Vascular endothelial growth factor and basic fibroblast growth factor expression in esophageal adenocarcinoma and Barrett esophagus

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Objective: This study was undertaken to investigate the role of the angiogenic factors vascular endothelial growth factor and basic fibroblast growth factor in the development and progression of Barrett esophagus and adenocarcinomas of the esophagus and gastroesophageal junction.

Methods: Vascular endothelial growth factor and basic fibroblast growth factor messenger RNA expression levels, relative to the control gene encoding β-actin, were measured by using a quantitative reverse transcription-polymerase chain reaction method (ABI 7700 Sequence Detector system) in specimens of Barrett intestinal metaplasia (n = 16), dysplasia (n = 11), adenocarcinoma (n = 15), and matching normal squamous esophageal tissues (n = 35). Vascular endothelial growth factor and basic fibroblast growth factor protein expression and CD31 microvessel density were assessed by means of immunohistochemistry in 25 tissue sections that included representative areas for each of these Barrett stages.

Results: Expression levels were significantly increased in adenocarcinoma compared with in either normal squamous mucosa (P < .0001 for both genes) or intestinal metaplasia (vascular endothelial growth factor, P = .002; basic fibroblast growth factor, P < .0001). Vascular endothelial growth factor levels were also significantly higher in cancer tissues compared with dysplasia tissues (P = .024, Mann-Whitney U test). Basic fibroblast growth factor expression was also significantly increased in Barrett dysplastic mucosa compared with in intestinal metaplasia or normal esophageal mucosa. Microvessel density was generally higher in adenocarcinoma compared with in preneoplastic Barrett tissues. The pattern of vascular endothelial growth factor and basic fibroblast growth factor protein expression was similar to the messenger RNA expression pattern, with the exception that mucin-containing goblet cells stained intensely for vascular endothelial growth factor and only weak vascular endothelial growth factor staining was present in some adenocarcinomas.
Conclusions: Vascular endothelial growth factor and basic fibroblast growth factor messenger RNA expression levels are significantly upregulated in esophageal and gastroesophageal junction adenocarcinomas, suggesting a role for these angiogenic factors in the development of these cancers. Vascular endothelial growth factor and basic fibroblast growth factor messenger RNA expression levels are also increased in some Barrett esophagus tissues, with this increase occurring at an earlier stage for basic fibroblast growth factor than for vascular endothelial growth factor. Basic fibroblast growth factor protein expression pattern is similar to the messenger RNA expression pattern, but unlike the messenger RNA findings, vascular endothelial growth factor protein expression is strongest in goblet cells.

The most significant risk factor for esophageal adenocarcinoma is the presence of Barrett esophagus, a disease in which the normal squamous lining of the distal esophagus is replaced with intestinalized columnar epithelium in response to chronic gastroesophageal reflux. Barrett esophagus is a multistage disease in which Barrett intestinal metaplasia (IM) progresses, in some patients, to low-grade dysplasia (LGD), high-grade dysplasia (HGD), and eventually adenocarcinoma. Regular surveillance endoscopy in patients with Barrett esophagus has resulted in an increase in the rate of early detection of cancer, but endoscopic surveillance fails to detect adenocarcinoma in many cases,1 and most cases of Barrett esophagus are undiagnosed and thus not included in a surveillance program. These clinical problems indicate that reliable diagnostic or prognostic biomarkers for Barrett esophagus and esophageal adenocarcinoma would be valuable.

The density of new vessel formation within and around tumors (tumor angiogenesis) and the expression of proangiogenic factors have been shown in many studies to be clinically significant biomarkers for esophageal squamous cell carcinoma (SCC),2-10 but few studies11-13 have investigated these factors in esophageal adenocarcinoma or Barrett esophagus. In this study we assessed the expression of 2 important proangiogenic factors, vascular endothelial growth factor (VEGF; also known as vascular permeability factor)14-17 and basic fibroblast growth factor (bFGF),18 in Barrett IM, dysplasia, and adenocarcinoma tissues to investigate the potential significance of these factors in the development and progression of this disease.

Materials and Methods
Tissue Samples for Reverse Transcription-Polymerase Chain Reaction
Seventy-seven tissue samples obtained at endoscopy and at the time of the operation from 38 patients with Barrett esophagus or adenocarcinoma of the esophagus or gastroesophageal junction were collected and immediately frozen in liquid nitrogen. Endoscopic biopsy specimens were obtained according to a protocol that required biopsy at 2-cm intervals from each quadrant (anterior, posterior, and right and left lateral positions) of the visible length of Barrett mucosa and an additional biopsy specimen from the normal-appearing squamous mucosa of the esophagus. Normal esophageal biopsy specimens were taken from the proximal margin of the operative resection specimens or from an area at least 4 cm proximal to the macroscopically abnormal epithelium on endoscopy. Part of the specimen or an adjacent specimen (from within 5 mm of the study specimen) was fixed in formalin and paraffin for histopathologic examination by expert Barrett pathologists. Frozen-section examination of the study tissue was performed if the diagnosis was uncertain. Specimens were classified as IM if IM but not dysplasia or cancer was present. Specimens were classified as dysplastic if either LGD or HGD was present. Dysplastic tissues were not divided into HGD and LGD groups because areas of LGD and HGD were commonly present in the same specimen. The fresh tumor specimens were macrodissected to ensure that the specimen analyzed contained mostly tumor cells.

By using these criteria, samples analyzed for VEGF and bFGF expression were Barrett IM (n = 16), Barrett dysplasia (n = 11), esophageal or gastroesophageal junction adenocarcinoma tissues (n = 15), and matching normal esophagus tissues from 35 of the patients (n = 35). The IM samples were all from patients who did not have dysplasia or adenocarcinoma. Four of the 11 dysplasia samples were from patients with adenocarcinoma. Of the 35 normal squamous esophageal samples, 22 were obtained from patients with Barrett metaplasia or dysplasia but no adenocarcinoma, and 13 were from patients with adenocarcinoma.

Approval for this study was obtained from the Institutional Review Board of the University of Southern California Keck School of Medicine, and written informed consent was obtained from participating patients.

RNA Extraction and cDNA Synthesis
Total RNA was isolated by using a single-step guanidinium isothiocyanate method with the QuickPrepMicro mRNA Purification Kit (Amersham Pharmacia Biotech Inc, Piscataway, NJ), according to the manufacturer’s instructions, and cDNAs were prepared as previously described.19,20

PCR Quantification of mRNA Expression
Quantitation of VEGF and bFGF cDNA and an internal reference cDNA (β-actin) was done by using a fluorescence detection tool.
method (ABI PRISM 7700 Sequence Detection System, Taqman; Perkin Elmer Applied Biosystems, Foster City, Calif), as previously described.20-22

The PCR reaction mixture consisted of 600 mmol/L of each primer; 200 mmol/L probe; 5 U of AmpliTaq Gold Polymerase; 200 pmol/L each of deoxyadenosine triphosphate, deoxyctydine triphosphate, and deoxyguanosine triphosphate; 400 μmol/L dUTP (2′-deoxyuridine 5′-triphosphate), 5.5 mmol/L MgCl₂; 1 U of AmpErase uracil N-glycosylase; and 1× Taqman Buffer A containing a reference dye to a final volume of 25 μL (all reagents were obtained from ABI Applied Biosystems, Foster City, Calif). Cycling conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Significant contamination with genomic DNA was excluded by amplifying non-reverse-transcribed RNA. Primer and probe sequences are available on request.

The reproducibility of the Taqman assay was tested by measuring VEGF mRNA expression in 5 tumor samples and 6 control RNAs (2 colon RNAs, 2 liver RNAs, and 2 lung RNAs; Stratagene, La Jolla, Calif) in triplicate.

### Immunohistochemistry

Five representative areas of each histopathologic stage (normal squamous esophagus, Barrett IM, LGD, HGD, and adenocarcinoma) were stained by means of immunohistochemistry to show the patterns of microvessel density, VEGF protein expression, and bFGF protein expression. Archival formalin-fixed, paraffin-embedded blocks were cut into 5-μm sections, mounted onto polyllysine-coated slides, dewaxed in xylene, and rehydrated in alcohol. Pretreatment by immersion in 10 mmol/L citrate buffer (pH 6.0) with microwave pressure cooker heating was performed for staining with the CD31 (platelet-endothelial adhesion molecule, clone JC/70A, 1:100 dilution; DAKO, Carpinteria, Calif) and bFGF (Ab-3, Clone 3H3, 1:50 dilution; Oncogene Research, San Diego, Calif) antibodies. Pretreatment by means of microwave heating in 10 mmol/L ethylenediamine tetraacetic acid (pH 8.0) was performed for staining with the VEGF (Clone JH121, 1:100 dilution, Ab-3; Neomarkers, Fremont, Calif) antibody. The sections were peroxidase blocked with 3% hydrogen peroxide in 0.05 mol/L TRIS-hydrochloric acid buffer, incubated for 15 minutes with normal horse serum, and incubated with primary antibody (all overnight at room temperature). Biotinylated horse anti-mouse secondary antibody (1:200 dilution for 40 minutes; Vector Labs, Burlingame, Calif), peroxidase-conjugated streptavidin complex reagent (1:100 dilution, 30 minutes, VectaStain Elite ABC Kit, Vector Labs), and 3.3′-diaminobenzidine (10 mg in 10 mL of TRIS buffer for 20 minutes) were used to visualize binding of the first antibody. Positive and negative controls were used for each antibody.

Areas of highest neovascularization, as assessed by means of CD31 positivity, were found by scanning each section at 100× magnification. Microvessel density was then quantified in these neovascular hot spots at 400× magnification. At least 2 different fields of hot spots were evaluated for each case to obtain the mean number of CD31+ microvessels per section. For counting positive-staining vessels, only those with at least one visible endothelial cell were considered.

The intensity of VEGF and bFGF cytoplasmic protein expression was graded as 0 (staining intensity not above background level), 1+ (weak uniform staining, slightly above background staining), 2+ (moderate uniform staining), or 3+ (strong uniform staining). The intensity classification for each section was based on the highest intensity staining area for the section. The staining patterns in both epithelial and endothelial cells were assessed.

### Statistical Analysis

VEGF and bFGF mRNA expression levels in adenocarcinoma, Barrett dysplasia, IM, and normal squamous esophageal tissues were compared by using the Kruskal-Wallis test to identify significant differences in expressions among all histopathologic groups. The Mann-Whitney U test was then used to compare the expression levels between 2 different groups. VEGF and bFGF expressions in premalignant Barrett and histologically normal squamous esophageal tissues from patients with Barrett esophagus but no cancer were compared with the expressions in these tissues from patients with cancer in a second analysis that used the same statistical methods. Reproducibility of the assay, as tested with triplicate VEGF assays in clinical specimens and control RNAs,

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### TABLE 1. Comparison of VEGF and bFGF mRNA expression levels in different tissues

<table>
<thead>
<tr>
<th>Pathology</th>
<th>No. of tissues</th>
<th>VEGF, median (range)</th>
<th>bFGF, median (range)</th>
<th>Tissues compared</th>
<th>VEGF P value</th>
<th>bFGF P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>15</td>
<td>57.6 (0.51-54.44)</td>
<td>7.13 (0.01-24)</td>
<td>Adenocarcinoma vs normal esophagus; adenocarcinoma vs dysplasia; adenocarcinoma vs IM</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Dysplasia vs normal esophagus</td>
<td></td>
<td></td>
<td></td>
<td>Dysplasia vs normal esophagus</td>
<td>.76</td>
<td>.007</td>
</tr>
<tr>
<td>IM vs normal esophagus</td>
<td>11</td>
<td>16.4 (2-616)</td>
<td>4.0 (0.18-18.54)</td>
<td>IM vs normal esophagus</td>
<td>.75</td>
<td>.009</td>
</tr>
<tr>
<td>Normal esophagus</td>
<td>35</td>
<td>17.8 (2-103)</td>
<td>1.0 (1.01-24)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
was assessed by calculating the coefficient of variance of the threshold cycle for each reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Results

RT-PCR Results

The median values and ranges of VEGF and bFGF mRNA expressions in adenocarcinoma (n = 15), Barrett dysplasia (n = 11), Barrett IM (n = 16), and normal squamous esophagus epithelial (n = 35) tissues are shown in Table 1. As shown, the RNA expression levels of both VEGF and bFGF were significantly upregulated in the group of adenocarcinoma tissues compared with in the group of normal squamous esophageal (P < .0001 for both genes) or Barrett IM tissues (P < .0001 for bFGF and P = .002 for VEGF). VEGF expression, but not bFGF expression, was also significantly higher in adenocarcinoma compared with that in dysplastic Barrett esophagus mucosa (P = .009 for VEGF).

bFGF mRNA expression levels were significantly higher in dysplasia tissues than in normal esophageal or IM tissues (Table 1). There was considerable variability in mRNA expression levels for both genes within each histopathologic stage, and very high measurements were recorded in some specimens from all tissue types.

The bFGF mRNA expression levels in the group of histologically normal squamous esophageal tissues from patients with adenocarcinoma (n = 13; median expression, 3.3) was significantly higher than the levels found in normal esophageal tissues from patients with Barrett IM or dysplasia but no cancer (n = 22; median expression, 0.21; P < .0001). There was no significant difference in VEGF RNA expression found between these 2 normal esophageal epithelium groups (P = .58), and there was no significant expression level difference found between dysplasia tissues from patients with cancer and dysplasia tissues from patients without cancer (P = .23 for both VEGF and bFGF). No IM specimens from patients with adenocarcinoma or dysplasia were studied.

Reproducibility of the Taqman Assay

The mean coefficient of variance of the PCR Taqman threshold cycles for RNAs run in triplicate for VEGF RNA expression was 0.39% (median, 0.30%) for the control specimens and 0.41% (median 0.26%) for the esophageal adenocarcinoma specimens (data available on request).

Immunohistochemistry Results

Representative immunohistochemistry sections are shown in Figure 1.

As expected, intense CD31 positivity was restricted to endothelial cells. Moderate or strong (2+ or 3+ intensity) microvessel CD31 immunoperoxidase staining with low background staining was present in all the sections. Table 2 shows the average numbers of CD31+ vessels found within each histopathologic tissue type. The microvessel density was generally higher in adenocarcinoma than in preneoplastic Barrett epithelium, with 4 of 5 tissues having microvessel counts higher than the median microvessel count for all 25 tissues studied (median, 10 vessels per high-power field), but 2 of 5 LGD and 1 of 5 HGD tissues also had microvessel counts of greater than the median value (Table 2). There was a high concentration of CD31+ vessels within the

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Normal esophagus</th>
<th>IM*</th>
<th>LGD*</th>
<th>HGD*</th>
<th>Adenocarcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31 (average number of vessels/high power field)†</td>
<td>9</td>
<td>6</td>
<td>2</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Case 1</td>
<td>12</td>
<td>7</td>
<td>6</td>
<td>8</td>
<td>13</td>
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<tr>
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<td>Case 3</td>
<td>14</td>
<td>8</td>
<td>14</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>Case 4</td>
<td>15</td>
<td>10</td>
<td>19</td>
<td>12</td>
<td>16</td>
</tr>
</tbody>
</table>

*Not including goblet cells in Barrett epithelium. Goblet cells generally stained intensely for VEGF and moderately for bFGF (see text).
†High-power field = 400 × magnification. Averages rounded to whole numbers.
subepithelium of the normal squamous mucosa (Table 2 and Figure 1) because the surface area of lamina propria, where the vessels are located, was much higher in squamous mucosa than in Barrett mucosa or adenocarcinoma. Tumor angiogenesis was heterogeneous, but areas of highest neo-vascularization (neovascular hot spots) were most frequently found at the margins of the adenocarcinomas.

VEGF staining was only weakly positive in 3 of 5 adenocarcinoma cases, but the intensity of staining in adenocarcinoma was nevertheless generally stronger than found in nongoblet columnar cells in Barrett esophagus or normal squamous mucosa (Table 2 and Figure 1). Mucin-containing goblet cells stained very intensely for VEGF. VEGF stained microvessel endothelial cells faintly. The concentration of VEGF-positive microvessels was highest in Barrett IM, followed by dysplastic Barrett, and lowest in adenocarcinoma.

bFGF showed intense staining in the malignant cells of adenocarcinoma, less intense but definite staining in LGD and HGD columnar cells, and faint staining in goblet cells (Table 2 and Figure 1). bFGF staining was present in the basal squamous layer in one normal esophagus case. bFGF positivity, including some moderately intense positivity, was found in endothelial cells in each histopathologic tissue type.

**Discussion**

This study demonstrates that mRNA expression levels of VEGF and bFGF are significantly upregulated in most adenocarcinomas of the esophagus or gastroesophageal junc-

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**Figure 1.** Representative immunohistochemistry sections: A, normal squamous esophagus; B, Barrett IM with goblet cells; C, esophageal adenocarcinoma; i, CD31 microvessel density, showing specific endothelial cell staining; ii, VEGF protein expression; iii, bFGF protein expression.
tion. There was considerable variation in expression levels for both genes at all histopathologic stages, but analysis of the grouped results for each stage showed that there was a highly significant increase in both VEGF and bFGF expression in adenocarcinoma tissues compared with normal esophageal or Barrett IM tissues. A study by Torres and colleagues found high new vessel densities in esophageal adenocarcinomas. Because VEGF and bFGF have critically important roles in inducing and maintaining angiogenesis, it is likely that the increased expression of these genes found in our study is causally associated with the tumor angiogenesis reported by Torres and colleagues.

As might be expected for a complex biologic process, there was no close correlation between the levels of VEGF and bFGF expression in individual patients in this study, indicating that there is little diagnostic value in measuring the expression of these genes for an individual patient. Assessment of a panel of informative genes should theoretically be more useful for individual patients.

Most of the patients with cancer had markedly increased levels of both VEGF and bFGF. Upregulation of both genes together suggests that their regulation might not be independent, a theory that is supported by the finding that bFGF can induce the expression of VEGF. In vitro angiogenesis assay studies have also demonstrated synergism between VEGF and bFGF.

In contrast to the similar marked upregulation in the expression of both genes in cancer tissues, there were some differences between the expression patterns of each gene in premalignant Barrett tissues. A significantly increased bFGF expression was found in the group of dysplastic Barrett tissues compared with either IM or normal esophagus groups, whereas for VEGF, there was no significant increase in expression in dysplastic tissues. Some individual patients had very high expression levels of either or both genes in IM or dysplastic epithelia. Because of the wide variability in gene expression between individual patients with the same histopathologic Barrett stage, it is acknowledged that analysis of tissues from a much larger number of patients could produce a different pattern of expression for the premalignant stages. Nevertheless, our results suggest that VEGF expression, although high in some Barrett esophagus specimens, is not consistently overexpressed until the Barrett epithelium acquires an invasive phenotype. This might occur at the time of the angiogenic switch, when the tumor recruits new capillaries and is thus able to expand. bFGF expression, in contrast, is upregulated more frequently at the dysplasia stage, before the development of invasive cancer and perhaps before the onset of the switch to an angiogenic phenotype. Interestingly, other studies report that tumor angiogenesis can be detected in hyperplastic and morphologically transformed but noninvasive cells.

bFGF expression levels were significantly higher in this study in the group of histologically normal squamous esophageal tissues from patients with cancer compared with the group of histologically normal squamous esophageal tissues from patients without cancer. These results indicate that some genetic changes precede the appearance of even the earliest morphologic changes in this disease. The normal esophageal biopsy specimens were from areas that were well separated from macroscopic disease, indicating that there is probably a very widespread oncogenic field effect in the esophagus in patients with cancer. Other studies have found similar evidence for the presence of a cancer field in esophageal adenocarcinoma.

A relatively small number of sections were stained for immunohistochemistry, and the protein expression findings are therefore not conclusive. Nevertheless, it is evident that bFGF mRNA and protein expression patterns are similar, with maximal expression in adenocarcinoma and dysplastic Barrett cells. In contrast to the mRNA findings and for unknown reasons, VEGF expression was maximal in the mucin in goblet cells. As expected, CD31 microvessel density, a measure of tumor angiogenesis, was generally higher in adenocarcinoma than in Barrett esophagus.

This study was not designed to investigate mechanisms regulating VEGF or bFGF. It can be noted, however, that some genetic alterations known to induce VEGF expression, including p53 mutations and erbB2/Her2/neu overexpression, are frequently present in adenocarcinomas of the esophagus and gastroesophageal junction; ras mutation status can influence VEGF expression, but the frequency of ras mutation in this disease is uncertain. There is also evidence that angiogenesis can be regulated by cyclooxygenase 2 (COX-2) expression, which is upregulated in both esophageal adenocarcinoma and Barrett esophagus. These observations suggest future areas for investigation in this disease, and they also suggest that combination therapies targeting both angiogenesis and angiogenic regulators, such as COX-2, ras, or erbB2/HER-2/neu, might be more effective than single-agent therapies alone. Ziche and associates noted that because the agents that can modulate angiogenesis are heterogeneous with respect to their chemical characteristics and biologic properties, quantitative measurement of angiogenesis factors would be helpful for selecting specific angiogenesis therapies and as an end point for antiangiogenic therapy. Our data indicate that the quantitative RT-PCR system used in this study would be suitable for this purpose.

The development of esophageal and gastroesophageal junction adenocarcinomas is associated with the expression of the angiogenic factors VEGF and bFGF. Some premalignant Barrett esophagus tissues also express high levels of these genes, with increased bFGF expression associated with the dysplastic Barrett phenotype. Further studies on
this important multistage carcinogenesis model are required to confirm the effect of VEGF and bFGF expression on angiogenesis itself, to determine the mechanisms regulating the expression of these genes, and to identify associations between angiogenesis and clinical outcomes.

References


Discussion

Dr Robert J. Keenan (Pittsburgh, Pa). Because your endothelial or vascular markers are really not specific from the standpoint of trying to determine the dysplastic changes from the adenocarcinoma-type changes, can you tell us how you might use this information if you have patients under surveillance who have Barrett esophagus without dysplastic changes to try to determine when you should intervene? The big controversy, of course, is that at some point we believe that if you get to the point of HGD, that in many cases this means that you already have adenocarcinoma somewhere in there, and people are talking about esophagectomy. Can you use this information in a way to try to help you determine who it is that is likely to go on to that kind of a course?

Dr Lord. Thank you. As you suggest is needed, many groups are looking for a biomarker that is specific for cancer, one that is present in malignant tissues but not in patients with high-grade dysplasia or other Barrett epithelia. Unfortunately, because there were some very high levels in dysplasia patients, we do not think that VEGF and basic FGF are going to be able to fulfill this role. We have found other genes, for example telomerase, the gamma retinoic acid receptor, and also to some extent COX-2, for which very abnormal levels are only found in patients with cancer, and at this stage those markers offer more promise. We have also conducted a multicenter serum study that looked at the methylated APC gene. So far, methylated APC has only been present in the serum in patients with cancer. It was not present in patients with premalignant Barrett esophagus or benign gastric diseases. So at the moment, we concede that the factors presented today by themselves do not offer enormous clinical utility, but there are other more promising factors. We think that ultimately there will be a panel of informative expression factors and with a large enough data set it will be possible to set limits above and below which we can reasonably accurately predict which patients have cancer and which patients are at higher risk for developing cancer.

Dr Nasser K. Altorki (New York, NY). I have noticed that your confidence intervals are quite large. Therefore although it is an interesting work, I think it needs to be validated by larger numbers. A few years back, we published an article on the expression of acidic and bFGF by means of immunohistochemistry and also by looking at the mRNA. We found that bFGF has a lot of mRNA expression, but we were not able to find it by means of immunohistochemistry. I wonder what happens to the mRNA. Is it stable, or is it just not transcribed? Did you have a chance to look at whether the mRNA is actually present and whether the protein is actually present?

Dr Lord. We are in the process of doing that, and I do not think we have sufficient information at the moment to answer your question adequately. Certainly there does seem to be a correlation between angiogenesis factor expression and microvessel density measured with CD31. In addition to your study, a paper by Torres and associates looked specifically at adenocarcinoma and squamous cell carcinoma of the esophagus. Torres and colleagues also found that microvessel density was significantly increased in adenocarcinoma tissues.

Dr David S. Schrump (Bethesda, Md). I have 2 related questions that are very brief.

I saw that you had acknowledged that p53, as well as HER-2/neu, mutations correlate with induction of VEGF and bFGF expression. Have you performed or do you plan to perform an analysis for p53, as well as your her-2/neu expression, in your mRNA samples to try to correlate the levels of VEGF and bFGF expression to these mutational events?

Dr Lord. Yes, we are interested in doing that. We do not intend to sequence these recently collected tissues themselves, but we do have now a group of approximately 50 adenocarcinomas that have been sequenced for at least exons 5 to 8 that were collected several years ago. The Danenberg laboratory where this work was done has recently developed a methodology to isolate RNA from paraffin sections, so we think that it will be possible to correlate the expression of these factors with the presence of a p53 mutation in the patients for whom we already have the p53 information. We also have information on COX-2 expression in a large number of tissues. So we do intend to pursue those interesting questions.