Vascular Endothelial Growth Factor Messenger RNA Expression Level Is Preserved in Liver Metastases Compared with Corresponding Primary Colorectal Cancer

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Abstract Purpose: Increased vascular endothelial growth factor (VEGF) expression is associated with colorectal cancer liver metastases. It is reasonable to expect that measurement of VEGF in liver metastases would provide the best prediction of therapy benefit for VEGF-targeted drugs, such as bevacizumab (Avastin). In this study, we evaluated how VEGF mRNA level in primary colorectal cancer was related to that in corresponding liver metastases. Thirty-one pairs of primary colorectal cancer and corresponding liver metastases were analyzed.

Experimental Design: Formalin-fixed, paraffin-embedded tumor specimens were dissected by using laser-captured microdissection. RNA was extracted and cDNA was prepared by reverse transcription. Quantitation of VEGF and internal reference gene (β-actin) was done using real-time PCR (Taqman PCR).

Results: There was no difference between median VEGF mRNA levels of primary colorectal cancer and liver metastases (median value 3.79 versus 3.97; P = 0.989). On an individual basis, there was a significant correlation in VEGF mRNA expression between primary colorectal cancer and corresponding liver metastases (r = 0.6627, P < 0.0001). In addition, the VEGF mRNA levels of the patients who had two or more liver metastatic tumors were significantly higher than those of the patient who had solitary liver metastatic tumor in both primary cancer (5.02 versus 3.34: P = 0.0483) and liver metastases (4.38 versus 3.25: P = 0.0358).

Conclusion: Good prediction of VEGF mRNA levels in liver metastases can be obtained by measuring those of primary colorectal cancer. The risk of multiple liver metastatic tumors might be predictable by measuring VEGF mRNA expression in primary colorectal cancer. Further study is required to confirm these preliminary results.

Colorectal cancer is the fourth most common malignancy and the second leading cause of cancer death in the United States (1). For most patients with colorectal cancer, liver metastases are the main cause of death. An essential process for promotion of metastases is angiogenesis, which is controlled by several angiogenic factors among which vascular endothelial growth factor (VEGF), also known as VEGF-A, is considered to be one of the most important. It has been reported that VEGF is strongly related to liver metastases of colorectal cancer and its expression levels are useful not only as a predictive marker for distant metastases but also as a prognostic marker (2–4). Takahashi et al. (2) reported that protein expressions of VEGF and its receptor, KDR, were higher in metastatic than in nonmetastatic neoplasms in colorectal cancer by using immunohistochemical staining. They also found that VEGF expression and vessel count were correlated with time to recurrence (5).

The latest efforts at molecularly targeted drug development have focused on VEGF and its receptors. A monoclonal antibody to VEGF (bevacizumab) has been developed and approved for colorectal cancer therapy in the United States, and it has been reported that treatment with bevacizumab plus 5-fluorouracil/leucovorin resulted in higher response rates and longer median survival compared with the 5-fluorouracil/leucovorin control arm in patients with metastatic colorectal cancer on phase II study (6).

Because controlling liver metastases is considered essential in the treatment of colorectal cancer, it is reasonable to expect that measurement of VEGF gene expression level in liver metastases would provide the best prediction of therapy benefit. Tamura et al. (7) reported that VEGF expression in pulmonary metastatic tumor was significantly correlated with patients' survival. However, in many if not most cases, only biopsies of
the patients' primary tumor are readily available for analysis. Thus, it would be important to know the relationship between levels of response determinants in the primary tumor and corresponding liver metastases to determine whether analysis of tissue biopsies of the primary tumor are useful for tumor response prediction.

In this study, we investigated the relationship between VEGF mRNA expressions in primary colorectal cancer and corresponding metastatic tumors and also between VEGF gene expression levels with the corresponding patients' clinical variables.

### Materials and Methods

**Patients and samples.** Thirty-one pairs of primary colorectal cancer and corresponding liver metastases were analyzed in this study (18 males and 13 females; median age 66 years [range 45–85 years]). These patients had undergone surgical resection of primary colorectal adenocarcinoma and liver metastasis between 1988 and 1999 at the Department of Gastroenterology, Tokyo Women’s Medical University, Tokyo, Japan. All of the patients were Japanese and written informed consent was obtained from every patient according to the institutional regulations. The characteristics of 31 patients are shown in Table 1. Seventeen patients had solitary liver metastases and 14 patients had multiple (two or more) metastases. Sixteen patients were synchronous metastases and 15 were metachronous metastases. Of the patients of metachronous liver metastases, four had received 5-fluorouracil-based chemotherapy as adjuvant therapy after the primary resection. Resections of the liver metastases in these patients were done at least 2 months after completion of the adjuvant chemotherapy. Adjacent normal colorectal mucosa and adjacent normal liver tissue were also evaluated as a control in each patient.

**Microdissection.** Formalin-fixed, paraffin-embedded tumor specimens and adjacent normal tissues were cut into serial sections with a thickness of 10 μm. For the pathologic diagnosis, one slide was stained with H&E and evaluated by a pathologist. Other sections were stained with nuclear fast red (American MasterTech Scientific, Inc., Lod, CA) to enable visualization of histology. Laser capture microdissection (P.A.L.M. Microlaser Technologies AG, Munich, Germany) was done in all the tumor samples to ensure that only tumor cells were dissected (8). Adjacent normal colorectal mucosa and liver tumor tissues were dissected from the slide using a scalpel.

**RNA isolation and cDNA synthesis.** RNA isolation from formalin-fixed paraffin-embedded specimens was done according to a proprietary procedure of Response Genetics, Inc. (U.S. patent no. 6,248,535). In brief, tissue samples were heated at 92 °C for 30 minutes in 4 mol/L DTT-GTIC/sarc [4 mol/L guanidinium isothiocyanate, 50 mmol/L Tris-HCl (pH 7.5), 25 mmol/L EDTA; Invitrogen, Carlsbad, CA]. To the tissue suspensions, 50 μL of 2 mol/L sodium acetate (pH 4.0), followed by 600 μL of freshly prepared phenol/chloroform/isomyl alcohol (250:50:1) were added. The suspensions were centrifuged at 13,000 rpm for 8 minutes in a chilled (8 °C) centrifuge. The upper aqueous phase was removed and combined with glyoxgen (10 μL) and 300 to 400 μL of isopropanol. The tubes were placed at −20 °C for 30 to 45 minutes to precipitate the RNA. Next, centrifugation at 13,000 rpm for 7 minutes in a chilled (8 °C) centrifuge, the supernatant was carefully poured off and the pellet was resuspended in 50 μL of 5 mmol/L Tris.

After RNA isolation, cDNA was prepared from each sample as described previously (9).

**Reverse transcription-PCR.** Quantification of VEGF and an internal reference gene (β-actin) was done using a fluorescence-based real-time detection method [ABI PRISM 7900 Sequence Detection System (Taqman); Applied Biosystems, Foster City, CA] as described previously (10, 11). The sequences of the primers and probe used are as follows: VEGF forward 5'-AGTTGGTTCAGGGTGCAC-3', reverse 5'-TCCATGAACTTCACACTTCTCG-3', probe 3'-FAM)ATGGCGACAAGGAGGACGCGAGATCA(TAMRA)-3'; β-actin forward 5'-TGAGCGGCGGCTACAGCTT-3', reverse 5'-TCTTAAATGTCAGCCGAGTTT-3', probe 5'-FAM)ACCACACCCGCGCCGCGGCGG(CMRA)-3'. VEGF primers and probe were designed in exons 1 and 2 so that all of VEGF isoforms, such as VEGF121, VEGF165, VEGF189, and VEGF206, were supposed to be measured. The PCR reaction mixture consisted 1,200 nmol/L of each primer, 200 nmol/L probe, 0.4 units of AmpliTaq Gold Polymerase, 200 nmol/L each of dATP, dCTP, dGTP, dTTP, 3.5 mmol/L MgCl₂, and 1 × Taqman buffer A containing a reference dye, to a final volume of 20 μL (all reagents were from PE Applied Biosystems, Foster City, CA). Cycling conditions were 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 46 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. Gene expression values (relative mRNA levels) are expressed as ratios (differences between the C_i values) between the genes of interest and an internal reference gene (β-actin) that provides a normalization factor for the amount of RNA isolated from a specimen.

**Statistical analysis.** To compare median mRNA levels of the primary colorectal cancer and corresponding liver metastases, the mRNA levels of corresponding adjacent normal tissues were assessed using the Wilcoxon signed-ranks test. The correlation between the mRNA levels of primary cancers and liver metastases was assessed using Spearman’s rank correlation. Statistical significance was set at P = 0.05. Comparisons between VEGF mRNA levels in patients with solitary metastases and those in patients with multiple metastases were assessed using Wilcoxon rank-sum test. All values were two sided.

### Results

**Median gene expression levels of primary tumor and liver metastases.** Median VEGF gene expression levels in primary colorectal cancer, corresponding liver metastases, and corresponding adjacent normal colon and liver tissue are shown in

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Frequency</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (y)</strong></td>
<td>66 (45–85)</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>18</td>
<td>58</td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>42</td>
</tr>
<tr>
<td><strong>Anatomic site</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right colon</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Transverse colon</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Left colon</td>
<td>13</td>
<td>42</td>
</tr>
<tr>
<td>Rectum</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>16</td>
<td>52</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>13</td>
<td>42</td>
</tr>
<tr>
<td>Mucinous</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td><strong>Dukes grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>65</td>
</tr>
<tr>
<td><strong>Number of liver metastases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solitary</td>
<td>17</td>
<td>55</td>
</tr>
<tr>
<td>Multiple</td>
<td>14</td>
<td>45</td>
</tr>
<tr>
<td>Liver synchronicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synchronous</td>
<td>16</td>
<td>52</td>
</tr>
<tr>
<td>Metachronous</td>
<td>15</td>
<td>48</td>
</tr>
</tbody>
</table>

Table 1. Demographic and clinical variables of patients with metastatic colorectal cancer
Table 2. There was no significant difference of median VEGF mRNA levels between primary colorectal cancer and liver metastases. \(3.79 \text{ versus } 3.97; P = 0.989; \text{Fig. 1}\).

**Correlation between primary colorectal cancer and liver metastases mRNA level.** When matched tissue sets were compared on an individual basis, there was a strong significant correlation for VEGF mRNA expression between primary colorectal cancer and corresponding liver metastases \(r_s = 0.6627, P < 0.0001; \text{Fig. 2}\). The correlation coefficient of VEGF expression between primary tumor and liver metastases was \(r_s = 0.7324, P = 0.0013\) in the synchronous metastases group and \(r_s = 0.6615, P = 0.01\) in the metachronous group.

**Gene expression of cancer tissue compared with adjacent normal tissue.** VEGF mRNA levels in primary colorectal cancer were significantly higher than that in adjacent normal colon mucosa \(3.79 \text{ versus } 1.73; P < 0.0001\). On the other hand, VEGF mRNA levels in liver metastases were significantly lower than those in normal adjacent liver tissue \(3.97 \text{ versus } 6.02; P = 0.0044\).

**VEGF expression analyzed by clinicopathologic factors.** When VEGF mRNA levels were compared between patients with solitary liver metastases and patients with two or more (multiple) metastases, VEGF mRNA levels of primary colorectal cancer in patients with multiple metastases were found to be significantly higher than those in patients with solitary metastases \(5.02 \text{ versus } 3.34; P = 0.083\). Patients with multiple metastases also had higher VEGF mRNA levels of liver metastases than patients with solitary metastases \(4.38 \text{ versus } 3.25; P = 0.0358; \text{Fig. 3}\). No significant difference of VEGF mRNA expression was seen between patients with synchronous metastases and patients with metachronous metastases, neither in primary colorectal cancer nor in liver metastases \(4.64 \text{ versus } 3.45; P = 0.1280; \text{liver}, 4.05 \text{ versus } 3.87; P = 0.9172\).

### Discussion

Recently, cancer chemotherapy has made dramatic progress in terms of new drugs specifically designed to target various molecules important for tumorigenesis. Antiangiogenic drugs are among the most important of these because of their potential for controlling tumor proliferation and metastases. Bevacizumab (Avastin), a recombinant humanized monoclonal antibody to VEGF, has been widely used in combination with other cytotoxic drugs (6). VEGF receptor has also been focused upon as a molecular target of chemotherapy, and several anti-VEGF receptor antibodies have been developed and are in clinical trials. Preclinical data on those antibodies have shown decreased VEGF-induced signaling, decreased angiogenesis, and decreased primary and metastatic growth in a variety of different tumor systems (12–15). Assuming that VEGF is indeed the real in vivo target of anti-VEGF drugs, it is reasonable to suppose that the effectiveness of these drugs will have some relationship to intratumoral levels of VEGF in individuals. To address this important question and perhaps, eventually, to make the most effective use of anti-VEGF drugs by designing individualized treatment, it will be important to know the level of VEGF expression at the primary tumor site as well as at metastatic sites. However, there is as yet very little data on the relationship between VEGF expression in primary tumor and that in metastases.

In this study, we found a strong and significant correlation between VEGF expression in primary colorectal cancer and that in corresponding liver metastases, not only in the median values but also between individual expression values in primary tumors and matched metastases from the same patient. We were able to find only one previous literature reference regarding comparative VEGF expression between primary tumor and metastases. Berney et al. (16) evaluated VEGF protein expression by immunohistochemistry in primary colorectal cancer tumors and the corresponding liver metastases and found that VEGF was significantly reduced in the metastatic liver tumors compared with primary tumors. The apparent discrepancy between our results obtained with reverse transcription-PCR and the immunohistochemistry data of Berney et al. (16) may be

<table>
<thead>
<tr>
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<th>Primary site</th>
<th>Metastatic liver site</th>
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<tbody>
<tr>
<td><strong>Cancer</strong></td>
<td>3.79 (1.04-13.10)</td>
<td>3.97 (0.69-12.42)</td>
</tr>
<tr>
<td><strong>Normal</strong></td>
<td>1.73 (0.67-4.16)</td>
<td>6.02 (1.25-13.36)</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>(&lt;0.0001)</td>
<td>(P = 0.0044)</td>
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Fig. 1. VEGF expression (mRNA levels) in primary colorectal cancer and liver metastases. No significant (\(P < 0.05\)) difference was seen in VEGF expression between primary colorectal cancer and liver metastases. Boxes, first and third quartiles (\(\text{median inside}\)); bars, range of values falling within 1.5-fold the interquartile range. Gene expression values were expressed as ratios between VEGF and an internal reference gene (\(\beta\)-actin).

**Table 2. Median VEGF gene expression levels in primary colorectal cancer, corresponding liver metastases, corresponding adjacent normal colon, and liver tissue**
be due to the different methodologies used in these studies. Immunohistochemistry technology is semiquantitative with limited accuracy (e.g., staining scored as low, medium, or high or percent of positively staining cells/total cells) and thus it may be difficult to obtain an accurate correlation coefficient between primary tumor and metastatic expressions. In contrast, real-time quantitative reverse transcription-PCR permits a precise quantification of gene expression (relative mRNA level), thus allowing accurate delineation of the correlation between primary tumor and metastases. However, other workers have found that immunohistochemical measurement of VEGF protein agreed well with the results of quantitative reverse transcription-PCR (17), both under steady-state conditions as well as when the regulation of VEGF gene expression is being manipulated, suggesting that at least in this set of patients, VEGF protein expression is not different between primary tumor and the corresponding metastasis. It is possible that in spite of similar gene expression, different posttranscriptional processing in the two tissues may cause some difference in protein levels. VEGF expression seems to be regulated by several factors, such as hypoxia, growth factors, and some cytokines (12), but it is not known whether such regulation at the posttranscriptional level occurs differently at metastatic sites that in the primary tumor. In any case, although such a differential regulation may change the absolute protein levels in one tissue compared with the other, it would not necessarily change the relative relationship (i.e., the correlation) between protein levels in matched pairs of primary and metastatic tissues.

Interestingly, although expression of VEGF in the normal tissue surrounding the primary tumor was lower than that of the tumor tissue, this was not the case in the liver where VEGF expression in normal liver tissue was actually higher than in the tumor metastases due to almost a 4-fold elevation of VEGF expression in normal liver compared with normal colon. This result underscores the importance of separating the tumor from nontumor tissue by microdissection when using homogeneous solution methodologies such as reverse transcription-PCR to determine tumor gene expression values. Whether the higher expression in surrounding normal liver tissue would have any effect on the activity of anti-VEGF drugs against liver metastases is entirely speculative.

A potentially important finding in this study was that VEGF mRNA expression in patients with multiple liver metastases was significantly higher in both primary tumors and metastatic sites than in patients with solitary liver metastases. Much previous literature reveals that the number of liver metastases is the major prognostic factor after the resection of colorectal metastases (18–21). Although there is no doubt that the best strategy to the patients with solitary metastases is surgical resection (22), the benefit of resection of four or more metastatic lesions is still controversial (19). In the clinical setting, cases are often encountered of patients in whom new liver tumors have recurred within a short period after the resection of liver metastatic tumors, suggesting that micrometastases that cannot be detected by image scanning already existed at the time of the first surgery. If patients with solitary metastases can be distinguished from those with multiple metastases by measuring VEGF expression in the primary tumor, it may result in a better strategy for treatment of liver metastases by avoidance of unnecessary surgery to patients who might have multiple liver metastases. Thus, it is important to confirm in a larger study our finding that primary tumor VEGF expression is indeed predictive of multiple metastases and if so, to determine more precise cutoff values for separating the patient groups.

Fig. 2. Correlation of VEGF mRNA between primary colorectal tumor and liver metastases. Significant correlation was seen between primary tumor and liver metastases ($r_s = 0.6627, P < 0.0001$). Gene expression values were expressed as ratios between the VEGF and an internal reference gene ($\beta$-actin).

Fig. 3. Comparison of VEGF mRNA expression between patients with multiple tumor and those with solitary tumor. Significant difference was seen both in primary tumor ($P = 0.045$) and liver metastases ($P = 0.0358$). Boxes, first and third quartiles (median inside); bars, range of values falling within 1.5-fold the interquartile range. Gene expression values were expressed as ratios between VEGF and an internal reference gene ($\beta$-actin).
In summary, we found that in this set of tumor specimens, there is a positive correlation between VEGF mRNA expression in liver metastases and in primary tumors, indicating that regulation of this gene is not altered appreciably during the metastatic process. Whether biopsies of the primary tumor may be as a used as a surrogate tissue for live metastases for optimizing patient treatment will require careful correlation with clinical outcomes.

References

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