posterior incision. Lungs were bimanually palpated for occult lesions and a lymph nodes sampling was performed. Complete resection (R0) was achieved in all patients.

Lung metastasis free survival (LMFS) was defined as the period of time between the diagnosis of the primary tumor and the diagnosis of metastatic spread to the lungs. Time to recurrence represented the period of time between PM and the first evidence of metastatic recurrence at any site. Time to pulmonary recurrence was defined as the time between PM and the first manifestation of pulmonary recurrence detected by CT scan.

Patients were postoperatively followed-up every three months during the first year and every six months during the following years. This study was approved by the ethics committee of the Medical University of Vienna (EK#: 1097/2014) and was performed according to the Declaration of Helsinki and the Good Scientific Practice guidelines of the Medical University of Vienna.

Immunohistochemistry and scoring

Formalin fixed, paraffin-embedded tissue specimens were analyzed using standard immunohistochemistry protocols. Tissue specimens were cut in 4 µm thick sections and heat mediated antigen retrieval was performed by microwave. For suppressing the endogenous peroxidase activity, the sections were incubated in 0.3% H_2O_2 for 30 min at room temperature. The following primary antibodies were used and incubated for 1 hour at 4 ℃: anti-COX-2 Clone CX-294 (DAKO) 1:50 and PGE2 EP4 Antibody (SantaCruz, USA) 1:25. The VECTASTAIN ABC Kit Mouse IgG and the VECTASTAIN ABC Kit Rabbit IgG (Vector Laboratories, Burlingame, California) were used according to the manufacturer's protocol. The reaction was visualized with DAB substrate (SIGMAFAST 3,3'-Diamino-benzidine tablets) and counterstained with hematoxylin. As negative

controls, the primary antibody was omitted. PGE2 and COX-2 staining failed in five cases, respectively, leaving 48 cases (91%) for further analyses.

Two independent blinded observers assessed the staining intensity as previously described (16). IHC scores were calculated by multiplying the intensity (0-negative, 1-weak, 2-moderate and 3-strong) by the percentage of positively stained tumor or stromal cells (0 to 100), resulting in IHC scores ranging from 0 to 300. In case that the two ratings differed, the stained section was discussed and re-evaluated. For some analysis the continuous IHC score was transformed into a dichotomous variable by calculating the median score for metastases and primary tumors, respectively. Tumor and stroma were defined as high-expressing (COX-2^{high}, PGE2^{high}) when IHC scores were equal or above the median and defined as low-expressing (COX-2^{low}, PGE2^{low}) when scores were below the median.

Statistical analysis

All data collected and used were evaluated using SPSS 21 (SPSS Inc., Chicago, USA). Kruskal-Wallis test was used to compare medians of two groups. Kaplan Meier curves and log rank test were used for comparison of survival functions. Cox-regression was used for multivariate analyses, including factors with a P value of <0.2 in univariate tests. Pearson correlation was applied to determine the relationship between IHC scores of pulmonary metastases and matched primaries. Chi-square test was used to compare binominal variables. If the expected frequency was below 5, Fisher's exact test was applied. All tests were calculated in a two-sided manner. P values of <0.05 were defined as statistically significant.

Results

Patient's characteristics

The study collective comprised 53 patients, 22 female and 31 male patients. The primary tumor site was colon in 59% and rectum in 41% of cases. Most of the patients were already in advanced tumor stages (n=44, 83% T3/T4) and more than half of the patients had histologically confirmed lymph node spreading at the time of their primary operation (60%). Eight of the 53 patients presented with concomitant distant metastases at the time of primary diagnosis (4 liver, 4 lung). 30% of the patients were curatively treated for liver metastases prior to PM. At the time of PM the median age was 65 years (range, 45–83 years). Most of our patients presented with singular pulmonary nodules (n=41; 77%). Resection was complete for all patients with negative resection margins. Only 1 patient had a positive intrathoracic lymph node metastasis according to the final histological report. Median follow up after PM was 28 months (range, 3–125 months).

COX-2 and PGE2 are highly expressed in lung metastases and corresponding primary tumors

Five patients had to be excluded from further analysis due to improper staining of COX-2 and PGE2. COX-2 was evident in 98% of pulmonary metastases (47/48). The calculated median IHC score was 125. Corresponding primaries evidenced detectable intratumoral COX-2 levels in 96.2% available specimens with a median IHC score of 180. Representative stainings of COX-2 in pulmonary metastases are shown in *Figure 1*, stainings of primary CRC specimens in *Figure S1*. The IHC scores between primary and pulmonary metastases did not correlate as evaluated by Pearson correlation (r=0.296, P=0.171).

All analyzed patients showed a positive expression of PGE2 in their pulmonary metastases (48/48) with a median IHC score of 210. PGE2 expression was also evident in all corresponding primary. PGE2 IHC score did not correlate between primary and pulmonary metastases (Pearson r correlation =–0.121, P=0.602). PGE2 stainings of pulmonary metastases and corresponding primaries are presented in *Figure 1* and *Figure S1*.

COX-2 and PGE2 are weakly expressed in peritumoral stroma of lung metastases and primary tumors

In contrast to the intralesional evaluations, stromal COX-2 expression was less common and COX-2 was only evident in 27% of pulmonary metastases (13/48). Thus, the calculated median IHC score was 0 (range, 0–90). Corresponding primaries showed a positive expression of stromal COX-2 in 28% of available specimens. Again, the IHC-levels of primary and pulmonary metastases did not correlate (Pearson r correlation =–0.06, P=0.791).

Eighty-three percent (40/48) of patients evidenced stromal expression of PGE2 in their pulmonary metastases with a median IHC score of 82.5. Stromal PGE2 expression was also evident in 83% corresponding primary, however, IHC scores between primary and pulmonary metastases did

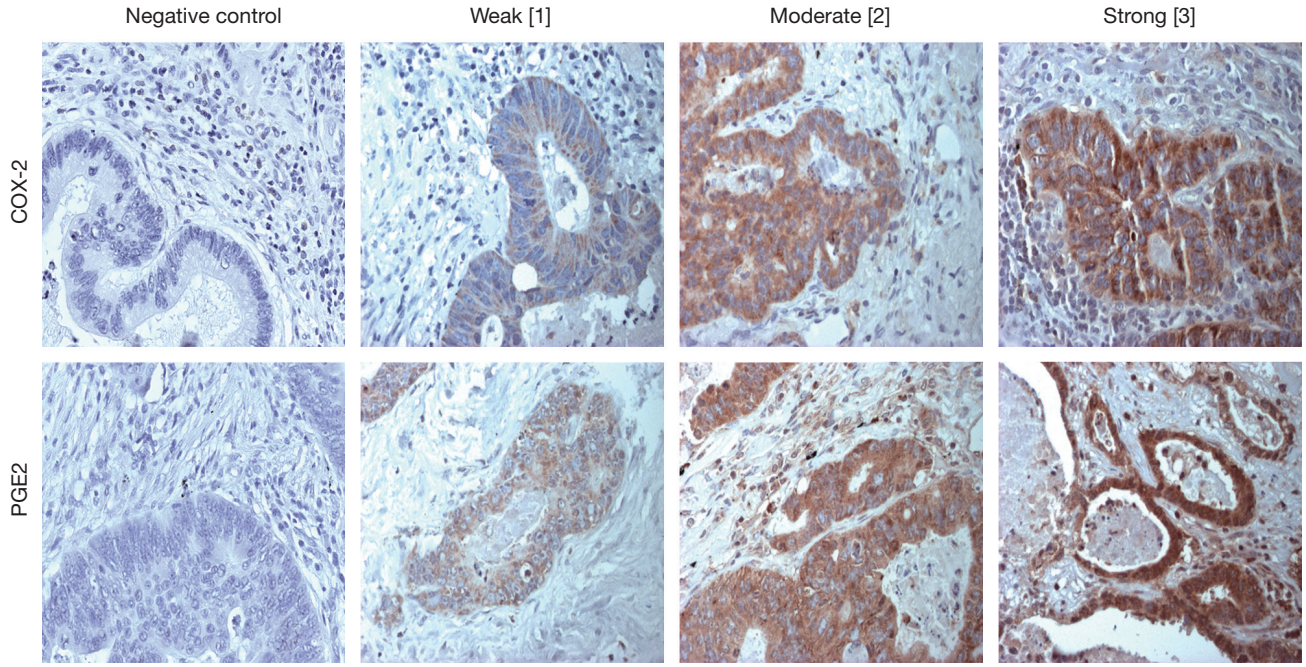

Figure 1 Expression of COX-2 and PGE2 in the tumor area of pulmonary metastases. Representative stainings of pulmonary metastases with different expression status (negative control; weak; moderate; strong) of cyclooxygenase-2 (COX-2, upper line) and prostaglandin-E2 (PGE2, lower line). Magnification 40×, DAB substrate, hematoxylin counterstain.

not correlate (Pearson r correlation =–0.322, P=0.143).

Representative stainings of stromal COX-2 and PGE2 expressions in pulmonary metastases are shown in *Figure 2* and for primary CRC specimens in *Figure S2*.

Distribution of COX-2 and PGE2 expression within clinicopathological characteristics

In order to facilitate the comparison between different COX-2 and PGE2 expression levels, patients with IHC scores equal or above the median were assigned to a high expression-group and those with IHC scores below the median were defined as low expressing. The impact of clinical characteristics on COX-2 and PGE2 expression was evaluated. No correlations between expression of these two inflammatory markers with gender, age, location of the primary tumor (colon *vs.* rectum), T stage, tumor grading, prior liver metastases and number of pulmonary nodules could be found (*Tables 1*,*2*). However, we found that patients who had not received chemotherapy before PM had higher IHC scores of COX-2 compared to patients with a prior chemotherapy (P=0.036).

Impact of COX-2 and PGE2 expression on outcome parameters

The impact of COX-2 and PGE2 expression on clinical outcome after PM was analysed next. Kaplan Meier curves showed similar survival curves for overall survival, but a trend towards prolonged time to pulmonary recurrence and time to any recurrence in the COX high-expressing group (*Figure 3*, *Table 3*). Expression level of PGE2 in pulmonary metastases tissue also had an impact on the time to pulmonary recurrence, the time to any recurrence and the overall survival. Again, Kaplan Meier analyses showed a favourable outcome of PGE2 high expressing tumors in terms of overall survival and time to recurrence (*Figure 3*, *Table 3*). The median time to lung specific recurrence was calculated as 13 months in the low-expression group, compared to 17 months in the high-expression group (P=0.096, log-rank test). The median time to tumor recurrence, irrespectively of the site of relapse, showed a significant difference between the low-expression (11 months) and the high-expression (17 months) group, respectively (P=0.041, log rank test). Overall survival in the low-expression

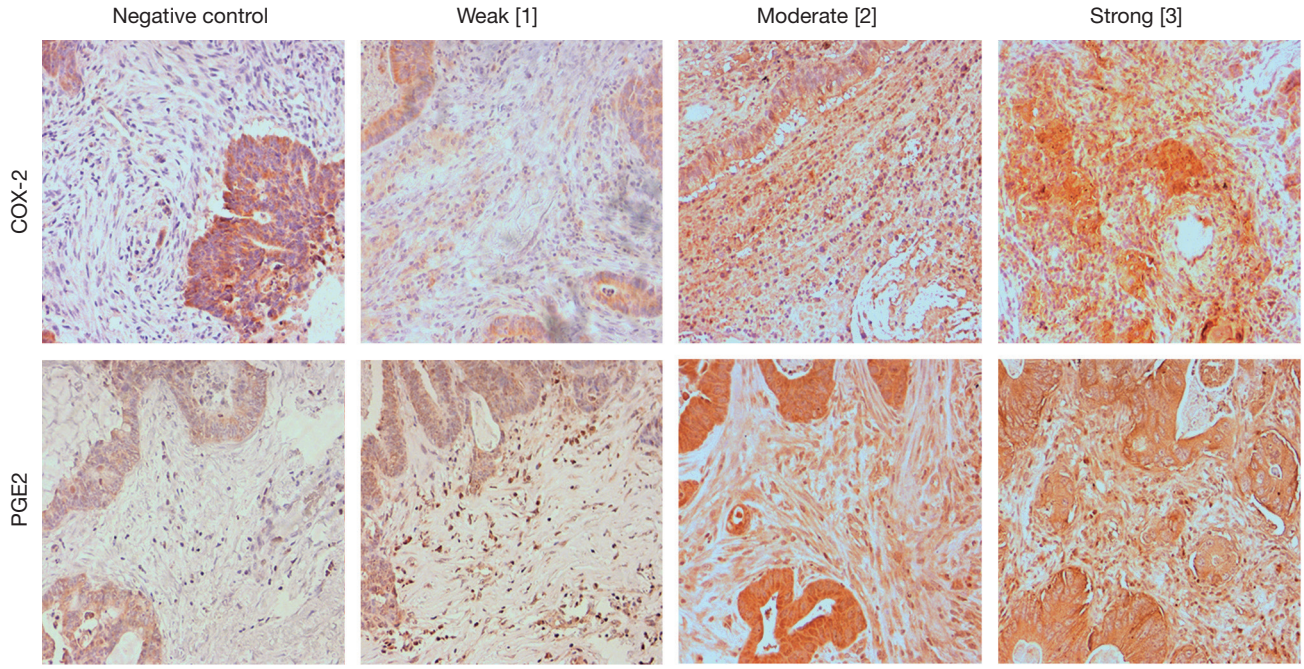

Figure 2 Stromal expression of COX-2 and PGE2 in pulmonary metastases (PM). Representative specimens of pulmonary metastases, showing different staining intensities (negative control; weak; moderate; strong) of cyclooxygenase-2 (COX-2, upper line) and prostaglandin-E2 (PGE2, lower line) in perimetastatic stroma. Magnification 20×, DAB substrate, hematoxylin counterstain.

group was 31 months, compared to 28 months in the high-expression group (P=0.055, log rank). COX-2, PGE2 and previously published prognostic factors of PM were included in a multivariate analysis (cut-off of P<0.2 in the univariate analysis). Neither COX-2 nor PGE were independent prognostic factors. However, the KRAS mutational status and the presence of lymphatic vessel invasion were associated with an impaired prognosis, as previously published (17,18).

No correlation of stromal COX-2 and PGE2 expression levels and clinical outcome

We further evaluated the impact of stromal expression of COX-2 and PGE2 in pulmonary metastases specimens on clinical outcome after PM. Kaplan Meier curves for stromal COX-2 and PGE2 expression are presented in *Figure 4*. Comparable to the data on tumoral COX-2 and PGE2 expression, there was a trend towards a better outcome in the high expression group. However, this trend did not reach the level of significance in univariate and multivariate analysis using log-rank tests (*Table 3*).

Conclusions

PM is an integral part of the treatment of oligometastatic stage IV CRC cancer. By removing all evident tumor spread PM represents a potential curative treatment. Published series of PM for CRC described 5-year survival rates ranging from 40% to 68% (7). Despite these encouraging overall survival rates, there is a broad distribution of outcomes varying from long-term remission to tumor recurrence within several weeks (19). Traditional selection criteria of PM fail to identify patients with an impaired overall prognosis, who would only marginally benefit from a local resection of pulmonary nodules. Thus, attempts have been made to focus on tumor biology when selecting patients for PM. Kawaguchi *et al.* proposed an observation period with a repeat CT-scan calculating the tumor growth before PM. A tumor-doubling time of >100 days led to an increased recurrence free survival with a hazard ratio of 5.89 (1.89–18.32) in patients without preoperative chemotherapy (20). In addition to that a variety of molecular markers reflecting tumor aggressiveness have been proposed such as B-cell lymphoma 2 (bcl-2), β-catenin, carcinoembryonic antigen

Table 1 summarizes demographic data and clinico-pathological characteristics of our patient collective, according to their cyclooxygenase-2 (COX-2, low-expression vs. high-expression) status in pulmonary metastases (PM) and perimetastatic stroma, as well as primary tumors

	COX-2 expression in PM								COX-2 expression in perimetastatic stroma					COX-2 expression in primary CRC							
	Improper staining	Included in analysis		Low-expression (<median)		High-expression (≥median)			Low-expression (<median)		High-expression (≥median)			Improper staining	Included in analysis		Low-expression (<median)		High-expression (≥median)		
	N=5	N=48	%	N=24	%	N=24	%	P value	N=24	%	N=24	%	P value	N=3	N=26	%	N=12	%	N=14	%	P value
Gender																					
Female	3	19	39.6	10	20.8	9	18.8	0.768[a]	15	31.3	4	8.3	0.522[c]	1	12	46.2	5	19.2	7	26.9	0.713[a]
Male	2	29	60.4	14	29.2	15	31.3		20	41.7	9	18.8		2	14	53.8	7	26.9	7	26.9	
Age (median)	54	65		65		65		0.885[b]/0.871[d]	64		70		0.486[b]/0.596[d]	59	65		68		62		1.000[b]/0.874[d]
Location																					
Colon	3	28	58.3	14	29.2	14	29.2	1.000[a]	20	41.7	8	16.7	1.000[c]	2	13	50	5	19.2	8	30.8	0.695[a]
Rectum	2	20	41.7	10	20.8	10	20.8		15	31.3	5	10.3		1	13	50	7	26.9	6	23.1	
Unknown	0	0			-						-			0	-	-	-	-	-	-	
T																					
1	1	0	0	0	0	0	0	0.385[c]	0	0	0	0	0.663[c]	0	1	3.8	1	3.8	0	0	0.199[c]
2	1	7	15.6	5	11.1	2	4.4		4	8.9	3	6.7		1	4	15.4	2	7.7	2	7.7	
3	2	33	73.3	14	31.1	19	42.2		24	53.3	9	20.0		1	17	65.4	9	34.6	8	30.8	
4	1	5	11.1	2	4.4	3	6.7		4	8.9	1	2.2		1	4	15.4	0	0	4	15.4	
Unknown	0	3			-						-			0	-	-	-	-	-	-	
N																					
N0	1	17	37.8	6	13.0	11	24.4	0.087[c]	12	26.7	5	11.1	1.000[c]	1	9	34.6	4	15.4	5	19.2	0.885[c]
N1	3	12	26.7	9	20.0	3	6.7		9	20.0	3	6.7		2	7	26.9	4	15.4	3	11.5	
N2	1	16	35.6	6	13.3	10	22.2		11	24.4	5	11.1		0	10	38.5	4	15.4	6	23.1	
unknown	0	3			-						-			0	-	-	-	-	-	-	
M																					
0	5	40	83.3	22	45.8	18	37.5	0.245[c]	30	62.5	10	20.8	0.664[c]	2	22	84.6	10	38.5	12	46.2	1.000[c]
1	0	8	16.7	2	4.2	6	12.5		5	10.4	3	6.3		1	4	15.4	2	7.7	2	7.7	
Unknown	0	0			-						-			0	-	-	-	-	-	-	
COX expression primary tumour																					
Low (<median)	1	11	47.8	6	26.1	5	21.7	0.684[c]	13	59.1	2	9.1	0.565[c]	-	-	-	-	-	-	-	-
High (≥median)	2	12	52.2	5	21.7	7	30.4		5	22.7	2	9.1		-	-	-	-	-	-	-	
Unknown	2	25			-						-			-	-	-	-	-	-	-	

Table 1 (*continued*)

Table 1 (*continued*)

	COX-2 expression in PM								COX-2 expression in perimetastatic stroma					COX-2 expression in primary CRC							
	Improper staining	Included in analysis		Low-expression (<median)		High-expression (≥median)			Low-expression (<median)		High-expression (≥median)			Improper staining	Included in analysis		Low-expression (<median)		High-expression (≥median)		
	N=5	N=48	%	N=24	%	N=24	%	P value	N=24	%	N=24	%	P value	N=3	N=26	%	N=12	%	N=14	%	P value
Previous liver metastases																					
No	2	35	72.9	18	37.5	17	35.4	1.000[a]	24	50.0	11	22.9	0.466[c]	1	17	65.4	8	30.8	9	34.6	1.000[c]
Yes	3	13	27.1	6	12.5	7	14.6		11	22.9	2	4.2		2	9	34.6	4	15.4	5	19.2	
Unknown	0	0			-						-			0	-	-	-	-	-	-	
Number of nodules																					
Singular	3	38	79.2	21	43.8	17	35.4	0.286[c]	28	58.3	10	20.8	1.000[c]	3	21	80.8	10	38.5	11	42.3	1.000[c]
Multiple	2	10	20.8	3	6.3	7	14.6		7	14.6	3	6.3		0	5	19.2	2	7.7	3	11.5	
Unknown	0	0			-						-			0	-	-	-	-	-	-	
KRAS status																					
Wildtype	1	23	54.8	13	31.0	10	23.8	1.000[a]	17	40.5	6	14.3	0.477[c]	2	15	57.7	7	26.9	8	30.8	1.000[a]
Mutant	3	19	45.2	10	23.8	9	21.4		16	38.1	3	7.1		1	11	42.3	5	19.2	6	23.1	
Unknown	1	6			-						-			0	-	-	-	-	-	-	
Chemotherapy before metastasectomy																					
No	0	11	22.9	2	4.2	9	18.8	0.036[c]	6	12.5	5	10.4	0.140[c]	0	6	23.1	2	7.7	4	15.4	0.652[c]
Yes	5	37	77.1	22	45.8	15	31.3		29	60.4	8	16.7		3	20	76.9	10	38.5	10	38.5	
Unknown	0	0			-						-			0	-	-	-	-	-	-	
Chemotherapy after metastasectomy																					
No	1	12	25.0	3	6.3	9	18.8	0.093[c]	7	14.6	5	10.4	0.263[c]	0	7	26.8	4	15.4	3	11.5	0.665[c]
Yes	4	36	75.0	21	43.8	15	31.3		28	58.3	8	16.7		3	19	73.1	8	30.8	11	42.3	
Unknown	0	0			-						-			0	-	-	-	-	-	-	

[a], Chi-square test; [b], Kruskal-Wallis test; [c], Fisker's exact test; [d], *t*-test.

Table 2 Shows the distribution of prostaglandin-E2 (PGE2, low-expression vs. high-expression) within demographic data and clinico-pathologic characteristics of our patient collective.

	PGE2 expression in PM								PGE2 expression in perimetastatic stroma					PGE2 expression in primary CRC							
	Improper staining	Included in analysis		Low-expression (<median)		High-expression (=>median)			Low-expression (<median)		High-expression (=>median)			Improper staining	Included in analysis		Low-expression (<median)		High-expression (=>median)		
	N=5	N=48	%	N=24	%	N=24	%	P value	N=24	%	N=24	%	P value	N=4	N=25	%	N=	%	N=	%	P value
Gender																					
Female	1	21	43.8	8	16.7	13	27.1	0.259[a]	10	20.8	11	22.9	1.000[a]	0	13	52	6	24	7	28	1.000[a]
Male	4	27	56.2	15	31.3	12	25.0		13	27.1	14	29.2		4	12	48	6	24	6	24	
Age (median)	73	63		62		64		0.495[b]/0.360[d]	61		63		0.749[b]/0.453[d]	66	62		61.5		62		0.913[b]/0.929[d]
Location																					
colon	3	28	58.3	11	22.9	17	35.4	0.241[a]	13	27.1	15	1.3	1.000[a]	2	13	52	7	28	6	24	0.695[a]
Rectum	2	20	41.7	12	25.0	8	16.7		10	20.8	10	20.8		2	12	48	5	20	7	28	
Unknown	0	0			–						–			0	–	–	–	–	–	–	
T																					
1	0	1	2.2	1	2.2	0	0	0.183[c]	1	2.2	0	0	0.630[c]	0	1	4	0	0	1	4	0.522[c]
2	0	8	15.6	6	13.3	2	4.4		3	6.7	5	11.1		1	4	16	3	12	1	4	
3	5	30	68.9	12	26.7	18	40.0		15	33.3	15	33.3		3	15	60	6	24	9	36	
4	0	6	13.3	3	6.7	3	6.7		4	8.9	2	4.4		0	5	20	3	12	2	8	
Unknown	0	3			–						–			0	–	–	–	–	–	–	
N																					
N0	1	17	37.8	8	17.8	9	20.0	0.982[a]	11	24.4	6	13.3	0.295[c]	1	9	36	3	12	6	24	0.679[c]
N1	1	14	31.1	7	15.6	7	15.6		5	11.1	9	20.0		2	7	28	4	16	3	12	
N2	3	14	31.1	7	15.6	7	15.6		7	15.6	7	15.6		1	9	36	5	20	4	16	
unknown	0	3			–						–			0	–	–	–	–	–		
M																					
0	3	42	87.5	21	43.8	21	43.8	0.668[c]	19	39.6	23	47.9	0.407[c]	4	20	80	11	44	9	36	0.322[c]
1	2	6	12.5	2	4.2	4	8.3		4	8.3	2	4.2		0	5	20	1	4	4	16	
unknown	0	0			–						–			0							
PGE2 expression primary tumour																					
Low (<median)	1	11	52.4	5	23.8	6	28.6	0.387[c]	5	22.7	5	22.7	0.172[c]	–	–	–	–	–	–	–	–
High (≥median)	3	10	47.6	7	33.3	3	14.3		10	45.5	2	9.1		–	–	–	–	–	–	–	
Unknown	1	27			–						–			–	–	–	–	–	–	–	

Table 2 (*continued*)

Table 2 (*continued*)

	PGE2 expression in PM								PGE2 expression in perimetastatic stroma					PGE2 expression in primary CRC							
	Improper staining	Included in analysis		Low-expression (<median)		High-expression (=>median)			Low-expression (<median)		High-expression (=>median)			Improper staining	Included in analysis		Low-expression (<median)		High-expression (=>median)		
	N=5	N=48	%	N=24	%	N=24	%	P value	N=24	%	N=24	%	P value	N=4	N=25	%	N=	%	N=	%	P value
Previous liver metastases																					
No	4	33	68.8	17	35.4	16	33.3	0.542[a]	15	31.3	18	37.5	0.757[a]	3	15	60	8	32	7	28	0.688[c]
Yes	1	15	31.3	6	12.5	9	18.8		8	16.7	7	14.6		1	10	40	4	16	6	24	
Unknown	0	0			–						–			0	–	–	–	–	–	–	
Number of nodules																					
Singular	4	37	77.1	18	37.5	19	39.6	1.000[c]	17	35.3	20	41.7	0.736[c]	3	21	84	12	48	9	36	0.096[c]
Multiple	1	11	22.9	5	10.4	6	12.5		6	12.5	5	10.4		1	4	16	0	0	4	16	
Unknown	0	0			–						–			0	–	–	–	–	–	–	
KRAS status																					
wildtype	2	22	53.7	12	29.3	10	24.4	1.000[a]	11	26.8	11	26.8	0.756[a]	4	13	52	7	28	6	24	0.695[a]
Mutant	3	19	46.3	10	24.4	9	22.0		11	26.8	8	19.5		0	12	48	5	20	7	28	
Unknown	0	7			–						–			0	–	–	–	–	–	–	
Chemotherapy before metastasectomy																					
No	1	10	20.8	3	6.3	7	14.6	0.292[c]	4	8.3	6	12.5	0.727[c]	0	6	24	2	8	4	16	0.645[c]
Yes	4	38	79.2	20	41.7	18	37.5		19	39.6	19	39.6		4	19	76	10	40	9	36	
Unknown	0	0			–						–			0	–	–	–	–	–	–	
Chemotherapy after metastasectomy																					
No	3	10	20.8	4	8.3	6	12.5	0.727[c]	5	10.4	5	10.4	1.000[c]	0	7	28	3	12	4	16	1.000[c]
Yes	2	38	79.2	19	39.6	19	39.6		18	37.5	20	41.7		4	18	72	9	36	9	36	
Unknown	0	0			–						–			0	–	–	–	–	–	–	

[a], Chi-square test; [b], Kruskal-Wallis test; [c], Fisker's exact test; [d], *t*-test.

Table 3 Univariate and multivariate analysis of overall survival, time to pulmonary recurrence and time to any recurrence

	Total		Overall survival (months)					Time to pulmonary recurrence (months)					Time to any recurrence (months)				
			Univariate analysis (log-rank)		Multivariate analysis (cox-regression)			Univariate analysis (log-rank)		Multivariate analysis (cox-regression)			Univariate analysis (log-rank)		Multivariate analysis (cox-regression)		
	N=53	%	Median	P value	Exp (B)	95 % CI	P value	Median	P value	Exp (B)	95 % CI	P value	Median	P value	Exp (B)	95 % CI	P value
Gender																	
Female	22	41.5	36	0.410				13.5	0.518				13	0.405			
Male	31	58.5	29					16					15				
Age (years)																	
<64 yrs	26	49.1	31	0.843				16.5	0.445				15.5	0.879			
≥64 yrs	27	50.9	30					13					11				
Location (primary tumor)																	
Colon	31	58.5	29	0.565				15	0.632				15	0.702			
Rectum	22	41.5	33					13.5					10.5				
T stage (primary tumor)																	
pT1 + pT2	9	17.0	31	0.643				15	0.685				15	0.630			
pT3 + pT4	44	83.0	30					17					13				
N stage (primary tumor)																	
pN0	18	34.0	29.5	0.122	0.690	0.178–2.672	0.591	17	0.814				17	0.961			
pN1 + pN2	35	60.4	31					13					13				
Previous liver metastasis																	
No	37	69.8	30	0.317				17	0.269				15	0.064	1.716	0.851–3.462	0.131
Yes	16	30.2	33					12					8.5				
COX-2 expression in PM																	
Low-expr.	24	45.3	36.5	0.800				15	0.287				14	0.588			
High-expr.	24	55.3	24					17					15				
Unknown	5	9.4	–					–					–				
COX-2 expression in perimetastatic stroma																	
Low-expr	35	66.0	36	0.642				17	0.733				17	0.710			
High-expr	13	24.5	22					8					8				
Unknown	5	9.4	–					–					–				
PGE2 expression in PM																	
Low-expr.	23	43.4	31	0.055	0.057	0.001–3.362	0.168	13	0.096	0.990	0.843–1.163	0.904	11	0.041	0.652	0.306–1.388	0.267
High-expr.	25	47.2	28					17					17				
Unknown	5	9.4	–					–					–				

Table 3 (*continued*)

Table 3 (*continued*)

	Total		Overall survival (months)					Time to pulmonary recurrence (months)					Time to any recurrence (months)				
			Univariate analysis (log-rank)		Multivariate analysis (cox-regression)			Univariate analysis (log-rank)		Multivariate analysis (cox-regression)			Univariate analysis (log-rank)		Multivariate analysis (cox-regression)		
	N=53	%	Median	P value	Exp (B)	95 % CI	P value	Median	P value	Exp (B)	95 % CI	P value	Median	P value	Exp (B)	95 % CI	P value
PGE2 expression in perimetastatic stroma																	
Low-expr	23	43.4	29	0.070	0.887	0.018–43.483	0.952	15	0.718				11	0.142	0.482	0.203–1.140	0.097
High-expr	25	47.2	37					15					13				
Unknown	5	9.4	–					–					–				
COX-2 expression in primary CRC																	
Low-expr	12	22.6	31.5	0.194	0.983	0.256–3.767	0.980	10.5	0.121	0.389	0.140–1.079	0.070	10.5	0.210			
High-expr	14	26.4	45					22.5					21.5				
Unknown	27	50.9	–					–					–				
KRAS status																	
Wildtype	24	45.3	36.5	0.432				18.5	0.131	3.589	1.243–10.358	0.018	15	0.311			
Mutant	22	41.5	33					15.5					15.5				
Unknown	7	13.2	–										–				
Chemotherapy before metastasectomy																	
No	11	20.8	30	0.898				17	0.538				17	0.414			
Yes	42	79.2	31					14.5					13				
unknown	–	–	–					–					–				
Chemotherapy after metastasectomy																	
No	13	24.5	23	0.379				13	0.548				13	0.993			
Yes	40	75.5	33					17					13				
unknown	–	–	–					–					–				
Number of nodules																	
Singular	41	77.4	36	0.331				15	0.611				13	0.865			
Multiple	12	22.6	24					16.5					12				
LMFS (months)																	
<36	36	67.9	30.5	0.792				16	0.929				14.5	0.926			
≥36	17	32.1	36					13					13				
Lymphatic invasion of pulmonary metastasis																	
No	33	62.3	35	0.057	18.769	1.956–180.115	0.011	17	0.261				16	0.186	2.452	1.042–5.770	0.040
Yes	20	37.7	22					8.5					8				

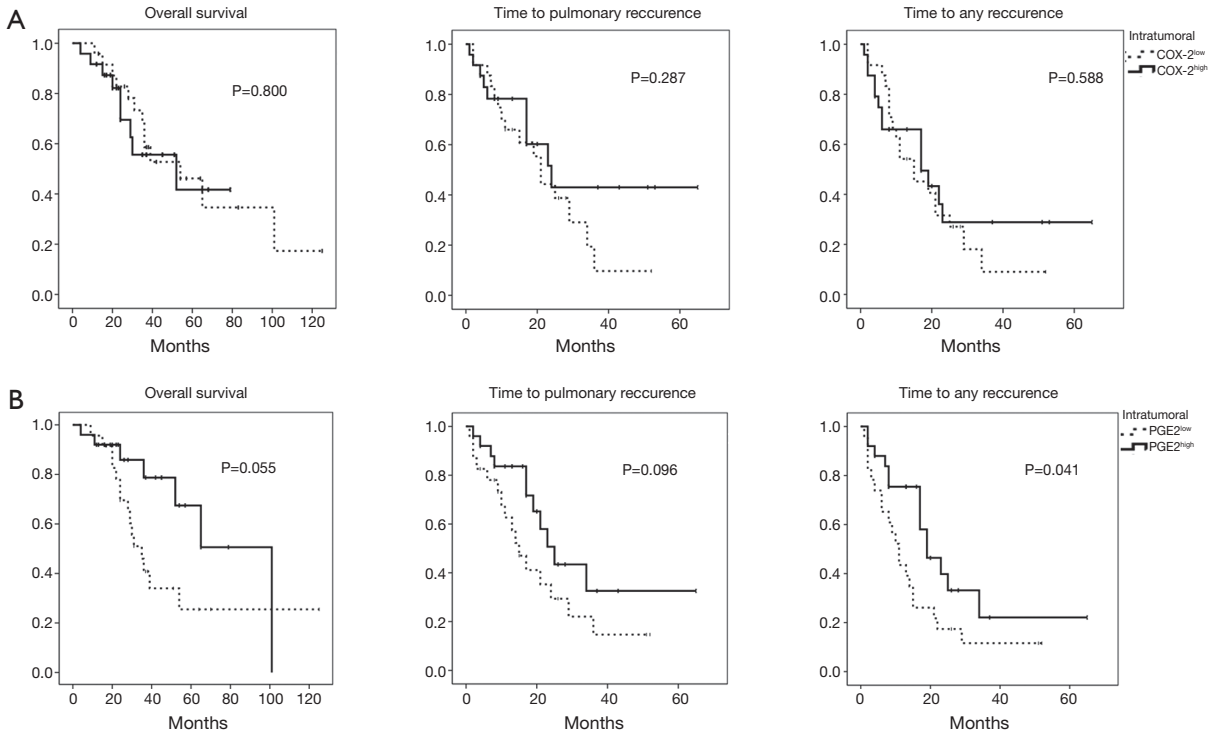

Figure 3 Overall survival, time to pulmonary recurrence and time to any recurrence of patients after pulmonary metastasectomy (PM), divided by low-expression and high-expression of (A) cyclooxygenase-2 (COX-2) and (B) prostaglandin-E2 (PGE2) in pulmonary metastases.

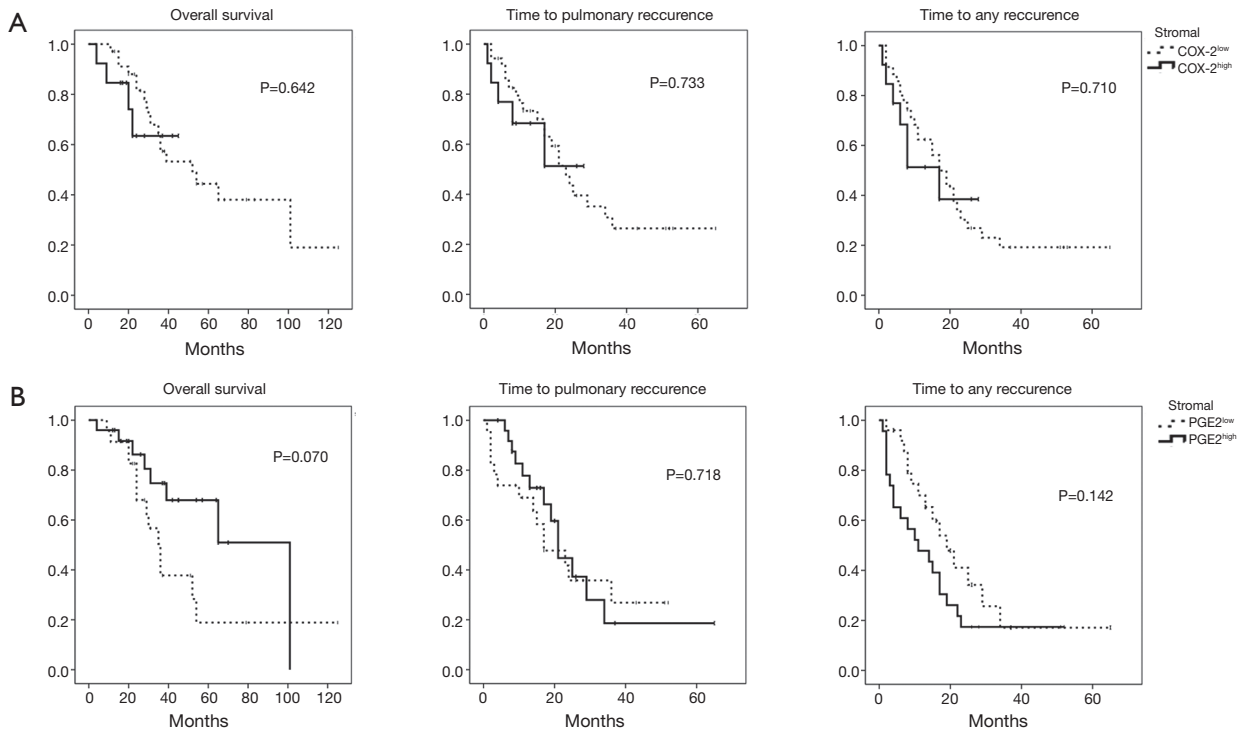

Figure 4 Overall survival, time to pulmonary recurrence and time to any recurrence of patients after pulmonary metastasectomy (PM), divided by low-expression and high-expression of (A) cyclooxygenase-2 (COX-2) and (B) prostaglandin-E2 (PGE2) in perimetastatic stroma (B).

(CEA), E-cadherin, excision repair cross-complementation group 1 (ERCC1), KRAS, lymphatic invasion, CD34, pleural invasion, vascular invasion and vascular endothelial growth factorα (VEGFα) (9). None of those markers has been implicated in the clinical practice due to either technical difficulties or lack of standardization.

Inflammation is nowadays recognized as a major contributing factor to tumor growth and progression. Several inflammatory mediators have been linked to cancer progression such as VEGF-A, CSFs, IL-1, IL-6, IL-8, or CXCL1 (21,22). On the other hand, the immune system is also considered as one of the key factors of the endogenous cancer defence. In CRC the density of tumor infiltrating mature T-cells [cluster of differentiation (CD) 3+], cytotoxic T-cells (CD8+) and memory-T-cells (CD45RO+) are strong positive prognostic factors (23). By modifying the local immune reaction a tumor can escape this anti-tumor activity. Manipulating this process of immunoediting is an essential factor in the development of immune checkpoint blocking compounds (24).

COX-2 is the inducible isoform of the cyclooxygenase enzyme family. It is responsible for the conversion of arachidonic acid into prostaglandins. COX-2 is upregulated in inflammatory processes as well as in cancerous tissue (25). Preclinical studies on COX-2 as a possible therapeutic target in CRC were promising. Depletion of COX led to an increased anti-cancer immune response and T cell-mediated tumor elimination in experimental mouse models of CRC. Moreover, COX inhibitors enhanced the efficacy of immunotherapy with anti-PD-1 blocking antibodies (26). Although there is clear evidence that COX-2 is involved in tumor development and progression, its role as a prognostic marker is still elusive. This has recently been highlighted in a metanalysis including 18 studies on primary CRC. The hazard ratio for overall survival was only 1.19 and the impact on disease free interval was not significant. In addition to that the majority of included publications found only an indeterminate association of COX and patient prognosis. Of note most studies comprised a mixture of primary CRC patients and no analysis on the prognostic role of COX-2 in pulmonary metastases (27). One has to be careful when transferring conclusions from primary tumors to metastasized tumor stages. Metastases are distinct from their primary with a diverse tumor biology. It is generally believed that metastases acquire a more aggressive tumor phenotype during metastasis.

COX-2 expression in published series of primary CRC ranged from 33 percent to 84 percent (28,29). In our study cohort of metastatic CRC COX-2 could be detected in nearly all of our cases using an anti-COX-2 clone CX-294 antibody. This antibody is highly selective to detect COX-2 in formalin fixed tissue samples and was shown to have excellent staining characteristics (30). The high rate of COX-2 staining in our patient cohort might also reflect the advanced tumor stage and an unfavourable tumor biology of our patients. More than half of our patients had positive lymph nodes and almost all were in stage T3/4 at the time of diagnosis.

The role of PGE2 in colorectal cancer is manifold and it impacts the function of cancer cells as well as the function of almost all immune effector cells. In azoxymethane (AOM) mouse models PGE2 treatment significantly increased colon tumor incidence and promoted metastasis (31,32). PGE2 directly binds to the cell surface of CRC tumor cells and mediates anti-apoptosis, migration and invasion. Dependent on the binding to different prostanoid receptor types PGE-2 can act as both pro- and anti-inflammatory. As a pro-inflammatory mediator it regulates the expression profile of dendritic cells and enhances T cell activation (33). This is especially important in CRC, a tumor type considered highly immunogenic. A high number of tumor infiltrating CD8+ cells can induce a potent tumor lytic response and were shown to be prognostic in primary and metastatic CRC (34,35). On the other hand PGE2 can promote the formation of inhibitory T-regs in a cancerous environment (36). The number of tumor infiltrating T-regs are thought to be a negative prognostic factor in CRC. The herein reported findings in CRC pulmonary metastases suggest that high-expression of PGE2 in pulmonary metastases tissues reflects a beneficial tumor biology and leads to a longer disease free interval after PM.

The role of site specific recurrence after metastasectomy for CRC has recently been a focus of research. It seems that a certain tumor biology determines the site of recurrence. KRAS mutations for example are associated with increased lung metastasis, whereas loss of Smad4 expression seems to predict liver metastasis (17,37,38). This is an important information since repeated pulmonary and hepatic metastasectomy can be offered to the patients with good results and low morbidity (39). We therefore determined the site-specific pattern of metastatic recurrence in our patients. Both, COX-2 and PGE2 overexpression did not point towards a predominance in pulmonary recurrence.

The herein reported study collective is based on a prospectively documented data base introduced in

2009 at our institution. Since that time 53 patients with CRC pulmonary spreading, who underwent curative metastasectomy, were included in the data base. This study was designed as a pilot study to evaluate the frequency and degree of COX-2 and PGE2 expression in pulmonary metastases from CRC and to assess its impact on outcome data. Although a prognostic trend was observed for COX-2 and PGE2 in most survival/follow-up calculations, it did not reach the level of statistical significance. We currently recruit patients within an international multi-institutional study protocol in order to evaluate the impact of proposed prognostic markers (e.g., COX-2 and PGE2) in a larger cohort.

In conclusion, this pilot study shows that COX-2 and PGE2 are uniformly overexpressed in pulmonary metastases from CRC. High expression of COX-2 and PGE2 seem to reflect a beneficial tumor biology with late tumor recurrence and prolonged overall survival after PM. Further studies are warranted to confirm these findings in a larger study cohort.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: This study was approved by the ethics committee of the Medical University of Vienna (EK#: 1097/2014) and was performed according to the Declaration of Helsinki and the Good Scientific Practice guidelines of the Medical University of Vienna.

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Supplementary

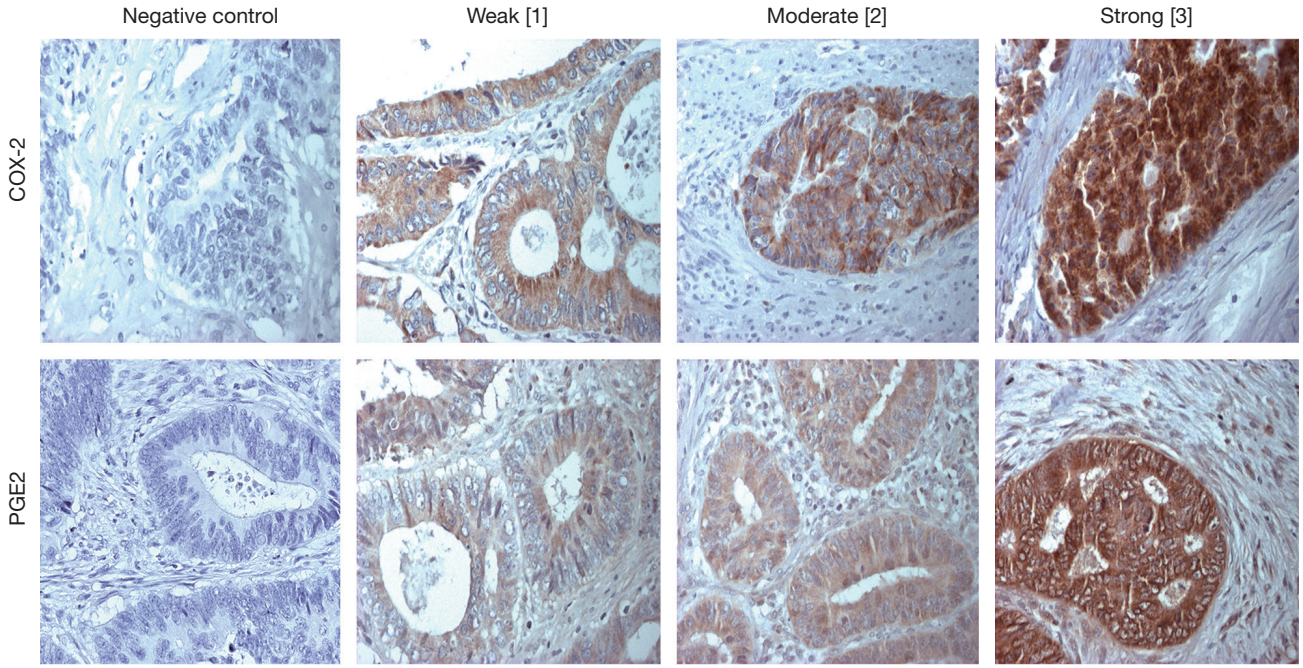

Figure S1 Expression of COX-2 and PGE2 in primary CRC. Representative slides of primary colorectal cancer (CRC) specimens, showing different intratumoral expressional status (negative control; weak; moderate; strong) of cyclooxygenase-2 (COX2, upper line) and prostaglandin-E2 (PGE2, lower line), Magnification 40×, DAB substrate, hematoxylin counterstain.

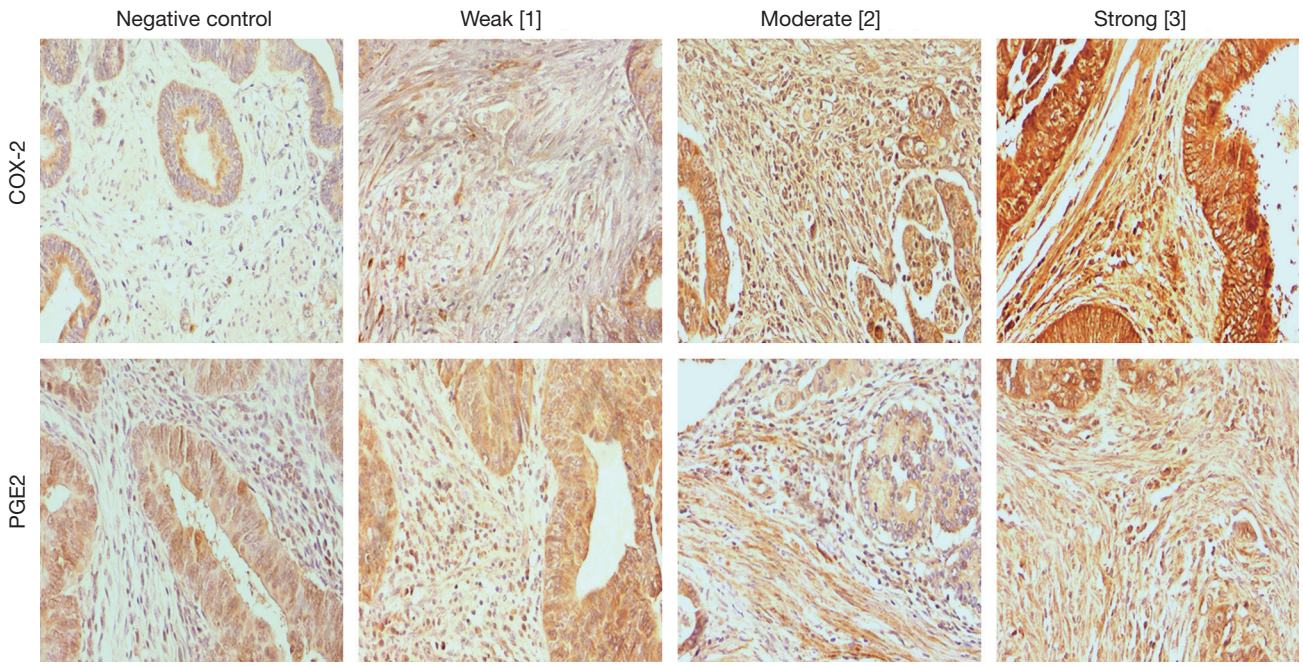

Figure S2 Stromal expression of COX-2 and PGE2 in primary colorectal cancer (CRC). Representative specimens of primary CRC samples, showing different expressional status (negative control; weak; moderate; strong) of cyclooxygenase-2 (COX2, upper line) and prostaglandin-E2 (PGE2, lower line) in peritumoral stroma. Magnification 20×, DAB substrate, hematoxylin counterstain.