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Imaging liver metastases of colorectal cancer patients with radiolabelled bevacizumab: Lack of correlation with VEGF-A expression

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ABSTRACT

Aim of the study: To investigate the correlation between tumour accumulation of In-111-bevacizumab and VEGF-A expression in patients with colorectal liver metastases.
Methods: Two weeks before resection of the liver metastases 12 patients were intravenously injected with In-111-labelled bevacizumab. Ten minutes and 7 d after injection a whole body scan was acquired. Seven days after the injection, 3D acquisition SPECT of the liver was performed.
Results: Enhanced uptake of In-111-bevacizumab in the liver metastases was observed in 9 of the 12 patients. The level of antibody accumulation in these lesions varied considerably.There was no correlation between the level of In-111-antibody accumulation and the level of VEGF-A expression in the tissue as determined by *in situ* hybridisation and ELISA.
Conclusions: In this study, we investigated the correlation between tumour accumulation of radiolabelled bevacizumab and VEGF-A expression in patients with colorectal liver metastases. No clear-cut correlation between the level of antibody accumulation and expression of VEGF-A was found.

1. Introduction

In order to grow and metastasise, tumours need a constant supply of oxygen and nutrients. For their growth beyond the size of 1–2 mm tumours are dependent on angiogenesis: the formation of new blood vessels from the existing ones.[1] Angiogenesis is a complex and dynamic process regulated by pro- and anti-angiogenic factors. Vascular endothelial growth factor A (VEGF-A) plays a pivotal role in angiogenesis. It promotes endothelial cell proliferation and migration, and enhances the vascular permeability of tumour vessels.[2] VEGF-A is frequently upregulated in tumours that are subject to hypoxia, but genetic factors may also contribute to enhanced VEGF-A expression.[3,4]

Since 2004, the humanised anti-VEGF-A monoclonal antibody (mAb), bevacizumab, derived from the murine mAb A4.6.1. has been approved for first-line treatment of patients with metastatic colorectal cancer in combination

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with 5-fluorouracil-based chemotherapy.[5] Bevacizumab binds all VEGF-A isoforms, which prevents interaction with the VEGF-A receptors VEGFR-1 and VEGFR-2, and thus inhibits VEGF-mediated effects on the tumour vascular network.[5,6]

In previous studies in murine tumour models, we and others have shown that radiolabelled bevacizumab specifically accumulates in VEGF-expressing tumours and that In-111-labelled bevacizumab allowed non-invasive detection of VEGF-A-expression.[7]

In this study, we investigated the potential of In-111-labelled bevacizumab to image the expression of VEGF-A in liver metastases of 12 patients with colorectal cancer. After scintigraphic imaging the liver metastases were resected. The VEGF-A expression in these resected liver metastases was determined by *in situ* hybridisation and ELISA.

2. Material and methods

2.1. Patients and study design

After informed consent, a total of 12 patients with colorectal liver metastases were enroled in this single centre study at the Radboud University Nijmegen Medical Centre. The study protocol was approved by the Institutional Review Board of the hospital. All patients were scheduled for resection of the colorectal liver metastases within 6 weeks after entering the study. Primary tumours had been removed at least 11 weeks before inclusion.

Exclusion criteria were pregnancy, lactation, any extra hepatic metastatic disease and prior chemotherapy treatment. During workup for surgical resection of the colorectal liver metastases a multislice CT scan of the chest and the abdomen was acquired on a 4-slice scanner (Somatom Volume Zoom, Siemens, Erlangen, Germany). In addition, a pre-operative FDG-PET scan was performed (Siemens ECAT Exact 47 or Siemens Biograph PET-CT Siemens, CTI, Knoxville, Tennessee, USA). The volume of the liver metastases was estimated based on the CT images using a dedicated software package (HepaVision, MeVis, Bremen, Germany).

2.2. Study drug

Bevacizumab (Avastin®, Roche, Basel, Switzerland) was conjugated to isothio-cyanatobenzyl-diethylenetriaminepentaacetic acid (DTPA) as described previously.[8] Kits containing 25 mg of bevacizumab-DTPA in 1.0 ml of 0.15 M citrate buffer, pH 5.5, ready for radiolabelling were produced and stored at –20 °C until use. Upon referral of a patient, a kit was labelled with 200 MBq In-111 (Mallinckrodt, Petten, the Netherlands). Radiochemical purity, as checked by instant thin layer chromatography (ITLC), always exceeded 95%.

2.3. Imaging

Ten to 14 d before surgical resection of the metastasis, 1 mg of bevacizumab, labelled with 200 MBq of In-111, was intravenously injected over a period of 10 min (1 ml/min) within 1 h after preparation.

Ten minutes and 7 d after injection of In-111-bevacizumab, a whole body scan was acquired using a double-headed gamma camera (Siemens Ecam, Hofmann Estates, IL) equipped with medium energy collimators (symmetric 15% window over 173 and 245 keV emission peaks, scan speed 5 cm/min immediately after injection and 10 cm/min at day 7). Images were stored digitally in a 256×1024 matrix. Seven days after injection, 3D SPECT (single photon emission computed tomograph) acquisition of the liver was performed allowing better visualisation of any uptake in liver metastases (2×32 views, 50 s per view, 128×128 matrix). The uptake in liver metastases was scored by comparing the uptake in the metastases to that in normal liver tissue. Uptake was scored as: 0 (equal or less uptake than surrounding normal liver tissue, 1 (slightly enhanced uptake), 2 (definite enhanced uptake) or 3 (clear hot spot in the liver).

In addition, the uptake of In-111-bevacizumab in the liver metastases was semi-quantitatively scored by drawing regions of interest over the liver metastases and adjacent normal liver tissue on the planar images. In-111-bevacizumab uptake in the ROI was expressed as the percentage increased uptake: (uptake in tumour–uptake in normal liver)/uptake in normal liver.

2.4. VEGF-A plasma concentration

Prior to injection of In-111-bevacizumab a blood sample was obtained to determine the plasma VEGF concentration (ng/ml). Plasma samples were stored at –80 °C within 1 h after venipuncture. A four-antibody sandwich ELISA was used, using duck anti-chicken and chicken anti-VEGF in the pre-analyte stage, and rabbit anti-VEGF and goat anti-rabbit in the post-analyte stage.[9]

2.5. *In situ* hybridisation

Spatial information on VEGF-A expression of all VEGF-A isoforms in tumour tissue was acquired by *in situ* hybridisation on formalin-fixed, paraffin-embedded tissue samples of all included patients. Sections (10 μm) were cut and hybridised with a digoxigenin-labelled antisense VEGF RNA probe as described previously.[8] A sense probe was used as control. Hybridised RNAs were detected using an anti-digoxygenin-alkaline-phosphatase antibody, which was visualised using NBT-BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate *p*-Toluidine Salt) using standard conditions.[10]

2.6. VEGF-A levels in tumour extract

From frozen metastases, thick cryosections were cut and pooled in RIPA buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1% NP-40, 0.5% sodiumdeoxycholate) and protease inhibitors Cocktail (Roche, Basel, Switzerland) containing heparin (5 U/ml) to release matrix-bound isoforms of VEGF-A. Sections were homogenised, incubated on ice for 20 min and centrifuged (800*g*, 20 min at 4 °C) to prepare a clear lysate. Protein concentrations were determined by the Bradford method (Biorad, Hercules, CA) and VEGF-A concentrations were measured by ELISA as described previously. This ELISA detects all VEGF-A isoforms.[9]

2.7. Immunohistochemical analysis

Vascular density of the liver metastases was studied in the tissue sections that were stained with an anti-CD31 monoclonal antibody (JC70A, dilution 1:40, Dako, Glostrup, Denmark).

A peroxidase-conjugated mouse antibody against smooth muscle actin (α-SMA, Sigma Chemical Co., St. Louis, USA) was used for detection of pericytes as a measure of vessel maturation stage. To detect hypoxic cells in the tumour, immunostainings for Glut1 expression were performed (A3536, dilution 1:200, Dako, Glostrup, Denmark).

Peritumoural vascular density, SMA expression and Glut1 expression were semi-quantitatively scored on a scale ranging from undetectable (–), low (±), moderate (+), high (++) to very high (+++).

Table 1 – Patients and tumour characteristics

Number of patients	
Total	12
Male	6
Female	6
Age	
Mean	63.2
Median	63.5
Range	50–75
TNM classification	
T1	0
T2	2
T3	9
T4	1
N0	7
N1	2
N2	3
M0	0
M1	12
Number of metastases	
1	5
2	6
3	1
⩾4	0
Mean	1.7
Median	1.5
Range	1–3
Size largest metastasis (cm)	
Mean	3.2
Median	3.3
Range	1.6–4.8
Volume metastases (ml)	
Mean	11.0
Median	9.7
Range	1.2–37.8

2.8. Statistical analysis

Correlations between the scintigraphic images and the laboratory results were determined by Pearson's (r) and Spearman's (ρ) correlation coefficients.

3. Results

3.1. Patient and tumour characteristics

Patient and tumour characteristics are listed in Table 1. A total of 12 patients were enroled in this study. The study population consisted of 6 males and 6 females with a median age of 64 years (range 50–75 years). The mean number of metastases in the liver was 1.7 ± 0.7. Five of the 12 patients had a solitary liver metastasis. Six patients had two lesions and one patient had three lesions. The mean diameter of the largest metastasis was 3.2 ± 1.0 cm, the mean volume of the largest liver metastases measured on CT was 11.0 ± 10.5 ml.

3.2. Scintigraphic imaging

The In-111-labelled bevacizumab images acquired 10 min after injection showed distribution of the radiolabelled antibody in the vascular system and blood-rich organs such as liver and spleen. On the images 7 d after injection, enhanced In-111-bevacizumab targeting was observed in the liver

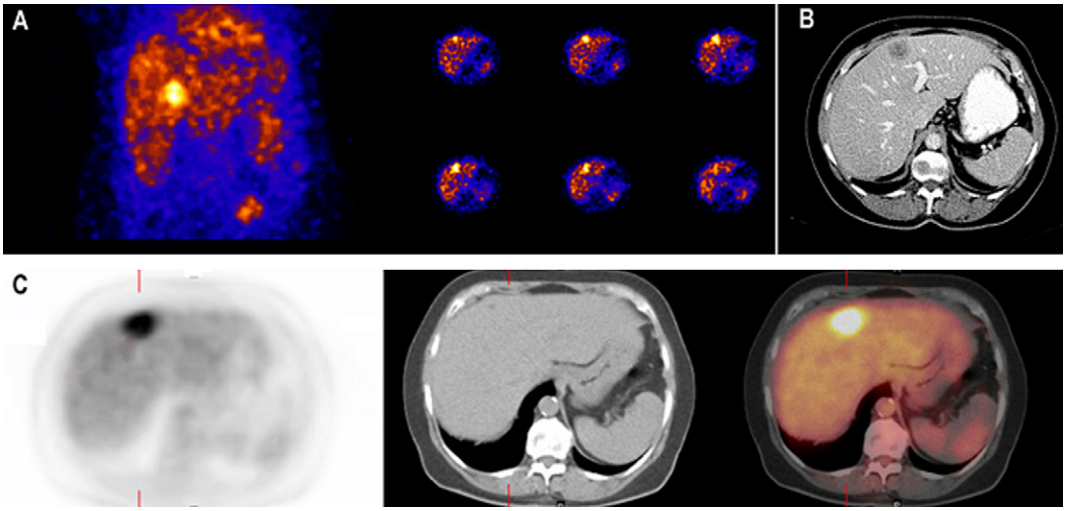

Fig. 1 – (A) In-111-bevacizumab scan: Scintigraphic imaging of a liver metastasis with In-111-bevacizumab. (B) 4-phase CT scan: Imaging of liver metastasis. (C) PET/CT scan: Combined FDG-PET scan and CT scan of the liver lesion.

metastases of 9 patients. A representative example of a scintigraphic image of a patient with enhanced uptake of In-111-bevacizumab in the liver metastasis is shown in Fig. 1.

Of the nine patients that showed positive imaging results, a clear hot spot was visible (score 3) in 4 patients, a definite increase (score 2) was observed in 2 patients and the uptake was slightly increased (score 1) in 3 patients (Table 2). The semi-quantitative score of uptake in liver metastases provided similar results as the visual scoring (Table 2).

Twenty liver metastases were studied in 12 patients. In all patients with more than one liver lesion only the largest metastasis was visible on the scintigraphic images.

There was no significant correlation between the level of In-111-bevacizumab uptake in the liver lesions, as derived from the image, and the diameter of the largest metastasis ($r = 0.62$, $p = 0.10$) or the volume of the largest metastasis ($r = 0.54$, $p = 0.17$).

3.3. Blood samples

The VEGF plasma levels as measured in the blood obtained before injection of the In-111-bevacizumab are listed in Table 2. Plasma VEGF levels ranged from 0.24 to 1.02 ng/ml. There was no correlation between the levels of VEGF in the plasma and the level of In-111-bevacizumab uptake in the liver metastases ($r = 0.06$, $p = 0.89$).

3.4. *In situ* hybridisation

The *in situ* hybridisation for VEGF-A on sections of the liver metastases was positive in 6 patients with high local levels of VEGF mRNA expression in the tissue samples of two of these patients (Table 2). Of these two patients, one had a high uptake of In-111-bevacizumab in the liver metastasis (score 3). The other patient did not have visible uptake of the radiolabelled antibody (score 0). VEGF-A levels as determined by *in situ* hybridisation correlated significantly with the VEGF-A levels in the tissues as determined by ELISA (Spearman's $\rho = 0.71$ with $p = 0.04$). The VEGF levels in plasma of these patients were 0.89 and 0.25 ng/ml, respectively. There was no correlation between the VEGF-A levels in the liver metastases as determined by *in situ* hybridisation and the level of VEGF in plasma or the uptake of In-111-bevacizumab in the lesion (Spearman's $\rho = 0.13$ and 0.43, respectively with $p = 0.76$ and 0.19, respectively).

3.5. VEGF-A levels in tumour extract

VEGF-A levels in tumour tissue extract were determined using an enzyme-linked immunosorbent assay. VEGF levels in tumour extracts varied from 0.54 to 28.3 ng/mg protein. There was no correlation between the VEGF levels in tumour extracts and the levels of antibody accumulation on the scintigraphic images ($r = 0.22$, $p = 0.67$).

3.6. Immunohistochemical analysis

Vascular density in the liver metastases, as determined by the expression of CD31, was moderate (+) to high (++) for all metastases. Glut1 expression in the liver metastases (which

Table 2 – Results of scintigraphic imaging, immunohistochemistry and ELISA

Patient	Number of lesions	Volume largest lesion (ml)	In-111-bevacizumab uptake in largest lesion[a]	In-111-bevacizumab uptake, Semi-quantitative score[b]	ISH[c]	VEGF-A in tumour extract (ng/mg protein)	Plasma VEGF level (ng/ml)	CD31[c]	α-SMA[c]	Glut1[c]
1	1	1.2	1	2.4	+	0.83	0.80	+	+++	+++
2	1	7.5	2	19	++	4.20	0.89	±	++	+++
3	2	8.6	2	16	–	NA	0.54	++	+++	++
4	3	10.8	3	55	+	2.00	1.02	++	+++	+
5	1	37.8	3	38	+	3.70	0.40	++	+++	+
6	2	24.5	1	36	–	1.70	0.24	+	+++	±
7	1	6.3	0	Not measurable	–	0.54	0.47	–	+++	+
8	2	3.1	0	Not measurable	++	28.3	0.25	+	+++	++
9	2	7.0	1	Not measurable	–	0.64	0.41	++	+++	±
10	2	14.9	3	37	+	4.30	0.70	±	+++	+
11	2	6.8	0	Not measurable	NA	NA	0.63	NA	NA	NA
12	1	4.1	3	23	–	NA	0.24	±	+++	+++

NA: Not analyzed.

a Uptake of In-111-antibody in liver metastases: no uptake (0), slightly enhanced uptake (1), enhanced uptake (2), hot spot (3).

b Semi-quantitative score: (uptake tumor–uptake normal liver tissue)/(uptake normal liver tissue).

c ISH, CD31, α-SMA, Glut1: undetectable (–), low (±), moderate (+), high (++), very high (+++).

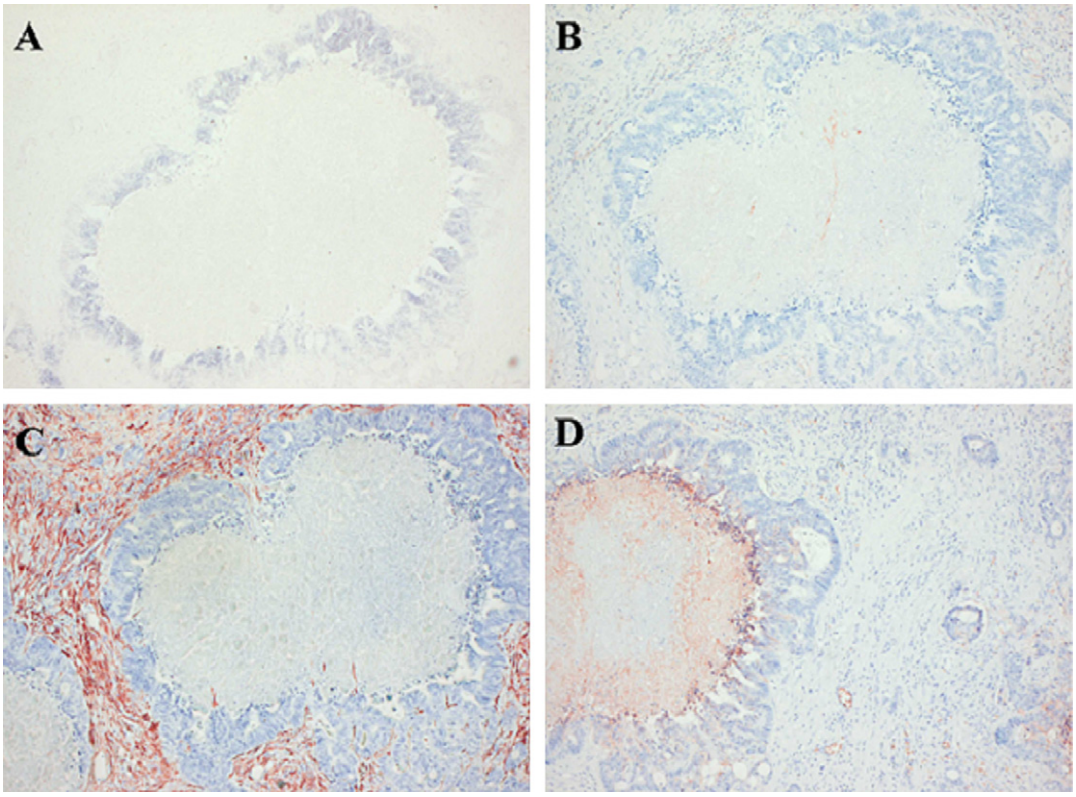

Fig. 2 – Immunohistochemistry.Tissue samples from patient with score 1 In-111-bevacizumab uptake on scintigraphic image. (A) Moderate VEGF expression with *in situ* hybridisation (+). (B). High CD31 expression (++). (C). Very high α-SMA expression (+++). (D). Moderate Glut1 expression (+).

is correlated with hypoxia) varied largely, ranging from low (±) to very high (+++). The expression of vessel-associated α-SMA was high to very high in all patients, indicating that most vessels in the liver metastases of these patients were mature vessels (see Fig. 2).

None of these three immunohistochemically determined expression levels correlated with the In-111-bevacizumab uptake on the scintigraphic scans (Spearman's $\rho = 0.25, 0.70, 0.55$ with $p = 0.55, 0.053, 0.16$, respectively).

4. Discussion

The primary aim of this study was to determine the correlation between tumour accumulation of In-111-bevacizumab and VEGF-A expression in patients with colorectal liver metastases using.

Bevacizumab, a humanised monoclonal antibody, inhibits the activity of VEGF-A on the tumour vascular network by binding and neutralising all VEGF-A isoforms. In combination with intravenous 5-fluorouracil-based chemotherapy, bevacizumab is indicated for first- or second-line treatment of patients with metastatic carcinoma of the colon or rectum. The response rate in patients treated with a combination of chemotherapy and bevacizumab is significantly higher (45%) than in patients treated with chemotherapy alone (35%). Similarly, addition of bevacizumab to the regimen of irinotecan, fluorouracil and leucovorin (IFL) increased the median response duration from 7.1 to 10.4 months.[11] However, only part of the patients treated with bevacizumab benefit from this therapy. Non-invasive determination of the VEGF-A expression in tumour lesions could potentially be used to select those patients who might benefit from therapy with bevacizumab. Therefore, in this study imaging of the VEGF-A expression with In-111-bevacizumab was studied in patients with metastatic colorectal cancer.

In this study, the level of VEGF-A expression was compared with the uptake of the radiolabelled antibody in the liver metastases. In nude mice with subcutaneous VEGF-A expressing human LS174T colorectal tumour xenografts, we showed that In-111-bevacizumab allowed specific detection of VEGF-A expression, whereas non-relevant antibodies did not accumulate in the tumour.[12] Intravenous injection of In-111-bevacizumab resulted in high and specific uptake in the tumour (20–25% ID/g). Specific accumulation of radiolabelled bevacizumab in human tumour xenografts in nude mice was also reported by Nagengast and colleagues. Although in this study the relation between tumour uptake of radiolabelled antibody and VEGF expression was not analysed.[7]

In this study the liver metastases were scintigraphically delineated in 9 of the 12 patients. In this group of nine patients antibody accumulation in the liver varied considerably. Despite extensive efforts to accurately determine VEGF-A expression in the lesions, no correlation was found between VEGF-A expression levels in the tumour samples as determined by *in situ* hybridisation, ELISA and antibody accumulation in the liver lesions.

To date at least seven splice variants of VEGF-A are known: $VEGF_{121}$ $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$ $VEGF_{189}$ and $VEGF_{206}$. Alternative splicing of a pseudo-exon 9 results in the generation of inactive $VEGF_{165b}$ variant.[13] Whereas $VEGF_{121}$ is a freely diffusible protein, $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the extracellular matrix (ECM). $VEGF_{165}$ (the predominant isoform of VEGF-A) has intermediate properties; it is secreted and a significant fraction is associated with the tumour cell surface and the ECM. Using mouse models with human VEGF-A negative MEL57 tumours, transfected with cDNA encoding VEGF-A specific isoforms, we recently demonstrated that the accumulation of the antibody in the tumour is due to interaction with VEGF-A isoforms that are associated with the tumour cell surface or ECM (submitted for publication).

When measuring the levels of VEGF-A in tumour extracts by ELISA or in tumour sections by *in situ* hybridisation all VEGF-A isoforms are measured. Since the freely diffusible $VEGF_{121}$ expression is not visualised by scintigraphic imaging, this could explain the discrepancy between the levels of VEGF-A as determined by *in situ* hybridisation or ELISA and the accumulation of radiolabelled antibody in the liver metastases visualised on the scintigraphic scan. Lack of bevacizumab accumulation in VEGF-A expressing lesions could therefore be due to the expression of the soluble isoform $VEGF_{121}$. However, this limits the suitability of radiolabelled bevacizumab as a tracer.

In contrast, a few liver metastases without any VEGF-A expression were visualised. Antibody accumulation in the tumour in the absence of VEGF-A expression could be due to enhanced vascular permeability of tumour vessels in these lesions. This will result in nonspecific enhanced extravasation of the radiolabelled antibody in the tumour, as reported first by Morrell and colleagues.[14] Theoretically, any IgG molecule would accumulate in these tumours.

Although no clear-cut correlation between the level of antibody accumulation an expression of VEGF-A was found, it would be very interesting to correlate the uptake of radiolabelled antibody to the clinical outcome of the patient in future studies.

In conclusion, in this study we investigated the correlation between tumour accumulation of radiolabelled bevacizumab and VEGF-A expression in patients with colorectal liver metastases. No clear-cut correlation between the level of antibody accumulation and expression of VEGF-A was found. This may be due to the inability to visualise the soluble $VEGF_{121}$ isoform and enhanced vascular permeability in tumours.

Conflict of interest statement

None declared.

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