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Original Article
Circulating levels of anti-angiogenic VEGF-A isoform (VEGF-A_{xxxb}) in colorectal cancer patients predicts tumour VEGF-A ratios

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Abstract: Purpose: Bevacizumab as an adjunct to chemotherapy improves survival for some patients with metastatic colorectal cancer. Immunohistochemical staining of samples from the registration ECOG E3200 trial of bevacizumab with FOLFOX demonstrated that only patients with carcinomas expressing low levels of VEGF-A_{165b}, an anti-angiogenic splice variant of the Vascular Endothelial Growth Factor family of proteins, benefited from bevacizumab treatment. To identify a more useful biomarker of response we tested the hypothesis that circulating VEGF-A_{165b} levels correlate with immunohistochemical staining. Experimental Design: 17 patients with biopsy proven colorectal adenocarcinoma had pre-operative blood samples drawn. They underwent resection and had post-resection blood drawn. The plasma was analysed for levels of VEGF-A_{xxxb} using enzyme-linked immunosorbent assay (ELISA) and the tumour blocks stained for VEGF-A_{xxxb} and pan-VEGF-A. The normalised ratio of VEGF-A_{xxxb} expression to that of panVEGF-A expression scored by IHC was calculated and correlated with plasma VEGF-A_{165b} levels. Results: Plasma levels of VEGF-A_{xxxb} significantly correlated with the VEGF-A_{xxxb}:panVEGF-A ratio (r=0.594, P<0.02) in colorectal cancers. Median plasma VEGF-A_{xxxb} levels were 151 pg/ml. The mean (1.5±0.17) and median, IQR (1.8, 1-2) IHC scores of the patients with greater than median plasma VEGF-A_{xxxb} levels were significantly greater than those with less than median plasma VEGF-A_{xxxb} levels (mean ± SEM=0.85±10.12, median, IQR=1, 0.54-1). Conclusion: These results suggest that plasma VEGF-A_{xxxb} levels could be an effective biomarker of response to Bevacizumab. These results indicate that a prospective trial is warranted to explore the use of plasma VEGF-A_{xxxb} levels to stratify patients for colorectal cancer treatment by bevacizumab.

Keywords: VEGF, bevacizumab, colorectal cancer, splicing, VEGF165b

Introduction
Tumour angiogenesis is one of the hallmarks of cancer [1], and is mediated by a variety of growth factors, the most widely studied of which is vascular endothelial growth factor (VEGF-A). High plasma levels of total VEGF-A may be observed in more advanced colorectal cancer [2] and the anti-VEGF-A monoclonal antibody Bevacizumab (Avastin®) increases median survival by a matter of weeks when given with chemotherapy in metastatic colorectal cancer [3, 4]. However, the response rate of patients to Bevacizumab is only around 10%, with responses not predicted by total VEGF-A levels [5]. VEGF-A is a multi-exon gene encoding multiple isoforms, identified by their amino acid number (e.g. VEGF-A_{165}, VEGF-A_{121}, etc.). Alternative splicing of exon 8 results in 2 families of proteins [6]. Depending on the resultant protein translated, the physiological outcome is either that of promotion or inhibition of microvessel formation. Proximal splice site (PSS) usage generates a short open reading frame (ORF) of 6 amino acids common to the pro-angiogenic VEGF-A_{xxx} isoforms. Alternative distal splice site (DSS) selection in exon 8 results in different 6 amino acid ORFs, resulting in proteins of the same length as the pro-angiogenic isoforms, but with a different terminal sequence. These VEGF-A_{xxxb} isoforms are anti-angiogenic in physiological systems [7], in experimental VEGF-A-driven angiogenesis [8], and in pathologic angiogenesis driven by VEGF-A [9], including in tumours [10-13].
The anti-angiogenic VEGF-A\textsubscript{\textit{xxx}}\textsubscript{b} isoforms are the predominant isoforms in normal human colon among other tissues [13]. Furthermore VEGF-A\textsubscript{\textit{xxx}}\textsubscript{b} isoforms are present in colonic adenoma and appear to be down regulated in colon cancer, but with substantial inter-patient variability [13]. The ratio of VEGF-A\textsubscript{\textit{xxx}}:VEGF-A\textsubscript{\textit{xxx}}\textsubscript{b} may have implications for anti-angiogenic therapy with Bevacizumab, as recently demonstrated in a study of patients who had been treated with chemotherapy with or without Bevacizumab. This study showed immunohistochemical (IHC) staining of tumours for VEGF-A\textsubscript{\textit{xxx}}\textsubscript{b} predicted disease free survival in patients being treated with Bevacizumab and oxaliplatin based chemotherapy [14]. This was using a subjective (although blinded) scoring system and required normal mucosa and tumour to be stained with both VEGF-A\textsubscript{\textit{xxx}}\textsubscript{b} and pan-VEGF antibodies. A more objective, non-invasive measure of VEGF-A\textsubscript{\textit{xxx}}\textsubscript{b} levels, for instance circulating VEGF-A\textsubscript{\textit{xxx}}\textsubscript{b} levels, would provide a cleaner and easier assessment criteria for bevaccizumab treatment.

We therefore set out to test the hypothesis that plasma VEGF-A\textsubscript{\textit{xxx}}\textsubscript{b} levels measured by ELISA can be used to predict the staining outcome.

**Materials and methods**

Plasma and tissues was obtained from 18 patients diagnosed with colorectal adenocarcinoma at the Bristol Royal Infirmary between May 2013 and December 2013. Ethics approval for the study was obtained from the North Somerset and South Bristol Research Ethics Committee (07/H0102/45) and protocols conformed to the tenets of the Declaration of Helsinki, as revised in 2008. A venous blood sample was obtained from each participant after informed written consent.

**Preparation**

During pre-operative assessment, 1 week before surgery, blood was drawn and sent up to the laboratory in EDTA-treated vacutainers, within 60 minutes from being drawn (identified for rapid processing using special labels). Post-operative plasma samples were collected by the same protocol 24-48 hours post-procedure. Pre-and post-operative blood samples were centrifuged for ten minutes at 3200 rpm and plasma was collected and frozen at -80°C. The colorectal carcinoma specimens were formalin-fixed and paraffin embedded. They were cut at 5 μm and stained using pro-angiogenic and anti-angiogenic antibodies using an automated immunohistochemistry process (Leica Bond III, Leica Biosystems).

**Immunohistochemistry**

All isoforms of VEGF-A, termed VEGF-A\textsubscript{total} or Pan-VEGF-A were examined by immunohistochemistry using a commercially available anti-VEGF-A antibody (A20, Santa Cruz, rabbit polyclonal) at 2 µg/ml in an automated machine (Leica Bond III, Leica Biosystems) in 1:100 EDTA buffer. This detects isoforms generated by both proximal splice site and distal splice site by identifying the C-terminus. One patient sample was lost during tissue processing. VEGF-A\textsubscript{\textit{xxx}}\textsubscript{b} expression was examined by immunohistochemistry using a mouse monoclonal IgG1 antibody raised to the terminal nine amino acids of the VEGF-A\textsubscript{165}b sequence. It was affinity purified against the antigen from conditioned media of hybridoma cells (Abcam MRVL56/1). It has previously been shown to have specificity for the VEGF-A\textsubscript{165}b isoform over the VEGF-A\textsubscript{165} isoforms and does not detect VEGF-A\textsubscript{165} or VEGF-A\textsubscript{121}, recombinant protein [15, 16]. This antibody has a VEGF-A\textsubscript{165}b association constant of 3x10⁴ (mol/L⁻¹s⁻¹) and dissociation constant of 0.011s⁻¹ but no affinity with VEGF-A\textsubscript{165} [10]. It also detects other VEGF-A\textsubscript{\textit{xxx}}\textsubscript{b} isoforms such as VEGF-A\textsubscript{121}b, VEGF-A\textsubscript{145}b, and VEGF-A\textsubscript{189}b in human tissues [16]. It was used at 37 µg/ml in 1:35 EDTA buffer. Negative controls received a matched concentration of IgG of the primary animal (mouse or rabbit).

Slides were independently assessed and staining intensity scored (1-4). All sections were examined, conducted by 3 different assessors, blinded to treatment (JB, GSF and DOB). The intensity of DAB staining was graded from 1-4 in the normal mucosal tissue and the most poorly differentiated tumour for each section. Stroma was not scored. Tissue staining was scored as follows:

<table>
<thead>
<tr>
<th>SCORE</th>
<th>DEGREE OF STAINING</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Weak staining</td>
</tr>
<tr>
<td>2</td>
<td>Moderate staining</td>
</tr>
<tr>
<td>3</td>
<td>Strong staining</td>
</tr>
<tr>
<td>4</td>
<td>Intense staining</td>
</tr>
</tbody>
</table>

Plasma VEGF-A_{xxb} predicts tumour VEGF isoform ratios

**Figure 1.** Immunohistochemical staining for VEGF-A_{xxb}, pan-VEGF and control (mouse IgG) from normal mucosa and from colorectal cancer samples. Staining intensity was scored from 1-4. Scale bar = 100 µm.
Plasma VEGF-\(A_{\text{xxx}}\)\(_b\) predicts tumour VEGF isoform ratios

Normal colonic mucosa adjacent to the tumour acted as a positive control and was used to determine the relative intensity of the VEGF-A isoforms between adenocarcinoma and normal mucosa. The ratio of the 2 scores was then calculated: 

\[
\frac{\text{VEGF-A}_{\text{xxx}} \text{normal/tumour}}{\text{panVEGF-A}_{\text{normal/tumour}}}
\]

**ELISA**

Plasma samples were assessed for expression of both VEGF-\(A_{\text{xxx}}\)\(_b\) and panVEGF-A, a measure of both VEGF-\(A_{\text{xxx}}\) and VEGF-\(A_{\text{xxx}}\)\(_b\) isoforms, using Enzyme-Linked Immunosorbent Assay (ELISA).

An Immunoassay 96 well plate (Thermo Life Sciences) was coated with a mouse anti-VEGF-\(A_{165}\)\(_b\) monoclonal antibody (Clone 56/1, MAB3045, R&D Systems) at a concentration of 10 µg/ml, covered with parafilm and protected from light, then left shaking at room temperature (RT) overnight (~16 hours).

panVEGF-A capture was mouse anti-VEGF-A monoclonal antibody at 2 µg/ml (MAB293, R&D Systems). The plate was then washed 3 times with 0.05% Tween/PBS (200 µl/well), then blocked with 1% BSA/PBS, at 200 µl/well, and left for 2 hours at RT. Plates were washed, and frozen plasma was thawed and added to each well at 100 µl/well.

Recombinant human VEGF-\(A_{165}\)\(_b\) (P/N#842338, R&D) or recombinant human VEGF-A (P/N# 840164, R&D) were used as a control, starting at a concentration of 4 ng/ml, at serial dilutions to generate a standard curve. Plates were washed and biotinylated goat polyclonal anti-VEGF-A (BAF293, R&D) was added at a concentration of 50 ng/ml, 100 µl/well and incubated at RT for 2 hours.

Plates were washed and HRP-streptavidin (P/N#890803, R&D) was diluted 1:200 and added at 100 µl/well. This was left at RT for 20 minutes without shaking and protected from light.

**Figure 2.** VEGF-\(A_{\text{xxx}}\)\(_b\) levels measured in plasma. A. Frequency distribution of sample values. B. VEGF-\(A_{\text{xxx}}\)\(_b\) levels before and after surgery. BDL= below detection limit. C. Correlation of VEGF-\(A_{\text{xxx}}\)\(_b\) levels before and after treatment. \(R=0.815, P<0.001\).
Plasma VEGF-A\textsubscript{xxx}b predicts tumour VEGF isoform ratios

Following a final wash, HRP substrate (A:B=1:1, DY999, R&D) was added in at 100 µl/well and incubated at RT in foil for 10-25 minutes. Addition of 1M H\textsubscript{2}SO\textsubscript{4} (50 µl/well) was used to halt the reaction.

The plate was read at 450 nm using the plate photo spectrometer, with secondary corrective reading of the plate at 540 nm or 620 nm to adjust for plate optical effect.

Statistics

Statistical analysis was performed using Prism6. A probability of <0.05 was considered significant. Spearman correlation coefficient was used to determine relationships. Differences were assessed using mann whitney U test (unpaired) or Wilcoxon paired test.

Results

Immunohistochemical staining

All 17 sections had both normal mucosa and tumour tissue. Figure 1 shows examples of both normal and tumour tissue stained with VEGF-A\textsubscript{xxx}b and pan VEGF staining, with examples of different scoring. VEGF-A\textsubscript{xxx}b was negatively correlated with panVEGF staining \(r=0.21, P=0.017\) but not correlated with AJCC score \(P=0.1\).

ELISA

VEGF-A\textsubscript{xxx}b was detected above the ELISA detection limit (15 pg.ml\textsuperscript{-1}) in 13/18 patients. Median plasma VEGF-A\textsubscript{xxx}b was 151.1 pg.ml\textsuperscript{-1}, (range, 0-1944), and the distribution of VEGF plasma levels was skewed, with a mean of 527±167 pg.ml\textsuperscript{-1} (Figure 2A). This is consistent with previously published values [13]. There was no significant difference between the VEGF-A\textsubscript{xxx}b levels before and after (median 170.9, range 1-2198 pg.ml\textsuperscript{-1}) surgery (Figure 2B), although they were highly correlated \(r=0.815, P<0.001\,\textit{Figure 2C}\).

The prognostic index calculated from the ECOG data was based on an \textit{a priori} hypothesis that the ratio of the intensity of VEGF-A\textsubscript{xxx}b\textsubscript{normal/tumour} to the panVEGF\textsubscript{normal/tumour} predicted response to bevacizumab. To determine whether this could be estimated based on plasma VEGF-A\textsubscript{xxx}b levels, we plotted the VEGF-A\textsubscript{xxx}b\textsubscript{normal/tumour}/panVEGF\textsubscript{normal/tumour} ratio against the plasma VEGF-A\textsubscript{xxx}b before surgery. There was a significant correlation (Figure 3A). The mean VEGF-A\textsubscript{xxx}b\textsubscript{normal/tumour}/panVEGF\textsubscript{normal/tumour} ratio of tumours from patients with a greater than median plasma VEGF-A\textsubscript{xxx}b was significantly greater than those with a lower than median VEGF-A\textsubscript{xxx}b plasma concentration (Figure 3B).

Discussion

The use of bevacizumab in treating metastatic and other colorectal cancer has raised considerable controversy and discussion. It is clear from a number of phase III clinical trials that the use of intravenous bevacizumab in combination with chemotherapy can provide an increase in both progression free and overall survival ranging from a few weeks to a couple of months. It is also clear that only a small subset of
patients can benefit from this treatment, and the search for a biomarker has been intense and largely fruitless. A few molecular markers have been identified by high throughput screening, but these tend not to have been validated in larger studies.

The identification of VEGF-A$_{xxx}$b levels as a biomarker was the first hypothesis driven, target mediated clue that tumours may have a molecular phenotype that could help targeting of bevacizumab therapy. That study used the tissue samples from the primary, independent registration trial of bevacizumab to show that the ratio of VEGF-A$_{xxx}$b to pan VEGF could be used to predict benefit of bevacizumab. In patients with a higher than average ratio of VEGF-A$_{xxx}$b to panVEGF there was no benefit in progression free survival, whereas in those with less than median showed a doubling of progression free survival. However although that clinical trial recruited over 1000 patients, samples for immunohistochemistry were only available for 287 patients. Of these, one third had been entered into a bevacizumab only arm that was stopped early due to poor outcomes. Thus only 179 samples were available for staining, and of these only 97 provided staining for both pan and VEGF-A$_{xxx}$b in both normal and tumour tissues. Staining was then assessed by observers and scored on a basis compared with normal tissue as an internal control. This process is subjective and difficult to standardise. We therefore sought to determine whether a circulating marker of VEGF-A$_{xxx}$b would correlate with the ratio, and could be used as potential marker for bevacizumab responsiveness.

The results shown here indicate that plasma VEGF-A$_{xxx}$b levels correlate with tissue VEGF-A$_{xxx}$b levels. This suggests that circulating VEGF-A$_{xxx}$b levels could be used to determine responsiveness to bevacizumab. To do so conclusively would require a prospective study of plasma VEGF-A$_{xxx}$b levels in patients treated with and without bevacizumab. This is an ethically complicated study, as it would deprive some patients of benefit from a standard of care treatment. An alternative would be to measure response rates in patients treated with bevacizumab and to determine whether they were greater in patients with high plasma VEGF-A$_{xxx}$b than in those with low VEGF-A$_{xxx}$b. The disadvantage with this process is that a very large number of patients would need to be entered into a trial to determine differences, as patients with high VEGF-A$_{xxx}$b have a better outcome irrespective of bevacizumab treatment. Thus the “response” rates, particularly for stable disease are likely to be higher in the high VEGF-A$_{xxx}$b group irrespective of bevacizumab.

The results shown here indicate that circulating VEGF-A$_{xxx}$b levels can be used as a surrogate of tumour relative VEGF-A$_{xxx}$b levels, and that this suggests that a prospective trial of bevacizumab treatment based on VEGF-A$_{xxx}$b levels is warranted, particularly in health care systems where bevacizumab is not available for metastatic colorectal cancer, and potentially for other cancers too.

We are in the molecular era of chemotherapeutics, with trials examining neoadjuvant chemotherapy and targeted receptor therapy, such as panitumumab in ras WT tumours in the FoxTROT study. If the VEGF angiogenic ratio proves to be a suitable biomarker of tumour response to bevacizumab, it begs the question of whether or not it will have a role in neoadjuvant treatment.

This opens the possibility of a radical change in the way operable colorectal cancer is managed (with targeted receptor therapy pre-resection) as well as tailored adjuvant therapy or successive therapy in progressive colorectal cancer cases.

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Disclosure of conflict of interest

Professor Harper and Bates are co-inventors on a patent describing VEGF-Axxx isoforms.

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