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Total cyclooxygenase-2 mRNA levels correlate with vascular endothelial growth factor mRNA levels, tumor angiogenesis and prognosis in non-small cell lung cancer patients

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Interaction between cancer cells and adjacent stromal cells is important to promote tumor development. Our aim was to study total COX-2 mRNA expression in both cancer cells and surrounding stromal cells and its association with angiogenic factor VEGF mRNA expression, tumor angiogenesis and prognosis in patients with NSCLC. COX-2 mRNA expression in both cancer cells and stromal tissue was analyzed using real-time quantitative (RTQ) RT-PCR in 60 NSCLC surgical specimens. Immunohistochemistry (IHC) was used to localize COX-2 protein in tumor specimens. Correlations between tumoral total COX-2 mRNA expression and VEGF mRNA expression (measured by RTQ RT-PCR), intratumoral microvessel counts (evaluated by IHC), other clinicopathologic variables, survival and relapse were tested. COX-2 protein expression was found in cancer as well as the surrounding stromal cells (including infiltrating inflammatory cells and endothelial cells of tumor-associated microvessels). VEGF protein expression was mainly located in cancer cells. There was a significant association between high tumoral total COX-2 mRNA expression and high VEGF mRNA expression ($p = 0.01$) or high intratumoral MVC ($p < 0.001$) but not other clinicopathologic variables, including tumor status and lymph node metastasis. Patients with higher tumoral total COX-2 mRNA expression had a statistically shorter survival time (median 15.0 ± 2.61 months) and relapse time (median 5.0 ± 1.37 months) than those with lower tumoral total COX-2 mRNA expression (median 40.0 ± 3.12 and 34.0 ± 3.11 months; $p < 0.0001$ and $p < 0.0001$, respectively, log-rank test). A significant difference in survival and relapse time was also seen between patients with high and low tumoral VEGF mRNA expression and between those with high and low intratumoral MVC ($p = 0.0046$ and $p = 0.0038$, respectively). After stratification by disease stage or histologic subtype, the prognostic significance of high total COX-2 mRNA expression was still apparent in both stage I and stage II–IV and in both squamous cell carcinoma and adenocarcinoma ($p \leq 0.01$ for all). Multivariate analysis using the Cox regression model with backward elimination showed that tumoral total COX-2 mRNA expression and lymph node status were the 2 most important independent prognostic predictors for survival and disease relapse. We report that total COX-2 mRNA expression in cancer cells and surrounding stromal cells correlates strongly and positively with VEGF mRNA expression, intratumoral MVC and adverse prognosis in NSCLC patients. This implies that COX-2 expression in both cancer cells and stromal cells within the tumor microenvironment may play an important role in upregulating the expression of the angiogenic factor VEGF and tumor angiogenesis in NSCLC and explains, in part, the adverse prognostic effect of COX-2 overexpression in patients with NSCLC.

Key words: COX-2 mRNA; VEGF mRNA; microvessel count; cancer cell; stromal cell; real-time quantitative RT-PCR; prognosis; lung cancer

Population-based studies have established that long-term intake of NSAIDs reduces the relative risk of developing colorectal cancers and several other human cancers.[1,2] Laboratory and animal studies have shown that COX-2 may be involved in carcinogenesis, tumor promotion and progression.[1,8] Oshima *et al.*[9] have shown that *COX-2* gene knockout can suppress intestinal polyp formation in $APC^{\Delta 716}$ knockout mice. In addition, inhibition of COX-2 reduced the incidence of colon cancer in rats treated with chemical carcinogens[10] and in *APC* gene knockout mice.[9]

COX is the key enzyme involved in the conversion of arachidonic acid to prostaglandins.[11,12] Two COX isoforms, COX-1 and COX-2, have been identified. *COX-1* is considered a housekeeping gene responsible for various physiologic functions, whereas *COX-2* is an inducible immediate-early gene, which can be induced by inflammatory stimuli,[13] growth factors[14,17] and tumor promoters.[18]

Constitutional overexpression of COX-2 has been reported in colorectal cancer, gastric cancer, pancreatic cancer, NSCLC, head-and-neck squamous cell cancer, malignant melanoma, breast cancer and bladder cancer.[19,26] The mechanism of the association between COX-2 overexpression and tumorigenesis remains unclear, though several *in vitro* studies have shown that COX-2 expression in cancer cell lines causes increased cell proliferation,[27] inhibition of apoptosis,[28] stimulation of angiogenesis[29] and suppression of immunosurveillance.[30] However, the *in vivo* relevance of these findings has not been fully determined.[18]

Several *in vitro* and animal studies have shown that COX-2 overexpression can stimulate angiogenesis, a process that is essential for tumor growth and metastasis.[29,31,32] Tsujii *et al.*[29] demonstrated that COX-2-overexpressing colorectal cancer cell lines secrete several angiogenic factors, including VEGF, bFGF and PDGF, and promote endothelial cell tube formation in coculture systems. Selective COX-2 inhibitors blocked neovascularization in a rat sponge model[31] and inhibited tumor growth and angiogenesis of gastrointestinal tumor xenografts in athymic mice.[32]

However, whether COX-2 participates in angiogenesis in human cancer remains unclear.[33,36] A positive correlation was reported between COX-2 expression, VEGF expression and tumor MVC in colorectal cancer[34] and between COX-2 expression and

Abbreviations: APC, adenomatous polyposis coli; bFGF, basic fibroblast growth factor; COX, cyclo-oxygenase; C_t, threshold cycle; 6FAM, 6-carboxyfluorescein; IHC, immunohistochemistry; ISH, *in situ* hybridization; MAb, monoclonal antibody; MVC, microvessel count; NSAID, nonsteroidal anti-inflammatory drug; NSCLC, non-small cell lung cancer; PDGF, platelet-derived growth factor; PGE_2/PGI_2, prostaglandin E_2/I_2; RTQ, real-time quantitative; TAMRA, 6-carboxytetramethylrhodamine; TBP, TATA box binding protein; VEGF, vascular endothelial growth factor.

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VEGF expression or MVC in head-and-neck squamous cell carcinoma[35] and endometrial carcinoma.[36] In terms of human lung cancer, investigations into COX-2 expression and tumor angiogenesis have been limited and have given conflicting results regarding the relationship between COX-2 upregulation and angiogenic factor expression.[37,38]

COX-2 overexpression has been associated with adverse prognosis in patients with colon, bladder, pancreatic, gastric and cervical cancers as well as head-and-neck squamous cell carcinoma[35,39,43] but not with prognostic significance in human malignant melanoma.[24] The few studies on the prognostic significance of COX-2 expression in NSCLC have given different results.[37,38,44] COX-2 expression was not associated with prognostic outcome in some studies[37,44] but was associated, with borderline statistical significance, with stage I adenocarcinoma in other studies.[38,44] In addition, there are no conclusive data on COX-2 expression and its prognostic significance in different histologic types of NSCLC.[38,45]

VEGF is the most potent mitogen for vascular endothelial cells,[46] and its expression has been strongly correlated with tumor angiogenesis in a variety of human cancers, including lung cancer.[47,50] However, to the best of our knowledge, there is no evidence that directly and positively links COX-2 expression, VEGF expression, MVC and outcome in NSCLC.

In a variety of human cancer biopsy specimens, COX-2 is overexpressed not only in cancer cells but also in stromal cells, including macrophages, fibroblasts and endothelial cells.[18,51] COX-2 expression is considered to be important in cytokine-induced angiogenesis in corneal angiogenesis models[33,51] and in promoting tumor-associated angiogenesis in *COX-2*$^{-/-}$ mice.[33,52] Most previous studies[37,38,44] used IHC staining or ISH to evaluate COX-2 expression in cancer cells themselves but did not assess expression in the stromal compartment.

In the present study, we used RTQ RT-PCR, one of the most accurate methods for quantifying gene copies and mRNA expression,[53] to assess total COX-2 mRNA expression in cancer and stromal cells in NSCLC and the relationship between tumoral total COX-2 mRNA expression, angiogenic factor VEGF mRNA expression (one of the most potent mitogens for vascular endothelial cells), MVC and patient outcome. Our aims were *(i)* to correlate tumoral total COX-2 mRNA expression with VEGF mRNA expression, tumoral MVC and other clinicopathologic variables; *(ii)* to correlate total COX-2 mRNA expression, VEGF mRNA expression and MVC with survival and relapse; *(iii)* to determine if there was a difference in prognostic significance of tumoral total COX-2 mRNA expression between early and late disease stages and between squamous cell carcinoma and adenocarcinoma; and *(iv)* to determine, by multivariate analysis, the most important independent prognostic factors in NSCLC.

Material and methods

Patients and tissue samples

Between January 1995 and July 1997, 70 sequential patients underwent surgical resection for NSCLC in our institution. Of these, 2 were excluded due to inappropriate specimen storage, 3 due to lack of clinical follow-up and 5 due to mortality from postoperation complications; 60 patients were therefore included in the study. These consisted of 40 men and 20 women, with a mean age of 62 ± 12 years. Among these patients, 23 had postoperation adjuvant chemotherapy and/or postoperation radiotherapy and 2 had neoadjuvant chemotherapy. Histologic classification of NSCLC was determined as recommended by the WHO,[54] and tumor staging was performed according to the TNM system recommended by the American Joint Committee on Cancer.[55] Paraffin-embedded, formalin-fixed surgical specimens were collected for IHC staining for COX-2 protein, VEGF protein and intratumoral microvessel endothelial cells. Tumor tissue (including cancer cells and stromal cells), obtained immediately after surgery, was placed in liquid nitrogen and stored frozen at –80°C for subsequent quantification of COX-2 mRNA and VEGF mRNA expression. Histopathology of the archived frozen tissues was confirmed by a pathologist to be similar to that of the paraffin-embedded tissues.

Patient survival time was calculated from the date of operation to the date of death. Relapse time was calculated from the date of operation to the date of local recurrence or distant metastasis. We arbitrarily defined *early relapse* as local recurrence or distal metastasis occurring earlier than the median value for relapse time and *short survival* as survival time less than the median value. The follow-up period lasted up to 60 months.

Quantification of COX-2 mRNA and VEGF mRNA expression using RTQ RT-PCR

An RNA extraction kit (RNeasy Mini-kit; Qiagen, Valencia, CA) was used to extract total RNA from frozen resected tumor tissue.

RTQ RT-PCR, a newly developed kinetic quantitative RT-PCR method (based on the TaqMan reaction), is considered to be one of the most sensitive and accurate methods for the quantification of nucleic acid (DNA and RNA) in tissue samples.[53] We used RTQ RT-PCR for the relative quantification of COX-2 mRNA and VEGF mRNA in tumor specimens, using TBP mRNA,[56] a low-abundance housekeeping gene, as an internal control. The real-time RT-PCR product of TBP was also used to assess RNA integrity.

Primers, probes and reference internal control mRNA

Primers and probes were designed using Primer Express software (Perkin-Elmer Applied Biosystems, Foster City, CA). Primers and probes were synthesized by, and purchased from, Perkin-Elmer Applied Biosystems. Based on the cDNA sequence (Genbank accession m59979), COX-2 mRNA contains 9 exons and 8 exon–exon junctions. Primers and the probe used for RTQ RT-PCR of COX-2 mRNA were (forward) 5′-ATGGAATTACCCAGTTTGTTGAATC-3′ (exon 6), (reverse) 5′-TGGAAGCCTGTGATACTTTCTGTACT-3′ (exon 7) and (probe) 5′-6FAM-TCCTACCACCAGCAACCCTGCCA-TAMRA-3′ (6FAM-reporter dye, TAMRA-quench dye), spanning the exon 6–7 junction, to avoid quantification of the PCR product contaminating COX-2 genomic DNA. Sequences of the primers and probe used for VEGF mRNA quantification, chosen using VEGF cDNA sequence data (accession m32977), were (forward) 5′-GCA CCC ATG GCA GAA GG-3′ (in exon 2), (reverse) 5′-CTC GAT TGG ATG GCA GTA GCT-3′ (in exon 3) and (probe) 5′-6FAM-ACG AAG TGG TGA AGT TCA TGG ATG TCT ATC AC-TAMRA -3′, spanning the exon 2–3 junction, to avoid quantification of the PCR product contaminating VEGF genomic DNA. TBP mRNA was used as internal control, and the sequence of the primers and probe, designed using the same method,[56] were (forward) 5′-CACGAACCACGGCACTGATT-3′, (reverse) 5′-TTTTGTTGCTGC CAGTCTGCAC-3′ and (probe) 5′-6FAM-TGTGCACAGGAG CCAAAGAGTGAAGA-TAMRA -3′.

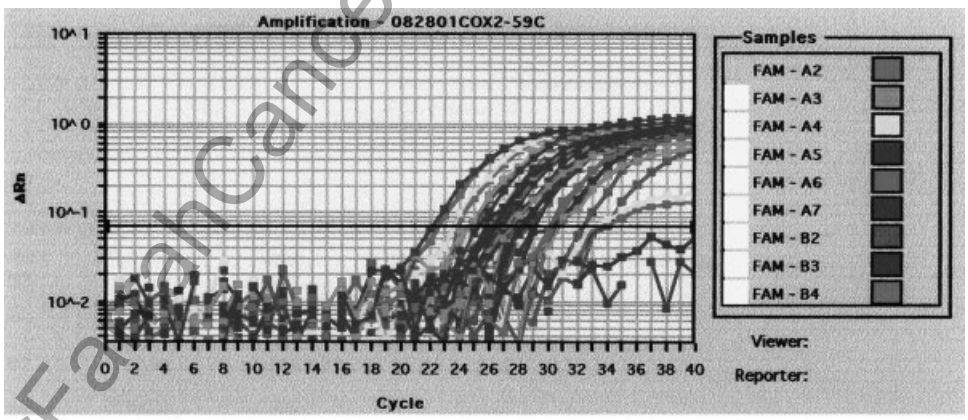
RT-PCR procedure

The amplification mixture (50 μl) contained 50 ng of sample RNA; 5 × TaqMan EZ buffer (10 μl); 25 mM manganese acetate (6 μl); 300 μM dATP, dCTP and dGTP; 600 μM dUTP; 5 units of rTth DNA polymerase; 0.5 units of AmpErase uracil *N*-glycosylase; 200 nM COX-2 (or VEGF) forward and reverse primers; and 100 nM COX-2 (or VEGF) probe. The rTth DNA polymerase had both RTase and Taq polymerase activity. Thermal cycling parameters were an initial step of 2 min at 50°C, 30 min at 60°C for reverse transcription, 5 min at 95°C for deactivation, then 40 cycles at 94°C for 20 sec and 59°C for 1 min for the melting and combined annealing and extension phases of the PCR. Each assay included duplicate standard curve samples, a no-template control and triplicate total RNA samples. All samples with a coefficient of variation >10% were retested.

TABLE I – CLINICOPATHOLOGIC CHARACTERISTICS AND TUMORAL TOTAL COX-2 mRNA EXPRESSION IN 60 PATIENTS WITH NSCLC

Variable	Number of cases	High total Cox-2 expression [1] (n = 30)	Low total Cox-2 expression (n = 30)	p^2
Total	60			
Age (years)				
≥ 63	33	17	16	0.795
< 63	27	13	14	
Sex				
Male	40	23	17	0.100
Female	20	7	13	
Histology				
Squamous cell carcinoma	26	14	12	0.602
Adenocarcinoma	34	16	18	
T status				
T1	15	8	7	0.766
T2–4	45	22	23	
N status				
N0	29	14	15	0.796
N1–3	31	16	15	
Stage				
I or II	33	15	18	0.463
IIIA, IIIB or IV	27	15	12	
Radiotherapy or chemotherapy				
Positive	25	11	14	0.432
Negative	35	19	16	

[1]A cut-off value ($-\Delta C_t$) of 0.045 was used to separate tumors with high and low tumoral total.–[2]p values derived from χ^2 test. $p < 0.05$ indicates statistical significance. COX-2 mRNA expression.



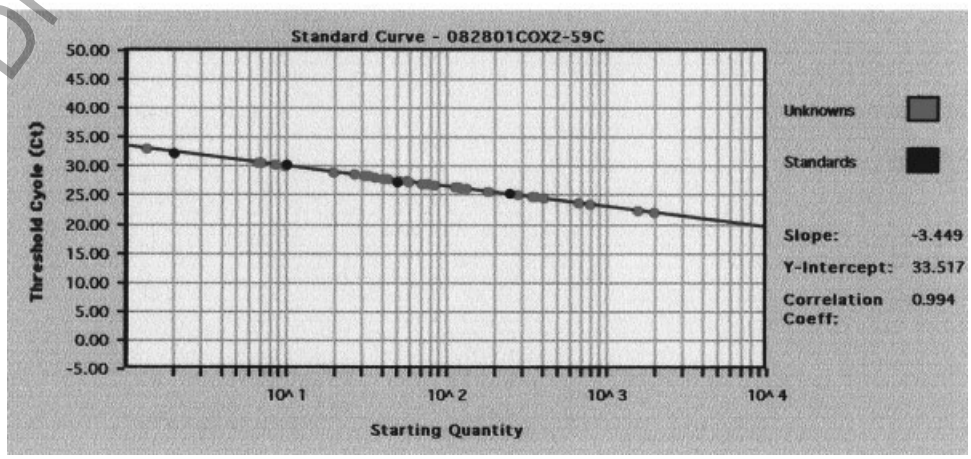


FIGURE 1 – Quantification of COX-2 mRNA expression in tumor samples by RTQ RT-PCR. *(a)* C_t values for COX-2 mRNA expression. *(b)* Standard curve. For each reaction, the fluorescence signal of the reporter dye (FAM) was divided by the fluorescence signal of the passive reference dye (ROX) to obtain a ratio defined as the normalized reported signal (Rn). ΔRn represents the normalized reporter signal (Rn) minus the baseline signal and is plotted against the cycle number. The C_t value represents the fractional cycle number at which a significant increase in Rn above a chosen threshold (horizontal black line) can first be detected. Standard curves show plots of C_t values against the log starting quantity of COX-2 mRNA. Black dots represent data for standard samples and red dots, data for unknown tissue samples.

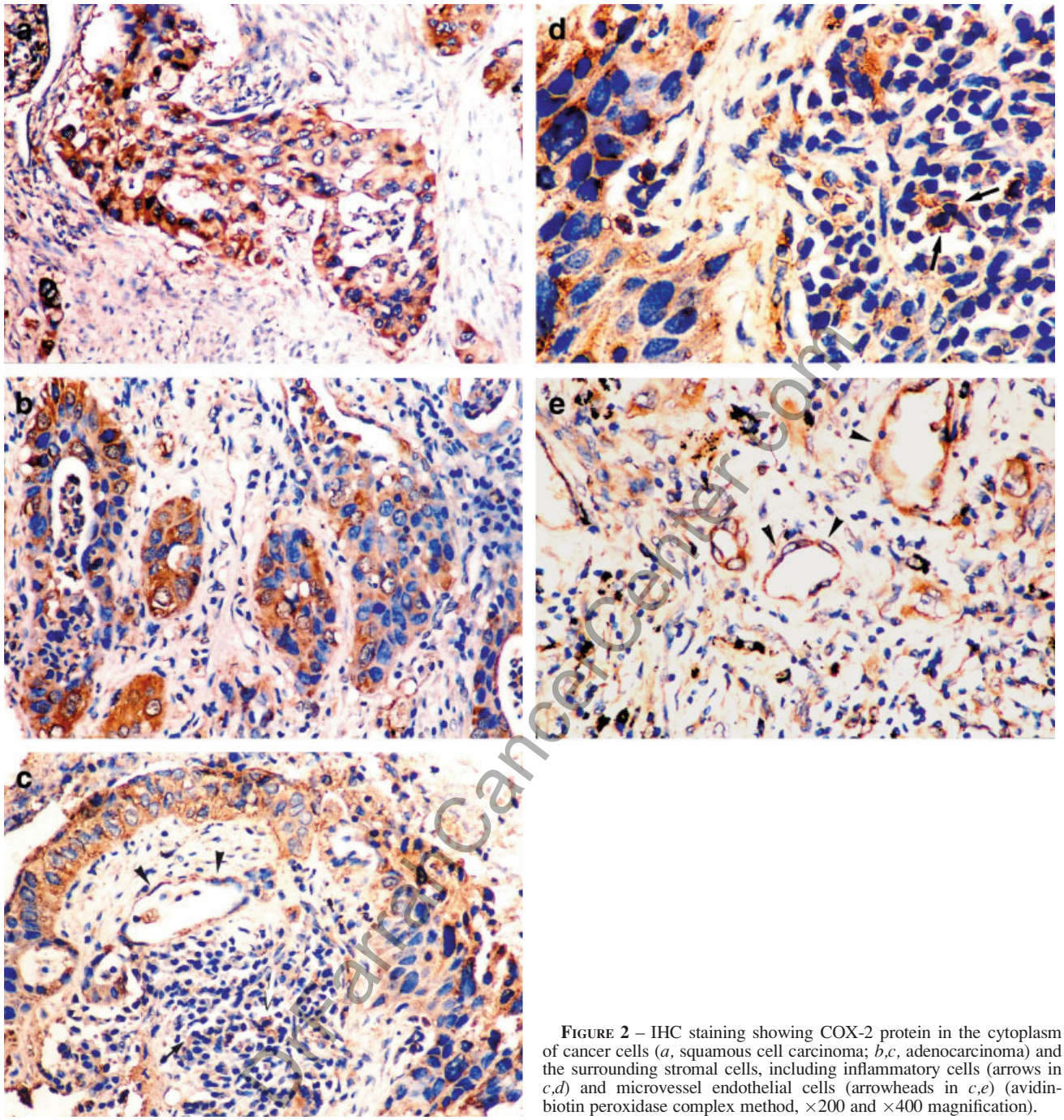


FIGURE 2 – IHC staining showing COX-2 protein in the cytoplasm of cancer cells (*a,* squamous cell carcinoma; *b,c,* adenocarcinoma) and the surrounding stromal cells, including inflammatory cells (arrows in *c,d*) and microvessel endothelial cells (arrowheads in *c,e*) (avidin-biotin peroxidase complex method, ×200 and ×400 magnification).

Detection of fluorescence emission and quantification of COX-2 and VEGF mRNA

Fluorescence emission from the reporter dye (6FAM, peak fluorescence emission 518 nm) was detected on-line in real time using an ABI prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems). The amount of COX-2 mRNA or VEGF mRNA in the tissue, standardized to TBP mRNA, was expressed as $-\Delta C_t = -[C_{t\ \text{COX-2 (or VEGF)}} - C_{t\ \text{TBP}}]$. The COX-2 or VEGF mRNA/TBP mRNA ratio was then calculated as $2^{-\Delta C_t} \times K$ (K, constant).

IHC staining for COX-2 protein, VEGF protein and microvessels

IHC staining for COX-2 protein and microvessels was performed using the avidin-biotin peroxidase method, with modifications as described previously.[57] Mouse MAbs against COX-2 protein (PG-27, 1:100 dilution; Oxford Biomedical Research, Oxford, UK), VEGF (1:50 dilution; Upstate Biotechnology, NY) or a polyclonal mouse anti-CD34 antiserum (1:100 dilution; Novocastra, Newcastle, UK; an endothelial cell marker) were used as primary antibodies. A colon cancer specimen known to overexpress COX-2 was used as the positive control for COX-2 protein expression, bronchial epithelium was used as the positive control for VEGF protein expression and the capillary surrounding the alveoli of normal lung tissue was used as the positive control for anti-CD34 staining. Negative controls were sections stained without the use of primary antibodies or using a control IgG instead of primary antibodies.

To evaluate the expression of VEGF protein, we established a score corresponding to *(i)* the percentage of positively staining

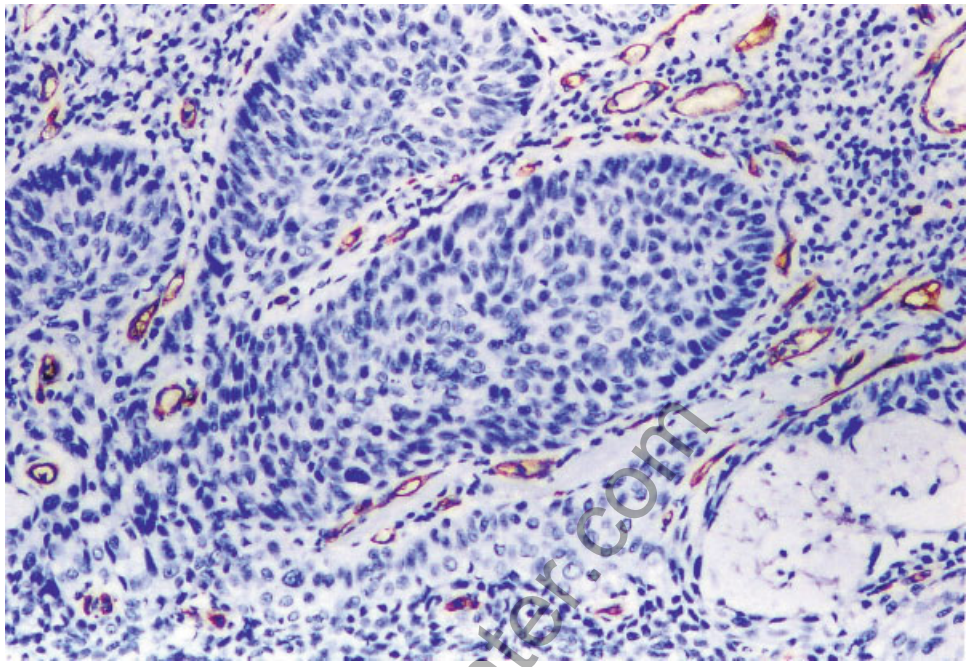


FIGURE 3 – IHC staining of microvessel endothelial cells (brown color) (avidin-biotin peroxidase complex method, ×200 magnification).

TABLE II – VEGF mRNA EXPRESSION, MVC AND PATIENT OUTCOMES IN TUMORS WITH HIGH OR LOW TOTAL COX-2 mRNA EXPRESSION

Variables	Tumoral COX-2 mRNA expression		p^1
	Low ($-\Delta_t < 0.045$)	High ($-\Delta_t \geq 0.045$)	
Number of cases	30	30	
VEGF mRNA expression[2]	8.24 ± 2.03	9.89 ± 2.40	0.006
MVC	92.6 ± 41.5	138.2 ± 52.3	<0.001
Survival (months)	40.0 ± 3.12	15.0 ± 2.61	<0.0001
Relapse (months)	34.0 ± 3.11	5.00 ± 1.37	<0.0001

[1]p values for difference of VEGF mRNA and MVC derived from independent 2-tailed t-test. Survival and relapse were expressed as median ± SE; p value for difference of survival and relapse derived from log-rank test. $p < 0.05$ indicates statistical significance.–[2]Value from $-\Delta C_t = -[C_{t\ (VEGF)} - C_{t(TBP)}]$.

cells and *(ii)* the staining intensity: grade 0, negative; grade 1, weak; grade 2, moderate; grade 3, high; grade 4, very high.[49] A similar semiquantitative IHC scoring method was also used for quantifying COX-2 protein expression in tumor cells or stromal cells. Scores ranged 0–3. Interobserver correlations for VEGF and COX-2 immunostaining were good ($r = 0.88$ and $r = 0.80$, respectively; $p < 0.001$).

Analysis of MVCs

Brown-immunostained endothelial cell clusters that were clearly separated from one another were considered as a single microvessel.[57] Microvessels in the area of most intense neovascularization were counted in 3 randomly chosen ×200 fields (×20 objective with ×10 ocular, 0.785 mm^2/field), and the average of 3 readings was taken as the MVC.

MVC was determined by 2 independent observers with no knowledge of the clinicopathologic variables, including COX-2 or VEGF mRNA expression. The interobserver correlation for MVC was good ($r = 0.86$, $p < 0.001$).

Statistical analysis

All statistical analyses were performed using SPSS (Chicago, IL) for Windows software (version 10.0). $-\Delta C_t$ and MVC values were analyzed as both continuous and dichotomous variables. Median values were used as the cut-off to distinguish between low and high levels of COX-2 or VEGF mRNA expression and between low and high MVCs. The independent sample 2-tailed t-test was used for comparison of VEGF mRNA expression and the MVC in tumors with high or low COX-2 mRNA expression. The χ^2 test[58] was used to compare categorical tumor variables. The correlation between tumoral COX-2 mRNA expression and VEGF mRNA or MVC was analyzed by linear regression.[58] The survival curve was obtained using the Kaplan-Meier method,[59] and differences in survival between patients with high and low COX-2 mRNA expression, high and low VEGF mRNA expression and high and low MVC were tested using the log-rank test.[58] The odds ratio was used to evaluate the relative risk of occurrence of relapse and mortality in patients with high tumoral COX-2 mRNA expression compared to patients with low expression. Multivariate analysis with the Cox regression model with backward elimination was used to identify the most important independent prognostic factors for predicting survival and relapse in NSCLC patients. $p < 0.05$ was considered statistically significant. When appropriate, the data are presented as means ± SD.

Results

Clinicopathologic variables

The clinicopathologic data for the 60 patients with NSCLC are listed in Table I. Using the medians for survival (27 months) and relapse time (14 months), 30 patients had short survival and 30, early disease relapse.

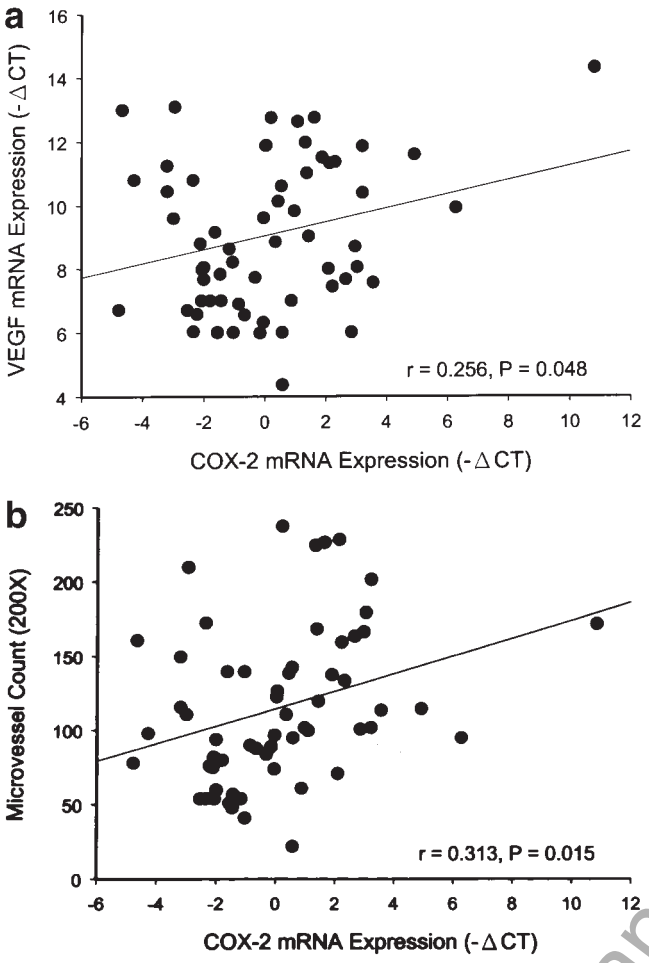


FIGURE 4 – Correlation of tumoral COX-2 mRNA expression with VEGF mRNA expression *(a)* and MVC *(b)* in NSCLC. Linear regression shows a significant positive correlation between tumoral COX-2 mRNA expression (*x* axis) and VEGF mRNA expression (*y* axis) ($r = 0.256$, $p = 0.048$) and between tumoral COX-2 mRNA expression (*x* axis) and MVC (*y* axis) ($r = 0.313$, $p = 0.015$).

Tumoral COX-2 and VEGF mRNA expression

COX-2 mRNA expression in tumor tissue (including both cancer cells and stromal cells) standardized to TBP mRNA was expressed as $-\Delta C_t = -(C_{t\ COX\text{-}2} - C_{t\ TBP})$. Figure 1 shows the C_t value for COX-2 mRNA expression and the corresponding standard curve. The $-\Delta C_t$ value for COX-2 mRNA expression in the 60 lung cancer tissue samples ranged from –4.79 to 10.85, with a mean ± SD of 0.11 ± 2.75 and a median of 0.045. Using the median value as the cut-off, 30 tumors showed high COX-2 mRNA expression and 30, low expression. When compared to other clinicopathologic variables, high COX-2 mRNA expression was not associated with histologic type, sex, age, tumor status, lymph node status, tumor stage or radiotherapy/chemotherapy status (Table I).

VEGF mRNA expression in tumor samples, expressed as $-\Delta C_t$, ranged 4.36–14.30, with a mean ± SD of 9.07 ± 2.36 and a median of 8.75. Using the median value as a cut-off point, 30 tumors had high VEGF mRNA expression and 30, low expression.

IHC staining for COX-2 protein, VEGF protein and microvessel endothelium

Both lung cancer cells and stromal cells stained positive for COX-2 protein expression. In lung cancer cells, COX-2 protein staining was scattered or diffuse in squamous cell carcinomas and adenocarcinomas (Fig. 2*a,b*). Inflammatory cells and microvessel endothelial cells in stroma also showed strong positive staining (Fig. 2*c–e*). High COX-2 protein expression (assessed by the semiquantitative IHC scoring method) was associated with high total COX-2 mRNA expression in tumor cells ($p = 0.113$, χ^2 test) and in stromal cells ($p = 0.176$, χ^2 test). The high total COX-2 protein expression (combination of tumor cell and stromal cell scores) was strongly and significantly associated with high total COX-2 mRNA expression, quantified by RTQ RT-PCR in lung cancers ($p = 0.019$, χ^2 test)

VEGF proteins were expressed predominantly in the cytoplasm of lung cancer cells of tumor specimens. Little staining was found in the stromal cells or tissue. VEGF expression in cancer cells was evaluated by a semiquantitative grading method, and the mean ± SD was 2.9 ± 0.97. There was a strong correlation between VEGF protein expression assessed by IHC and tumoral VEGF mRNA expression quantified by RTQ RT-PCR ($r = 0.91$, $p < 0.001$).

Endothelial cells of the microvessels stained positive with CD34 MAb (Fig. 3). MVC in lung cancer tissue ranged 22–238 (per ×200 field), the mean ± SD being 115 ± 52.1 and the median being 102. Using the median value as the cut-off, 31 tumors had high MVC and 29, low MVC.

Correlation between total COX-2 and VEGF mRNA expression

VEGF mRNA expression in tumors with high total COX-2 mRNA expression was significantly higher than in those with low total COX-2 mRNA expression ($-\Delta C_t = 9.89 \pm 2.40$ *vs.* 8.24 ± 2.03, respectively; $p = 0.006$, independent sample *t*-test) (Table II). Tumors with high total COX-2 mRNA expression showed a greater frequency of high VEGF mRNA expression ($-\Delta C_{t\ VEGF} \geq 8.75$, 20 of 30) than those with low total COX-2 mRNA expression (10 of 30; $p = 0.01$, χ^2 test). When COX-2 or VEGF mRNA expression was treated as a continuous variable, linear regression showed that tumoral total COX-2 mRNA expression correlated positively with VEGF mRNA expression ($r = 0.256$, $p = 0.048$) (Fig. 4*a*).

Correlation between total COX-2 mRNA expression and MVC

The MVC in tumors with high total COX-2 mRNA expression was 138.2 ± 52.3, significantly higher than that in tumors with low total COX-2 mRNA expression (92.6 ± 41.5, $p < 0.001$, independent sample *t*-test) (Table II). A high MVC (≥102, ×200) was seen in 23 of 30 tumors with high total COX-2 mRNA expression but in only 8 of 30 with low total COX-2 mRNA expression ($p < 0.001$, χ^2 test). Linear regression also showed that there was a significant positive correlation between tumoral total COX-2 mRNA expression and intratumoral MVC ($r = 0.313$, $p = 0.015$) (Fig. 4*b*).

COX-2 mRNA, VEGF mRNA, MVC and prognosis

As shown in Table II, the median survival time for patients with tumors with high total COX-2 mRNA expression ($-\Delta C_t \geq 0.045$) was 15.0 ± 2.61 months, significantly shorter than that for patients with tumors with low total COX-2 mRNA expression (40.0 ± 3.12 months, $p < 0.0001$) (Fig. 5*a*).

After stratification of surgicopathologic stages into stage I and stage II–IV, patients with tumors with high total COX-2 mRNA expression still had a significant shorter survival than patients with tumors with low total COX-2 mRNA expression ($p = 0.0015$ for stage I, $p < 0.0001$ for stage II–IV, log-rank test) (Fig. 5*b,c*). After stratification by histologic types, the survival difference between patients with tumors with high and low total COX-2 mRNA expression was still significant ($p = 0.017$ for squamous cell carcinoma, $p = 0.0001$ for adenocarcinoma, log-rank test) (Fig. 5*d,e*).

High COX-2 protein expression in tumor cells was associated with short patient survival ($p = 0.03$). In contrast, the association between high COX-2 protein expression in stromal cells and short

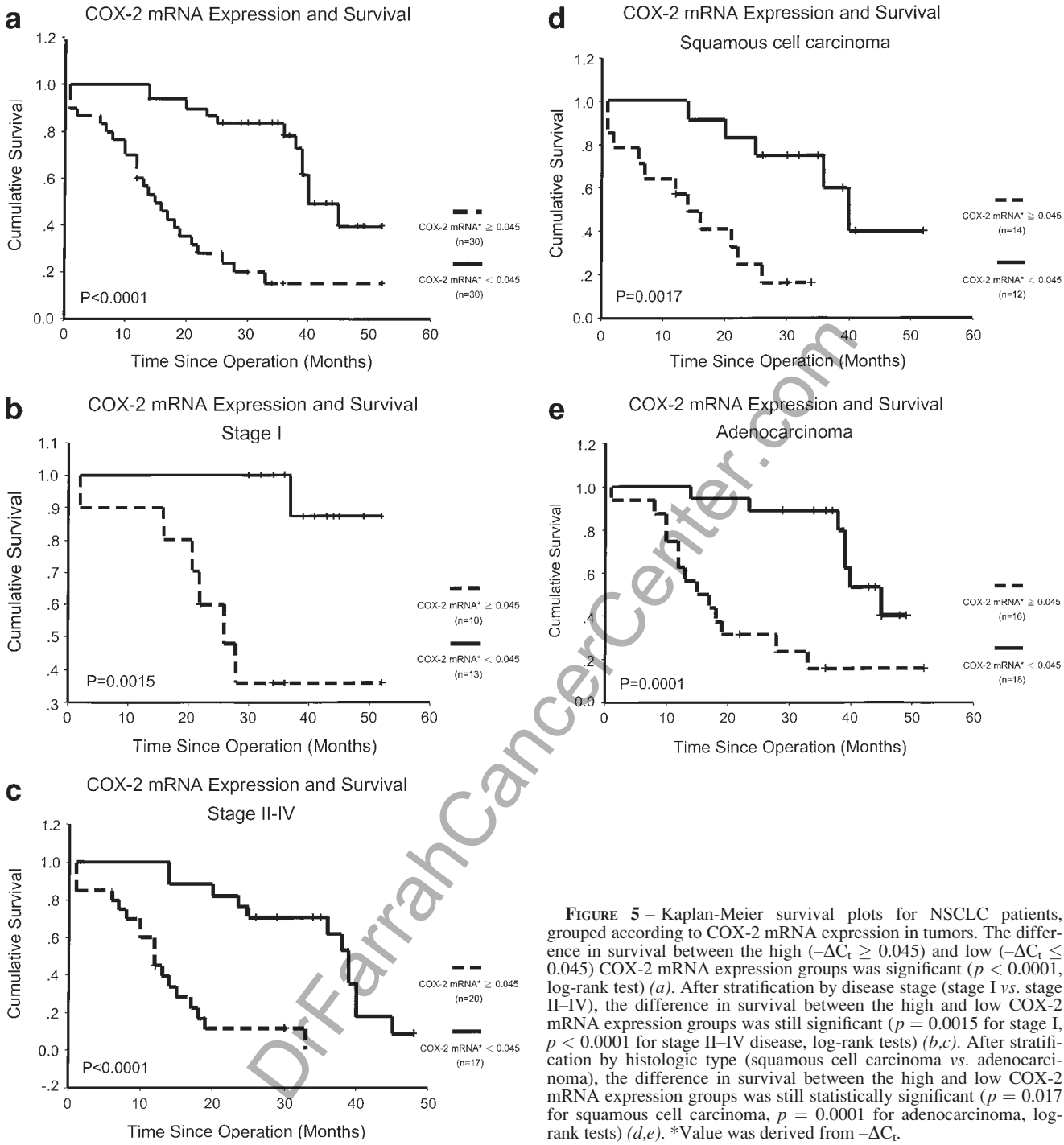


FIGURE 5 – Kaplan-Meier survival plots for NSCLC patients, grouped according to COX-2 mRNA expression in tumors. The difference in survival between the high ($-\Delta C_t \geq 0.045$) and low ($-\Delta C_t \leq 0.045$) COX-2 mRNA expression groups was significant ($p < 0.0001$, log-rank test) *(a)*. After stratification by disease stage (stage I *vs.* stage II–IV), the difference in survival between the high and low COX-2 mRNA expression groups was still significant ($p = 0.0015$ for stage I, $p < 0.0001$ for stage II–IV disease, log-rank tests) *(b,c)*. After stratification by histologic type (squamous cell carcinoma *vs.* adenocarcinoma), the difference in survival between the high and low COX-2 mRNA expression groups was still statistically significant ($p = 0.017$ for squamous cell carcinoma, $p = 0.0001$ for adenocarcinoma, log-rank tests) *(d,e)*. *Value was derived from $-\Delta C_t$.

patient survival did not reach statistical significance ($p = 0.28$). However, when we combined COX-2 IHC scores in these 2 compartments, the results showed that high total COX-2 expression (in both tumor and stromal cells) was strongly and significantly associated with short survival ($p = 0.0016$).

Tumoral VEGF mRNA expression and MVC both correlated negatively with survival. Survival for patients with high tumoral VEGF mRNA or a high intratumoral MVC was significantly shorter than for those with tumors with low VEGF mRNA expression or a low intratumoral MVC ($p = 0.0046$ for VEGF mRNA expression and $p = 0.0038$ for MVC, log-rank test) (Fig. 6*a,b*).

Tumoral total COX-2 mRNA expression also correlated with disease relapse in NSCLC. The median relapse time for patients with tumors with high total COX-2 mRNA expression was 5.0 ± 1.37 months, significantly shorter than that for patients with tumors with low total COX-2 mRNA expression (34.0 ± 3.10 months; $p < 0.0001$, log-rank test) (Table II). This difference in relapse time was still significant after stratification by disease stage ($p = 0.0084$ for stage I, $p < 0.0001$ for stage II–IV) or by histologic type ($p = 0.0097$ for squamous cell carcinoma, $p < 0.0001$ for adenocarcinoma).

Patients with tumors with high VEGF mRNA or a high intratumoral MVC also had a significantly shorter relapse time than those

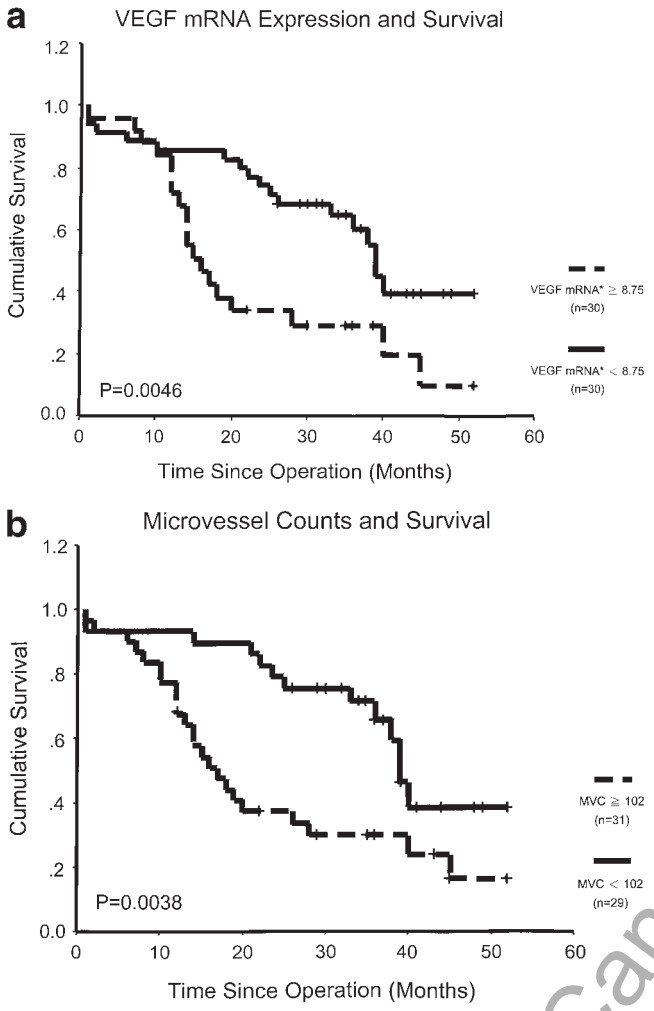


FIGURE 6 – Kaplan-Meier survival plots for NSCLC patients grouped according to high or low tumoral VEGF mRNA expression *(a)* and high or low intratumoral MVC *(b)*. The difference in survival between the high and low tumoral VEGF mRNA expression groups ($-\Delta C_t \geq$ or $<$ 8.75) (median survival $\pm$ SE, 16.0 $\pm$ 3.12 months *vs.* 39.0 $\pm$ 1.28 months) or high and low intratumoral MVC groups ($\geq$102 *vs.* $<$ 102, $\times$200) (median survival $\pm$ SE, 17.0 $\pm$ 2.69 months *vs.* 39.0 $\pm$ 1.11 months) was statistically significant (p = 0.0046 and p = 0.0038, respectively, log-rank test).

with tumors with low VEGF mRNA expression or a low intratumoral MVC (p = 0.0033 for VEGF mRNA expression, p = 0.0117 for MVC).

In addition, we used the odds ratio to evaluate the relative risk of occurrence of relapse or mortality in patients with high tumoral total COX-2 mRNA expression compared to those with low tumoral total COX-2 mRNA expression (Table III). The relative risk of patients with tumors with high total COX-2 mRNA expression having a disease relapse or mortality tended to be higher in those with late disease stages (stage III–IV) (odds ratios = 5.87 and 12.64, respectively), while the relative risk for disease relapse tended to be higher in patients with adenocarcinoma (odds ratio = 6.44).

Multivariate analysis using the Cox regression model with backward elimination showed that, among the potential prognostic factors (age, sex, tumor status, nodal status, disease stage, COX-2 mRNA expression, VEGF mRNA expression and MVC), tumoral total COX-2 mRNA expression and lymph node metastasis were the most important for survival ($p < 0.0001$ and $p < 0.0001$, respectively) and disease relapse (local recurrence or distal metastasis; $p < 0.0001$ and $p = 0.0006$, respectively) (Table IV).

Discussion

In our study, using IHC staining, we found that elevated COX-2 protein was expressed not only in lung cancer cells but also in tumor-infiltrating inflammatory cells and microvessel endothelial cells. In contrast, VEGF protein was mainly expressed in cancer cells. We used RTQ RT-PCR to evaluate total COX-2 mRNA expression in cancer and surrounding stromal cells in NSCLC and investigated the relationship between total COX-2 mRNA expression and VEGF mRNA expression (one of the most potent endothelial cell-specific angiogenic factors), intratumoral MVC or prognosis in NSCLC. Our results showed that tumoral total COX-2 mRNA expression (in cancer and stromal cells) correlated positively with VEGF mRNA expression and MVC. High tumoral COX-2 mRNA expression was also significantly associated with early relapse and short survival. Stratification analysis showed that the prognostic significance was valid in both stage I and stage II–IV disease and in both squamous cell carcinoma and adenocarcinoma. Nevertheless, the relative risk of patients with high tumoral total COX-2 mRNA expression having a disease relapse and mortality tended to be higher in advanced disease, and the relative risk of patients with high tumoral total COX-2 mRNA expression having a disease relapse tended to be higher in adenocarcinoma. Thus, our *in vivo* evidence positively links total tumoral COX-2 mRNA expression in both cancer and stromal cells with VEGF mRNA expression, intratumoral MVC and prognosis in patients with NSCLC and shows that the prognostic significance of COX-2 mRNA expression is valid in early (stage I) and late (stage II-IV) disease and in squamous cell carcinoma and adenocarcinoma.

The mechanism by which COX-2 contributes to tumor angiogenesis has been investigated in several *in vitro* and animal studies.[29,31,32] Tsujii *et al.*[29] used an endothelial cell/colon cancer coculture system and showed that COX-2-overexpressing CaCo-2 and HCA-7 cells stimulate endothelial cell motility and tube formation by increasing the production of proangiogenic factors, including VEGF, bFGF and PDGF, and that this effect is blocked by NS-398, a selective COX-2 inhibitor. COX-2 metabolic products, such as PGE_2, PGI_2 and thromboxane A_2, increase endothelial cell migration and experimental angiogenesis.[60,61] COX-2 also plays a role in the activation of survival genes, such as *Bcl-2* and serine threonine kinase *Akt,* which may contribute to apoptosis resistance in endothelial cells.[28,62]

Several *in vitro* studies have suggested that VEGF is one of the most important factors involved in the stimulation of tumor angiogenesis by COX-2.[9,52,63] Oshima *et al.*[9] showed that treatment of $APC^{\Delta 716}$ mice with a specific COX-2 inhibitor resulted in a dose-dependent reduction in the number and size of colon polyps and in a significant decrease in VEGF production. Gallo *et al.*[63] showed that COX-2 activation in the epidermal tumor cell lines CA-431 and SCC-91 causes rapid induction of VEGF mRNA and protein production in neoplastic cells. $COX\text{-}2^{-/-}$ mouse fibroblasts also showed a 94% reduction in VEGF protein levels compared to wild-type fibroblasts.[52] However, only a few *in vivo* studies have demonstrated upregulation of VEGF expression and subsequent tumor angiogenesis by COX-2 overexpression in human cancer; this has been shown in colorectal cancer,[34] head-and-neck squamous cell carcinoma[35] and endometrial carcinoma.[36]

Our study shows that tumoral total COX-2 mRNA expression in cancer and stromal cells correlates positively with VEGF mRNA expression, intratumoral MVC and prognosis in NSCLC. This provides *in vivo* evidence that COX-2 overexpression in cancer cells and stromal cells plays an important role in upregulation of VEGF expression and enhancement of tumor angiogenesis in NSCLC.

Our results also support, in part, the findings of Marrogi *et al.,*[37] who showed a correlation between expression of nitric oxide synthase, COX-2 and VEGF protein in NSCLC cancer cells but no

TABLE III – RELATIVE RISK (RR)[1] AND 95% CONFIDENCE INTERVAL (CI) OF RELAPSE AND MORTALITY FOR PATIENTS WITH HIGH COMPARED TO LOW TOTAL TUMORAL COX-2 mRNA EXPRESSION

	Number	RR (95% CI) for relapse	RR (95% CI) for mortality
Total	60	4.71 (2.41–9.19)	5.68 (2.70–11.96)
Stage			
1–2	33	4.15 (1.64–10.47)	5.63 (1.88–16.84)
3–4	27	5.87 (2.10–16.38)	12.64 (2.17–59.00)
Histology			
Squamous cell carcinoma	26	3.28 (1.24–8.55)	6.20 (1.70–22.67)
Adenocarinoma	34	6.44 (2.51–16.51)	5.57 (2.11–14.74)

[1]RR for early relapse and short survival derived from odds ratio of Cox regression analysis.

TABLE IV – MULTIVARIATE ANALYSIS WITH COX REGRESSION MODEL FOR PATIENT SURVIVAL AND DISEASE RELAPSE[1]

	B	SE	Exp(B)	95% CI	p^2
Survival					
Total COX-2 mRNA expression	2.405	0.467	11.084	3.16–31.13	<0.0001
Lymph node status	−1.639	0.428	0.194	0.07–0.48	<0.0001
Relapse					
Total COX-2 mRNA expression	2.038	0.392	7.675	3.56–16.56	<0.0001
Lymph node status	−1.26	0.369	0.284	0.14–0.58	0.0006

[1]Total COX-2 mRNA expression and lymph node status were the two factors that remained statistically significant in the backward elimination analysis.[2]$p<0.05$ is statistically significant.

correlation between these factors and patient survival. In contrast, Khuri *et al.*[38] studied COX-2 mRNA expression using ISH in patients with NSCLC cells and found no correlation between COX-2 mRNA expression and expression of angiogenic factors, including VEGF, IL-8, bFGF and MVC. This difference may be due to the different methods used to detect COX-2 expression. Khuri *et al.*[38] used ISH to detect COX-2 mRNA expression in cancer cells, while we used RTQ RT-PCR to detect total COX-2 mRNA expression in cancer cells and stromal tissue in lung cancer specimens. In our IHC staining, COX-2 protein was found in the cancer cells, stromal inflammatory cells and microvascular endothelial cells. A similar distribution of COX-2 protein has been shown in colon, breast and prostate cancers as well as malignant melanoma.[33,51,52] The cancer cell–stromal cell interaction has been considered important for tumor growth and progression.[18,64] COX-2 might also facilitate tumor progression through effects on cancer cells themselves (cell autonomous effects) and on the stromal microenvironment (landscaping effects).[18] Thus, evaluation of COX-2 expression in both cancer and stromal cells might be a more reasonable method to use in future investigations. RTQ RT-PCR can provide a way to accurately quantitate COX-2 mRNA expression in both cancer cells and the stromal cell compartment in solid tumors. This difference in methods to assess COX-2 expression in tumor specimens (cancer cells *vs.* cancer cells plus stromal cells) may explain, in part, the difference in results between our and other studies.

COX-2 overexpression has been associated with an adverse prognosis in a variety of human cancers, including colorectal cancer, cervical cancer, pancreatic cancer, mesothelioma and invasive bladder cancer.[35,39,42,65] In our study, we also showed that survival time and relapse time were significantly shorter in NSCLC patients with tumors with high total COX-2 mRNA expression in cancer cells and stromal cells. Several studies have investigated the prognostic significance of COX-2 expression in NSCLC but with different results.[37,38,44] Marrogi *et al.*[37] showed no significant correlation between COX-2 protein expression in cancer cells and survival. Achiwa *et al.*[44] showed no prognostic significance of COX-2 expression in cancer cells in their entire cohort of patients with resected lung adenocarcinoma but a significant relationship between elevated COX-2 expression and short survival in the stage I subgroup. Khuri *et al.*[38] showed lack of a good association between COX-2 overexpression in cancer cells and survival in stage I NSCLC ($p = 0.106$).

In our study, after stratification by disease stage (stage I *vs.* stage II–IV), the prognostic significance (survival and relapse) of total COX-2 mRNA overexpression was still valid in both early and late disease stage subgroups. This implies that COX-2 expression might have an effect on facilitating tumor progression and metastasis in both the early and late stages of NSCLC. However, although one study emphasized that adenocarcinoma is the main histologic subtype with high COX-2 protein expression in NSCLC,[45] our data and those of others[38] did not show any difference in COX-2 expression between the 2 main histologic types of NSCLC, *i.e.,* squamous cell carcinoma and adenocarcinoma. Furthermore, few studies have compared the prognostic significance of tumoral COX-2 expression in squamous cell carcinoma and adenocarcinoma. Our data showed that, after stratification by these 2 histologic types, the prognostic effect (survival and disease relapse) of total COX-2 mRNA expression was still statistically significant in both subgroups. The relative risk of disease relapse for patients with high tumoral COX-2 mRNA (compared to low COX-2 mRNA) was 4.71 for total, 4.15 for early stage, 5.87 for advanced stage, 3.26 for squamous cell carcinoma and 6.44 for adenocarcinoma. The relative risk of mortality for patients with high tumoral COX-2 mRNA was 5.68 for total, 5.63 for early stage, 12.64 for late stage, 6.20 for squamous cell carcinoma and 5.57 for adenocarcinoma. The relative risk for patients with high tumoral COX-2 mRNA expression having a relapse and mortality tended to be higher with advanced disease stage, while the relative risk of disease relapse tended to be higher in adenocarcinoma, though the trend did not reach statistical significance. This information might be important in the choice of adjuvant therapy with selective COX-2 inhibitors for NSCLC patients.

Our results showed that total tumoral COX-2 mRNA expression did not correlate with tumor size, lymph node status or other classical clinicopathologic prognostic factors. This implies that tumor COX-2 mRNA expression may be an independent prognostic factor for patients with NSCLC. Multivariate analysis using the Cox regression model confirmed that COX-2 mRNA expression and lymph node metastasis were the 2 most important prognostic predictors for survival and relapse.

We conclude that total COX-2 mRNA expression in the cancer and stromal compartments correlates significantly with angiogenic factor VEGF mRNA expression and with intratumoral MVC. This implies that COX-2 expression in cancer cells themselves and surrounding stromal cells may play an important role in the regulation of VEGF expression and tumor angiogenesis and may explain, in part, the adverse effect of COX-2 overexpression on prognosis in NSCLC. Our results also suggest that COX-2 expression may be used as a prognostic indicator and as a therapeutic target (NSAIDs) in NSCLC patients.

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