Correlations between Cyclooxygenase-2 Expression and Angiogenic Factors in Primary Tumors and Liver Metastases in Colorectal Cancer

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Background: Angiogenesis is required for growth and metastasis of colorectal cancer (CRC), and several positive regulators of tumor angiogenesis have been identified. Cyclooxygenase-2 (COX-2), known to be elevated in several human cancers, regulates angiogenesis by inducing angiogenic factors. The aim of this study was to clarify the levels and evaluate the relationships of COX-2, vascular endothelial growth factor A and C, thymidine phosphorylase (TP) and microvascular density (MVD) in paired tissue specimens between primary CRC and corresponding metastatic liver cancer.

Methods: Tissue samples from pairs of primary tumors and corresponding metastatic liver tumors from 44 patients with CRC were immunohistochemically evaluated for COX-2, VEGF-A, VEGF-C, TP and MVD.

Results: The primary and corresponding metastatic liver tumors tended to show concordant immunoreactivity for COX-2 ($P = 0.005$, $rs = 0.428$), VEGF-A ($P = 0.039$, $rs = 0.314$), TP ($P = 0.005$, $rs = 0.422$) and MVD ($P = 0.046$, $rs = 0.304$) by Spearman rank test. The rate of COX-2 immunoreactivity was higher in liver metastases than in primary tumors ($P = 0.002$), while the rate of VEGF-A was higher in primary tumors than in liver metastases ($P = 0.0004$). The incidence of TP immunoreactivity and the level of MVD did not differ between primary and metastatic liver tumors ($P = 0.247$; $P = 0.229$). Significant correlations were found between COX-2 immunoreactivity and VEGF-A immunoreactivity in metastatic liver tumors ($P = 0.033$) as well as in primary tumors ($P = 0.008$).

Conclusion: The positive correlations between COX-2, VEGF-A, TP and MVD in primary CRC and liver metastasis as demonstrated here will help to predict the angiogenic activity of liver metastasis by analyzing primary tumors, allowing for individualized cancer treatment options.

Key words: primary colorectal cancer – liver metastases – angiogenesis – cyclooxygenase-2

INTRODUCTION

Colorectal cancer (CRC) is one of the most common causes of cancer death in the world, accounting for more than 150,000 new cases and 55,000 deaths every year in the USA and approximately 125,000 mortalities each year in the European Union (1,2). An increasing trend in the incidence of this cancer has been noted in Asian nations, including Japan (3). Liver metastasis of CRC is an important prognostic factor and occurs in 20–25% of CRC patients (4). Five-year survival rate for patients with resection of hepatic metastases reaches 30–40%, while the resection rate is reported to be about 30% (5).

It has been shown that tumor angiogenesis plays a critical role in tumor growth, invasion, metastasis and recurrence. Without the ability to recruit new blood vessels, most tumors would never grow beyond 1–2 mm in diameter and would remain localized to the primary site (6–9). Several studies have identified growth factors that promote angiogenesis, such as cyclooxygenase-2 (COX-2), vascular endothelial growth factors A and C (VEGF-A and VEGF-C) and thymidine phosphorylase (TP). These are reportedly useful prognostic markers in many carcinomas, including CRC (10–12). VEGF is a potent mitogen for vascular endothelial
cells and its expression has been strongly correlated with tumor angiogenesis and lymph node metastasis in CRC (13,14).

Cyclooxygenase-2 (COX-2), a key enzyme involved in the conversion of arachidonic acid to prostaglandin H, synthesizes inflammatory mediators that induce the chronic inflammatory state, thereby promoting tumor growth and metastasis formation in CRC (15–18). Sano et al. (19) reported that human CRC overexpressed COX-2 and numerous experimental studies have indicated that COX-2 regulates cancer-induced angiogenesis by stimulating the release of VEGF and increasing the production of PGs. There is also sufficient evidence to suggest that COX-2 inhibitors suppress angiogenesis in CRC, and new research has focused on angiogenesis control as an approach for cancer treatment (16,20–24).

The aim of this study was to clarify the levels and evaluate the relationships of COX-2, VEGF-A, VEGF-C, TP and microvascular density (MVD) in paired tissue specimens between primary CRC and corresponding metastatic liver tumors. This research will help elucidate the angiogenic potential of liver metastases by analyzing primary tumors. In addition, the present observations suggest that knowledge of molecular features may help determine prognosis or anticancer drug sensitivity in patients with liver metastasis by analyzing primary CRC.

MATERIALS AND METHODS

PATIENTS AND TISSUE SAMPLES

The surgically resected specimens used for this study were obtained from 44 patients with CRC at the Department of Surgical Oncology, Tokyo Medical and Dental University and Kudanzaka Hospital, Japan, from October 1991 to June 2005. This study was approved by the institutional Review Boards of the Tokyo Medical and Dental University and Kudanzaka Hospital. Primary colorectal tumors and corresponding liver metastases were obtained from each patient. Twenty-five specimens were diagnosed with metachronous and 19 with synchronous liver metastases. There were 32 males and 12 females, with a mean age of 61 years (SD 8.9 years; 42–76 years). In all cases, archival hematoxylin and eosin-stained (H&E) slides of the primary tumor and respective liver metastases were retrieved and reviewed to confirm pathological features, as well as to select suitable tissue blocks for immunohistochemical analysis.

IMMUNOHISTOCHEMISTRY

IMMUNOHISTOCHEMICAL STAINING FOR COX-2 ANTIGEN, VEGF-A ANTIGEN, VEGF-C ANTIGEN AND CD34 ANTIGEN

COX-2 antigen, VEGF-A antigen, VEGF-C antigen and CD34 antigen were stained using the universal immunoenzyme polymer method, as described in our previous publications (14,25). To block the intrinsic biotin-binding activity in liver metastases, hepatic tissue slides were treated with avidin–biotin blocking kit reagents (Vector Laboratories Inc., Burlingame, CA, USA) for 15 min. We used the following primary antibodies: a mouse monoclonal antibody against human COX-2 (Cayman Chemical Co., Ann Arbor, MI, USA; dilution 1:250; for 2 h at room temperature), a rabbit polyclonal antibody against human VEGF-A (CH-190 Santa Cruz Biotechnology; dilution 1:200; for 1 h at room temperature), a rabbit polyclonal anti-VEGF-C (CH-190 Santa Cruz Biotechnology; dilution 1:50; overnight at 4°C) and a mouse monoclonal antibody against human CD34 (CD34 Class II Clone QBEnd-10, Dako Cytomation, Denmark A/S; dilution 1:50; for 1.5 h at room temperature).

Negative controls used nonspecific IgG as the primary antibody.

IMMUNOHISTOCHEMICAL STAINING FOR TP ANTIGEN

The universal immunoenzyme polymer method was used for immunostaining. Sections (3 μm) were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-lysine coated slides, dewaxed in xylene, and rehydrated through a graded series of ethanol. After deparaffinization, antigen retrieval treatment was performed by heating in a microwave oven (500 W) twice for 5 min each in 10 mmol/l sodium citrate buffer (pH 6.0), followed by treatment with 3% hydrogen peroxide in methanol solution for 20 min in order to quench endogenous peroxidase activity. Nonspecific binding was blocked by treating slides with 1% Blocking Ace for 30 min. To block intrinsic biotin-binding activity, hepatic tissue slides were treated with avidin–biotin blocking kit reagents (Vector Laboratories) for 15 min. Thereafter, slides were incubated with mouse monoclonal antibodies against human TP (dilution 1:1000; 1C6-203, Roche) for 90 min at room temperature for TP. Slides were then incubated with labeled polymer (N-Histofine Simple Stain MAX PO MULTI; Nichirei Co., Tokyo, Japan) for 30 min at room temperature. Color development was performed using 0.02% 3,3’-diaminobenzidine tetrahydrochloride (Sigma) and 0.06% hydrogen peroxide in 50 mmol/l Tris–HCl (pH 7.6) for 5 min. Finally, slides were counterstained with 1% Meyer’s hematoxylin. As a negative control for TP staining, tissue sections were treated with normal serum instead of primary antibody.

In order to evaluate the staining intensity of COX-2, VEGF, TP and MVD, tissue samples (human primary CRC) from our laboratory of pathology were used as positive controls, as described in our previous studies (10,14,25).

EVALUATION OF STAINING

COX-2

All sections were scored under a light microscope by a researcher who was blinded to clinical data. For COX-2
assessment, the entire tissue section was scanned to assign the scores. Staining intensity was scored as 0 (negative), 1 (weak), 2 (medium) or 3 (strong). Extent of staining was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%) or 4 (76–100%), according to the proportion of positive areas in relation to the whole carcinoma area. The sum of intensity and extent scores was used as the final immunoreactivity score (0–7) for COX-2. Tumors having a final staining score of 4 were considered positive for primary CRC and liver metastases. Results obtained using this scoring method correlated well between independent evaluators, as observed in previous studies (25–27).

**VEGF-A and VEGF-C**

Cytoplasmic VEGF-A and VEGF-C immunoreactivity was evaluated according to positive tumor cell percentage (extension) as follows: 0, if ≤1%; 1, if >1% and ≤20%; 2, if >20% and ≤50%; 3, if >50% and ≤80%; or 4, if >80%. For staining intensity: 1 = weak; 2 = moderate; or 3 = strong. Final classification was obtained by combining the two score values (sum) as follows: negative (sum range 0–2), low (3–5) or high (6–7) expression. VEGF-A and C were considered positive when the final score was ≥5 for the primary and liver metastasis samples (28).

**TP**

Tumors were assessed for TP based on the proportion of cell staining. The proportion of cells stained was categorized as 0, no staining; 1, <25% positive staining; 2, 25–75% positive staining; or 3, >75% positive staining. A cutoff point of 25% was selected and the patients were separated into two groups; samples were considered TP positive when >25% of the tumor cells were stained and TP negative when <25% of the cells were stained (29).

**MVD**

MVD was assessed according to international consensus. The entire section was scanned systematically at ×100 magnification in order to identify the most intense areas of neovascularization within the tumor. After three areas with the highest number of capillaries and small venules were identified, microvessels were counted at ×200 magnification using a ×20 objective, and the average of count in three fields was calculated. Samples with a count of ≥97 were considered positive for primary CRC and samples with a count of ≥91 were considered positive for liver metastases (30).

**Statistical Analysis**

All statistical analyses were carried out using StatView Software (version 5.0). Correlations between expression of COX-2, VEGF-A, VEGF-C, TP and MVD and various clinicopathological parameters were assessed by χ² test. Differences in tumor marker scores between primary and metastatic liver tumors were assessed using Wilcoxon signed rank test. Correlations between VEGF-A, VEGF-C, COX-2, TP and MVD scores in CRC and those in liver metastases were determined by Spearman rank test. Statistical significance was set at P = 0.05.

**RESULTS**

Scores for the tumor markers were assessed in 44 pairs of primary CRC and corresponding liver metastases. No significant differences in COX-2, VEGF-A, VEGF-C, TP and MVD expression were observed for clinical pathological features such as age, location of primary tumor, time (synchronous or metachronous), vascular or lymph node invasion, Duke’s stage or tumor grade. In this study, only COX-2 and TP expression were significantly correlated with gender and histopathological type, respectively, and VEGF-C expression was positively correlated with depth of invasion (Table 1).

Representative COX-2 staining scores are shown in Figure 1.

**Differences in Individual Tumor Markers Expression Scores between Primary Tumor and Liver Metastases**

Higher expression scores were observed for COX-2 and VEGF-A, while lower expression scores were observed for VEGF-C, in primary CRC when compared with liver metastases.

As shown in Table 2, COX-2 expression was significantly higher in liver metastases than in the corresponding primary tumor (89 and 66%, respectively; P = 0.002). At the same time, VEGF-A score in the primary tumor was significantly higher than in liver metastases (68 and 30%, respectively; P = 0.0004). TP and MVD scores did not differ significantly between liver metastases and primary tumors (P = 0.247 and P = 0.229, respectively). Only VEGF-C in primary tumors showed higher expression when compared with liver metastases (P = 0.02).

**Individual Correlations in Tumor Marker Scores between Primary Tumors and Liver Metastases**

In individual patients, the status of COX-2, VEGF-A, TP and MVD were significantly correlated between primary tumors and corresponding metastatic liver tumors (P = 0.005; rs = 0.428 (Fig. 2); P = 0.039, rs = 0.314; P = 0.005; rs = 0.422; P = 0.046, rs = 0.304, respectively) by Spearman rank test. No positive correlations between VEGF-C score in primary tumors and liver metastases were noted (rs = 0.108, P = 0.477; Table 2).

**Correlations Between Two Different Tumor Markers in Primary Tumors and Liver Metastases**

Significant correlations were found between COX-2 and VEGF-A scores, as shown in Fig. 3, in primary tumors.
A similar pattern was noted when a comparison was made between VEGF-A and TP scores in both primary tumors (rs = 0.479, P = 0.001) and liver metastases (rs = 0.35, P = 0.021). COX-2 was also significantly correlated with TP and MVD (rs = 0.404, P = 0.008; rs = 0.336, P = 0.027, respectively); however these correlations were only seen in the primary tumor. No other correlations among the tumor markers were noted.

**DISCUSSION**

Angiogenesis is essential for the growth of primary tumors, the development of metastasis and the continued growth of
This process involves the proliferation of host endothelial cells, and is likely to be regulated by several growth factors. Recent studies have demonstrated increased COX-2 expression in up to 80% of CRC and 40% of pre-malignant adenomas. This may indicate involvement in colorectal tumors and their metastatic spread to distant organs. COX-2 expression has been implicated in the regulation of VEGF and TP in human colonic cancer tissues. In the present study, VEGF-A and COX-2 were closely correlated in both primary tumors and liver metastases. We also showed that COX-2 is up-regulated in liver metastases.

These findings are supported by a similar study performed by Chen et al. (37), who evaluated 17 cases of liver metastasis and their corresponding primary tumors. They found that metastatic liver tumors from primary CRC tumors tend to have higher COX-2 expression than the primary tumors. They noted this by observing elevated levels of PGE, a COX enzyme-catalyzed product, in CRC patients with liver metastases. Pai et al. (39) reported PGE induced VEGF gene expression in rat gastric microvascular endothelial cells. Taken together, these findings suggest that there is a positive feedback loop for continuous local production of VEGF and COX-2 in endothelial cells. This mechanism would explain the correlation between VEGF-A and COX-2 in liver metastases and primary CRC found in this study.

Statistical analysis demonstrated that VEGF-A scores were higher in primary tumors in comparison to liver metastases. Various published papers have demonstrated that VEGF-A is up-regulated in primary CRC cells while being down-regulated in their corresponding liver metastases. This could be due to a local down-regulation of VEGF-A expression, at either the transcriptional or post-transcriptional level. It has been suggested that hypoxia may provide the stimulus that up-regulates VEGF-A transcription and malignancy. However, the relatively high levels of oxygen in hepatic parenchyma mean that VEGF-A is less of an angiogenic driving force at these sites than at the primary tumor. These points still need to be addressed with regard to biological regulatory function.

TP and VEGF-A are coexpressed in various human cancers. These two potent angiogenic molecules, which have different specificities with respect to endothelial stimulation, could have a cooperative role in neovascularization, according to research focusing on hypoxia. Our results also indicated that levels of VEGF and TP are significantly correlated in primary tumors as well as liver metastases.

Table 2. Correlation between individual tumor marker in primary CRC and corresponding liver metastases

<table>
<thead>
<tr>
<th></th>
<th>Primary CRC</th>
<th>Liver metastases</th>
<th>P-value (Wilcoxon)</th>
<th>Correlation and P-value (Spearman)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td>66</td>
<td>89</td>
<td>0.002</td>
<td>Positive (P = 0.005, rs = 0.428)</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>69</td>
<td>30</td>
<td>0.0004</td>
<td>Positive (P = 0.039, rs = 0.314)</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>45</td>
<td>73</td>
<td>0.02</td>
<td>None (P = 0.477, rs = 0.108)</td>
</tr>
<tr>
<td>TP</td>
<td>86</td>
<td>77</td>
<td>0.247</td>
<td>Positive (P = 0.005, rs = 0.422)</td>
</tr>
<tr>
<td>MVD</td>
<td>52</td>
<td>45</td>
<td>0.229</td>
<td>Positive (P = 0.046, rs = 0.304)</td>
</tr>
</tbody>
</table>

CRC, colorectal cancer; MVD, microvascular density.
Several authors have confirmed that angiogenic factors are up-regulated in various human tumors and that their expression is typically correlated with high MVD and poor outcome (45). Interestingly, MVD was positively correlated with only COX-2 scores in the primary tumor. The variability of our results may be explained by the high stage of tumors evaluated in this study (Duke’s III and IV). As suggested by Couvelard et al. (46), low MVD is seen in advanced carcinomas, as vessels can progressively regress during tumor progress. Another factor in these discrepancies may be in the methodology for measurement of MVD, as well as the mechanism or mechanisms by which they are involved in primary tumor vascularization and progression.

Based on the present data, we may be able to predict the angiogenic activity of liver metastases by analyzing the primary tumor, as VEGF-A, COX-2, TP and MVD expression are positively correlated between primary CRC and liver metastasis. However, further study is needed to evaluate the combination of different markers with the aim of improving reliability and sensitivity.

Conflict of interest statement
None declared.

References