Identification of serum angiopoietin-2 as a biomarker for clinical outcome of colorectal cancer patients treated with bevacizumab-containing therapy

Y. Goede¹,², O. Coutelle¹,², J. Neuneier¹, A. Reinacher-Schick², R. Schnell¹, T. C. Koslowsky⁴, M. Reinhard¹, R. Schnell³, T. C. Koslowsky⁴, M. R. Weihrauch¹ and U. T. Hacker*,¹,8

¹Department of Internal Medicine I, Center of Integrated Oncology Cologne-Bonn, University Hospital Cologne, Kerpener Straße 62, Cologne 50924, Germany; ²Department of Internal Medicine, University Hospital Bochum, In der Schornau 23–25, Bochum 44892, Germany; ³Oncology and Hematology Practice, Kölnner Strasse 9, Frechen 50226, Germany; ⁴Department of Surgery, St Elisabeth Hospital, Wertenstr. 1, Cologne 50935, Germany; ⁵Institute for Medical Microbiology, Immunology and Hygiene, University of Cologne, Goldenfelds Strasse 19–21, Cologne 50935, Germany; ⁶Department of Pathology, University of Cologne, Kerpener Straße 62, Cologne 50924, Germany; ⁷Joint Research Division Vascular Biology, Medical Faculty Mannheim (CBTM), Heidelberg University and German Cancer Research Center Heidelberg (DKFZ-ZMBG Alliance), Im Neuenheimer Feld 280; Heidelberg 69120, Germany

BACKGROUND: The combination of chemotherapy with the vascular endothelial growth factor (VEGF) antibody bevacizumab is a standard of care in advanced colorectal cancer (CRC). However, biomarkers predicting outcome of bevacizumab-containing treatment are lacking. As angiopoietin-2 (Ang-2) is a key regulator of vascular remodelling in concert with VEGF, we investigated its role as a biomarker in metastatic CRC.

METHODS: Serum Ang-2 levels were measured in 33 healthy volunteers and 90 patients with CRC. Of these, 34 had metastatic disease and received bevacizumab-containing therapy. To determine the tissue of origin of Ang-2, quantitative real-time PCR was performed on microdissected cryosections of human CRC and in a murine xenograft model of CRC using species-specific amplification.

RESULTS: Ang-2 originated from the stromal compartment of CRC tissues. Serum Ang-2 levels were significantly elevated in patients with metastatic CRC compared with healthy controls. Amongst patients receiving bevacizumab-containing treatment, low pre-therapeutic serum Ang-2 levels were associated with a significant better response rate (82% vs 31%; P < 0.01), a prolonged median progression-free survival (14.1 vs 8.5 months; P < 0.01) and a reduction of 91% in the hazard of death (P < 0.005).

CONCLUSION: Serum Ang-2 is a candidate biomarker for outcome of patients with metastatic CRC treated with bevacizumab-containing therapy, and it should be further validated to customise combined chemotherapeutic and anti-angiogenic treatment.

British Journal of Cancer (2010) 103, 1407 – 1414. doi:10.1038/sj.bjc.6605925  www.bjcancer.com
Published online 5 October 2010 © 2010 Cancer Research UK

Keywords: colorectal cancer; angiopoietin-2; biomarker; chemotherapy; bevacizumab

The overall survival (OS) time of patients with metastatic colorectal cancer (CRC) has increased owing to novel therapeutic strategies combining chemotherapy with monoclonal antibodies against vascular endothelial growth factor (VEGF) or epidermal growth factor receptor (EGFR). Whereas mutations in the k-ras oncogene predict outcome to EGFR antibody treatment in patients with metastatic CRC (Lievre et al., 2006; Karapetis et al., 2008), equivalent biomarkers for the VEGF antibody, bevacizumab, are currently lacking (Jubb et al., 2006b; Sessa et al., 2008), mainly because the specific molecular determinants of clinical response and resistance to the drug are unknown. Similarly, there are currently no established biomarkers predicting outcome to chemotherapy in CRC (De Roock et al., 2009).

Originally, it was anticipated that traditional markers of tumour angiogenesis would predict outcome to bevacizumab. However, neither VEGF expression levels nor tumour microvessel density (MVD) were found to be predictive of treatment response, disease progression or death in CRC patients receiving chemotherapy plus the antibody (Jubb et al., 2006a). Newer studies evaluated alternative surrogates of neovascularisation such as circulating endothelial cells and phosphorylated VEGF receptor in cancers other than CRC (Murukesh et al., 2010). Results were promising, but assay requirements prevent widespread application in larger trials.

The therapeutic blockade of VEGF by bevacizumab in CRC patients induces complex changes in the stromal compartment of the tumour lesion, including the loss of chaotic microvessels, remodelling of the vascular wall and a reduction in the interstitial fluid pressure (Willett et al., 2004). Such stromal alterations are part of the vascular ‘normalisation’ process induced by bevacizumab and contribute to more efficient delivery of chemotherapeutic agents (Jain, 2005; Jain et al., 2006). Recently, dynamic
contrast-enhanced magnetic resonance imaging was proposed to assess the extent of vascular normalisation and predict clinical outcome to VEGF-targeting treatment (Sorensen et al, 2009; Murukesh et al, 2010). However, molecular markers reflecting the normalisation status of the tumour vascular bed as part of the tumour stroma have not been explored in this regard so far.

The molecular alterations of tumour cells are traditionally regarded as the major determinants of clinical response to chemotherapy. However, the above observations suggest that the vascular microenvironment of tumour cells could be equally important for efficacious cytostatic treatment. Surrogates of vascular normalisation, therefore, might predict outcome to chemotherapy as well as to VEGF-targeting therapy.

Angiopoietin-2 (Ang-2) is an inhibitory ligand of the Tie-2 receptor that is stored in the Weibel–Palade bodies of endothelial cells (Fiedler et al, 2004) and disrupts the integrity of the blood vessel wall, thus counteracting vascular normalisation (Maisonpierre et al, 1997; Scharpfenecker et al, 2005; Falcon et al, 2009; Reiss et al, 2009). On the basis of this, we expected Ang-2 to be predominantly expressed in the stromal compartment of CRC, and hypothesised that low and high serum Ang-2 levels in patients with metastatic CRC would predict different outcomes to bevacizumab-containing treatment.

**MATERIALS AND METHODS**

**Microdissection**

For microdissection analysis, cyrosections were prepared from human adenocarcinoma samples. Discrete areas of tumour or stromal tissue were microdissected using a laser microbeam (P.A.L.M., Bernried, Germany). Microdissected tissue areas (each 100 μm in diameter, 30 per slide for stroma and tumour each) were laser catapulted into a microfuge tube.

**Xenografts**

Animal experiments were performed in accordance with the German animal protection law. The colon carcinoma cell line LS174T was purchased from ATCC (Manassas, VA, USA) to establish xenografts in nude mice. Cultured LS174T cells (5 × 10⁶) were injected subcutaneously into the flank region of male BALB/cA nude mice (Taconic, Ry, Denmark). Animals were killed after tumour size had reached 10 mm in diameter.

**Cell cultures**

The colon carcinoma cell lines LS174T, HT29, DLD-1 and SW948 were cultured in VLE RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% FCS. Human umbilical vein endothelial cells were purchased from Promocell (Heidelberg, Germany) and cultured in endothelial cell growth medium (Promocell). All cell lines were maintained at 37°C and 5% CO₂ except for one experiment involving exposure to controlled hypoxia (1% O₂, 5% CO₂, balanced N₂) for 24 h.

**Quantitative real-time PCR**

The RNA was extracted from tissue microdissections using the NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany) and reverse transcribed using the RevertAid cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany). For gene expression analysis in xenografts, tumours were suspended in RNAlater (Qiagen, (Fermentas, St Leon-Rot, Germany). For gene expression analysis and reverse transcribed using the RevertAid cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany). For gene expression analysis in xenografts, tumours were suspended in RNAlater (Qiagen, (Fermentas, St Leon-Rot, Germany). For gene expression analysis and reverse transcribed using the RevertAid cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany). For gene expression analysis in xenografts, tumours were suspended in RNAlater (Qiagen, (Fermentas, St Leon-Rot, Germany). For gene expression analysis and reverse transcribed using the RevertAid cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany). For gene expression analysis in xenografts, tumours were suspended in RNAlater (Qiagen, (Fermentas, St Leon-Rot, Germany). For gene expression analysis and reverse transcribed using the RevertAid cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany). As published (Thijssen et al, 2004). Universal GAPDH primers were as follows: Forward 5’-TGC(A/C)TCTGCG ACCACCAACT-3’, Reverse 5’(C/T)GGCTGCTTCACCACCTTC-3’.

**Western blot**

For western blot (WB) analysis, cytosolic extracts of cultured cells were prepared as described previously (Kashkar et al, 2002). Equal amounts (100 μg) of protein were separated by SDS-PAGE, transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), and probed with Ang-2 antibody (F18, Santa Cruz Biotec, Santa Cruz, CA, USA). Primary antibodies were detected using a horseradish-peroxidase-conjugated secondary antibody (1:2000; Dako, Hamburg, Germany) and visualised with the ECL system (Amersham Biosciences, Hamburg, Germany). Culture supernatants were analysed for Ang-2 protein concentrations using Quantikine Immunoassays (R&D Systems, Wiesbaden, Germany).

**Clinical samples**

A total of 90 patients with colorectal adenocarcinoma and 33 healthy volunteers were studied between September 2005 and November 2008. One cohort of 56 patients had newly diagnosed CRC of various stages (UICC I–IV). After obtaining informed consent, serum samples and tumour tissues were collected at the time of primary resection (sampling from September 2005 to August 2006). A second cohort of 34 patients had primary (n = 25) or relapsed (n = 9) CRC of advanced stage and received a combination treatment of bevacizumab and chemotherapy either in the context of a clinical trial (AOI trial KKR 0604, n = 15) conducted by the Department of Internal Medicine of the University Hospital Bochum (Reinacher-Schick et al, 2008) or without participating in a clinical trial (n = 19) at the Center of Integrated Oncology Cologne-Bonn. Following approval by the Ethics Committees, serum samples from these patients were taken prior to treatment (sampling from March 2006 to April 2008). Where available, paraffin blocks of tumour tissue were retrieved from the local pathology archives.

Demographical, clinical and histopathological baseline parameters were documented in all patients. For patients receiving bevacizumab-containing therapy, the clinical response after 2 months of treatment was assessed according to response evaluation criteria in solid tumours (RECIST). Patients were continuously monitored during the course of treatment and disease progression and deaths occurring during and after therapy were recorded.

**Enzyme-linked immunosorbent assay**

Quantikine Immunoassays (R&D Systems) were used to measure protein concentrations of Ang-2 and VEGF in serum samples according to the manufacturer’s instructions.

**Immunohistochemistry**

Sections of paraffin tissue blocks of CRC specimens were processed for histological analysis as previously described (Eberhard et al, 2000). Immunohistochemistry (IHC) for Ang-2 was carried out with the following antibodies: MAB 0983 (R&D Systems), N18 and F18 (Santa Cruz Biotec). To assess non-specific antibody binding, Ang-2 antibodies were blocked by pre-incubation with recombinant Ang-2 (ratio 1:5, R&D systems) prior to incubation of histological sections. A biotinylated secondary antibody, streptavidin peroxidase complex, and diamobenzidine as the substrate (Zymed, Carlsbad, CA, USA) were used to visualise binding of the primary antibody (followed by...
Serum angiopoietin-2 in colorectal cancer
V Goede et al

RESULTS

Ang-2 is expressed in the stromal compartment of CRC but not in the tumour cells

Extensive immunohistochemical analysis of Ang-2 with three frequently reported commercial Ang-2 antibodies produced ambiguous results concerning the tissue localisation in human CRC because of poor antibody specificity (Supplementary Figure 1). Therefore, to identify the tissue of origin of Ang-2 in CRC, laser-captured microdissection was used to isolate the tumour and stromal compartments from tissue sections of CRC patients for quantitative real-time PCR (Figure 1A). The Ang-2 mRNA was clearly detectable in the dissected stromal compartment, but not in the tumour cell compartment (Figure 1B).

Xenografts of CRC in nude mice were generated to further verify the stromal origin of Ang-2. In these animals, the stromal compartment is of murine origin and the tumour cell compartment is of human origin (LS174T colon carcinoma cells). Using species-specific RT–PCR, Ang-2 mRNA expression was found to be exclusively of stromal (murine) origin (Figure 1C). Correspondingly, no significant amount of Ang-2 protein was detectable in cytosolic extracts and culture supernatants of various colon carcinoma cell lines (LS174T, HT29, DLD-1 and SW948) cultured under normoxia or hypoxia to mimic the conditions inside the tumour (Supplementary Figure 2).

Serum Ang-2 levels are elevated in CRC patients with metastatic disease

Blood serum concentrations of Ang-2 were studied in a total of 90 patients with colorectal adenocarcinoma and 33 healthy volunteers. The patient demographics are summarised in Table 1. In UICC stage IV patients, serum Ang-2 levels were significantly higher compared with patients with UICC stage I–III or healthy controls (3.9 vs 2.3 ng ml\(^{-1}\), P = 0.001 and 3.9 vs 2.4 ng ml\(^{-1}\), P = 0.006, respectively) (Figure 2). In contrast, serum Ang-2 levels did not differ significantly between UICC stage I–III patients and controls.

Serum Ang-2 levels identify CRC patients of different clinical outcomes to bevacizumab-containing therapy

On enrolment, 34 patients with primary or relapsed CRC of UICC stage IV were treated with bevacizumab in combination with
Serum angiopoietin-2 in colorectal cancer
V Goede et al

Table I Demographics of studied patients

| N (%) |                           |
|-------|---------------------------|
| Patients | 90 (100.0) |
| Sex     |                           |
| Male    | 54 (60.0)    |
| Female  | 36 (40.0)    |
| Age     |                           |
| Median (years) | 69 |
| Range (years) | 45–86 |
| <65 years | 31 (34.4) |
| ≥65 years | 59 (65.6) |
| Stage (UICC) |           |
| I       | 3 (3.3)       |
| II      | 30 (33.3)     |
| III     | 15 (16.7)     |
| IV      | 42 (46.7)     |
| Systemic therapy |          |
| Unfolloweda | 56 (62.2) |
| Followedb | 34 (37.8) |
| Controls | 33            |

Abbreviations: BV = bevacizumab; CRC = colorectal cancer; UICC = Union for International Cancer Control. aAt enrolment. bPatients with primary CRC who underwent tumour resection and were not followed up for outcome of any further treatment. cPatients with primary or relapsed CRC who received BV-containing treatment and were followed up for treatment outcome.

Figure 2 Serum levels of Ang-2 in CRC (n = 90). Serum Ang-2 in healthy controls, non-metastatic (stage I–III) and metastatic disease (stage IV). Ang-2, angiopoietin-2.

DISCUSSION

Bevacizumab is a VEGF-targeting antibody that is widely used in combination with chemotherapy to treat metastatic CRC (Cercek and Saltz, 2008; McCormack and Keam, 2008). Although much has been learned about its mechanisms of action, suitable biomarkers predicting patients who are likely to benefit from bevacizumab treatment remain elusive (Jubb et al., 2008). With this in mind, we investigated whether serum Ang-2 levels could serve as biomarkers for predicting treatment response and survival in patients with CRC.

We found that serum Ang-2 levels were lower in treatment responders compared with non-responders (3.3 vs 5.8 ng ml\(^{-1}\); P = 0.008). Disease control was significantly better in patients with low serum Ang-2 levels than in patients with high serum Ang-2 (median PFS: 14.1 vs 8.5 months; P = 0.009). The overall response rate (OR) in the two groups was 82 and 31% (P = 0.005), respectively (Figure 3A). Mean serum Ang-2 concentrations were lower in treatment responders compared with non-responders (3.3 vs 5.8 ng ml\(^{-1}\); P = 0.008). Disease control was significantly better in patients with low serum Ang-2 levels than in patients with high serum Ang-2 (median PFS: 14.1 vs 8.5 months; P = 0.009) (Figure 3B). There was a 63% reduction in the hazard of progression for patients with low serum Ang-2 compared with those with high serum Ang-2 (HR 0.37; 95% CI: 0.17–0.80; P = 0.01). Overall survival was remarkably prolonged in patients with low serum Ang-2 levels (median OS: not reached) compared with the group of patients with high serum Ang-2 (median OS: 16.2 months; P = 0.004). Survival rates at 1.5 years were 94 versus 53% in the low and high serum Ang-2 group, respectively (Figure 3C), and the hazard of death in low Ang-2 patients was reduced by 91% compared with patients with high serum Ang-2 levels (HR 0.09; 95% CI: 0.01–0.70; P = 0.02). Differences in survival remained significant when patients with primary and relapsed CRC were analysed separately (Supplementary Table 3), and in a multivariate analysis of variables with potential impact on OR, PFS and OS (gender, age, site, treatment regimen and treatment line), serum Ang-2 was confirmed as an independent prognostic marker for all three end points (HR 0.003, P = 0.005 and P = 0.003, respectively).

In contrast to serum Ang-2 levels, there was no significant association between OR, PFS or OS and low vs high serum VEGF or tumour PC, respectively, using cutoff values as determined by ROC analysis and Youden’s index. Similarly, tumour MVD was not associated with these end points except for OR (Table 2, Figure 4).

Although VEGF is primarily produced by tumour cells, its target structure is the tumour vasculature embedded in the stromal compartment, where the therapeutic effects of the antibody involve extensive changes such as blood vessel pruning and reorganisation of the chaotic tumour vasculature (Willett et al., 2004). Thus, stromal factors controlling the responsiveness of blood vessels to VEGF withdrawal rather than determinants of VEGF availability are attractive candidates as outcome predictors for bevacizumab treatment. Potentially, stromal factors are also outcome predictors of chemotherapy, because the delivery of cytostatic
| Sex | Age (years) | ECOG | Metas. (sites) | Regimen | Line | Initial response | Time to progress (months) | Time to death (months) | Ang-2 (ng ml⁻¹) | VEGF (ng ml⁻¹) | MVD (per HPF) | PC (%) |
|-----|-------------|------|----------------|---------|-------|----------------|--------------------------|------------------------|----------------|----------------|---------------|-------|
| M   | 63          | 1    | 1              | FOLFIRI+BV | 1st   | Yes           | —                        | —                      | 0.7            | 0.05           | NA            | NA   |
| M   | 67          | 0    | 1              | XELIRI+BV | 1st   | Yes           | —                        | —                      | 2.4            | 0.19           | 80            | 29   |
| M   | 57          | 1    | 1              | FOLFIRI+BV | 2nd   | Yes           | —                        | —                      | 2.4            | 0.20           | 29            | 56   |
| M   | 69          | 1    | 2              | FOLFIRI+BV | 1st   | Yes           | —                        | —                      | 2.9            | 0.25           | 42            | 20   |
| F   | 69          | 1    | 1              | FOLFOX+BV | 1st   | Yes           | —                        | —                      | 4.1            | 0.31           | 58            | 56   |
| M   | 59          | 1    | 1              | XELOX+BV  | 1st   | No            | —                        | —                      | 3.8            | 0.20           | NA            | NA   |
| M   | 70          | 0    | 1              | XELOX+BV  | 1st   | Yes           | 20                       | —                      | 3.3            | 0.02           | NA            | NA   |
| F   | 54          | 1    | 1              | FOLFIRI+BV | 2nd   | Yes           | 17                       | —                      | 2.1            | 0.10           | 53            | 60   |
| F   | 57          | 1    | 2              | FOLFIRI+BV | 1st   | Yes           | 17                       | —                      | 4.4            | 0.08           | 28            | 24   |
| M   | 69          | 1    | 1              | FOLFIRI+BV | 1st   | Yes           | 14                       | —                      | 2.3            | 0.62           | 49            | 24   |
| M   | 70          | 1    | 1              | FOLFIRI+BV | 2nd   | Yes           | 13                       | —                      | 1.8            | 0.20           | NA            | NA   |
| M   | 58          | 0    | 1              | XELOX+BV  | 1st   | Yes           | 13                       | —                      | 2.7            | 0.00*          | NA            | NA   |
| M   | 73          | 1    | 1              | FOLFIRI+BV | 1st   | Yes           | 13                       | —                      | 2.8            | 0.13           | 65            | 36   |
| F   | 70          | 1    | 1              | FOLFIRI+BV | 1st   | Yes           | 13                       | —                      | 3.2            | 0.64           | NA            | NA   |
| F   | 45          | 0    | 1              | XELIRI+BV | 1st   | Yes           | 13                       | —                      | 5.2            | 0.00*          | NA            | NA   |
| M   | 54          | 1    | 1              | FOLFIRI+BV | 1st   | Yes           | 12                       | —                      | 1.9            | 0.25           | NA            | NA   |
| F   | 75          | 1    | 1              | XELIRI+BV | 1st   | Yes           | 11                       | —                      | 5.9            | 0.07           | 50            | 72   |
| F   | 57          | 1    | 1              | XELOX+BV  | 1st   | Yes           | 10                       | —                      | 3.4            | 0.00*          | NA            | NA   |
| F   | 60          | 1    | 1              | FOLFIRI+BV | 2nd   | No            | 14                       | —                      | 2.0            | 0.25           | 25            | 64   |
| F   | 72          | 1    | 1              | FOLFIRI+BV | 2nd   | No            | 10                       | —                      | 2.9            | 0.33           | NA            | NA   |
| F   | 75          | 0    | 1              | XELOX+BV  | 1st   | No            | 10                       | —                      | 6.3            | 0.16           | NA            | NA   |
| F   | 77          | 1    | 3              | XELOX+BV  | 1st   | No            | 8                        | —                      | 6.5            | 0.10           | 47            | 40   |
| M   | 71          | 1    | 1              | XELOX+BV  | 1st   | No            | 8                        | —                      | 10.8           | 0.03           | 29            | 72   |
| M   | 72          | 0    | 1              | XELIRI+BV | 1st   | No            | 10                       | 14                     | 4.5            | 0.02           | 26            | 13   |
| F   | 52          | 2    | 1              | XELIRI+BV | 1st   | No            | 10                       | 11                     | 11.1           | 0.02           | 36            | 52   |
| M   | 83          | 1    | 1              | XELO+BV  | 1st   | Yes           | 8                        | 10                     | 3.7            | 0.10           | 31            | 64   |
| F   | 60          | 1    | 1              | FOLFOX+BV | 2nd   | No            | 7                        | 7                      | 2.4            | 0.15           | NA            | NA   |
| F   | 70          | 2    | 2              | FOLFIRI+BV | 2nd   | Yes           | 7                        | 9                      | 7.6            | 0.29           | 93            | 44   |
| F   | 72          | 1    | 1              | FOLFIRI+BV | 1st   | No            | 6                        | 6                      | 3.5            | 0.08           | 30            | 52   |
| M   | 57          | 0    | 2              | XELIRI+BV | 1st   | No            | 6                        | 16                     | 6.1            | 0.36           | NA            | NA   |
| M   | 77          | 0    | 3              | XELIRI+BV | 1st   | No            | 5                        | 6                      | 12.1           | 0.19           | 45            | 56   |
| F   | 51          | 0    | 2              | XELOX+BV  | 1st   | No            | 4                        | 19                     | 4.4            | 0.02           | NA            | NA   |
| F   | 80          | 2    | 2              | XELOX+BV  | 2nd   | No            | 4                        | 4                      | 7.7            | 0.39           | NA            | NA   |

Abbreviations: 5-FU/5-FU = 5-fluorouracil/folinic acid; Ang-2 = angiopoietin-2 (serum concentration); BV = bevacizumab; ECOG = Eastern Cooperative Oncology Group; F = female; FOLFIRI = 5-fluorouracil/folinic acid/irinotecan; FOLFOX = 5-fluorouracil/folinic acid/oxaliplatin; HPF = high power field; M = male; Metas. = metastasis (number of organs with metastases); MVD = microvessel density; NA = not analyzed; PC = pericyte coverage; VEGF = vascular endothelial growth factor (serum concentration); XEL = capecitabine; XELIRI = capecitabine/irinotecan; XELOX = capecitabine/oxaliplatin. *Concentration not measurable. —, No disease progression or death.
Serum angiopoietin-2 in colorectal cancer
V Goede et al

Figure 3  Outcome to bevacizumab-containing therapy in CRC by serum Ang-2 (n = 34). (A) Response rate by serum Ang-2. (B) PFS by serum Ang-2. (C) OS by serum Ang-2. Ang-2, angiopoietin-2.

drugs to tumour cells is controlled by the vascular tumour microenvironment.

Angiopoietin-2 has been proposed as a gatekeeper of VEGF function and vascular remodelling. (Hanahan, 1997; Augustin et al., 2009). We here identified Ang-2 as a stromal factor in CRC. In tumour lesions of CRC patients and in a murine xenograft model of CRC, Ang-2 mRNA was expressed exclusively in the tumour stromal compartment, but not in the tumour cell compartment itself. Although these findings are at odds with previous immunohistochemical studies reporting Ang-2 expression in the tumour cell compartment of CRC (Ochiumi et al., 2004; Ogawa et al., 2004; Chung et al., 2006; Gu et al., 2006), the published immunohistochemical data should be interpreted with caution owing to the limited specificity of the available antibodies. Indeed, careful analysis of the tissue localisation of Ang-2 expression in cancer entities and tumour models other than CRC has called into question the tumour cell origin of Ang-2 (Zhang et al., 2003).

To further elucidate the clinical impact of stromal-derived Ang-2, we measured serum Ang-2 concentrations in CRC patients. Serum Ang-2 levels were significantly elevated in patients with metastatic disease. Indeed, Ang-2 has been shown to promote metastatic growth (Imanishi et al., 2007). Although one can only speculate as to what extent the stromal expression of Ang-2 contributes to serum Ang-2 concentrations in patients with advanced CRC, serum levels of Ang-2 in such patients were found to be significantly higher than in healthy individuals. It seems likely, therefore, that serum Ang-2 levels reflect the level of Ang-2 expression in the tumour stromal compartment.

Elevated serum concentrations of Ang-2 have also been reported for patients with cancers other than CRC, such as non-small-cell lung cancer and melanoma, in which high serum Ang-2 levels correlate with disease stage and poor OS (Park et al., 2007; Helfrich et al., 2009). However, the relationship between serum Ang-2 levels and clinical outcome in patients treated with VEGF-targeting drugs and chemotherapeutic agents has not been explored before, and this study is the first to investigate the impact of pre-therapeutic serum Ang-2 concentrations on the clinical outcome in patients with metastatic CRC under bevacizumab-containing therapy. Compared with high serum Ang-2 levels, low serum Ang-2 was associated with an outstanding response rate (>80%), better disease control and excellent OS (>90% after 18 months). In accordance with previous reports (Jubb et al., 2006a), VEGF and tumour MVD were not similarly correlated to these end points. Similarly, the pericyte content of CRC was not linked to treatment outcome and did not correlate with Ang-2 serum concentrations, indicating that serum Ang-2 is probably not simply a surrogate of blood vessel morphology.

On the basis of experimental models, Ang-2 has been described as an opponent of vascular normalisation which prevents blood vessels from becoming structurally and functionally stabilised (Maisonpierre et al., 1997; Scharpfenecker et al., 2005; Falcon et al., 2009; Reiss et al., 2009). Conceivably, normalisation of tumour vessels by bevacizumab-mediated blockade of VEGF may be more difficult to achieve and chemotherapeutic drugs cannot be delivered appropriately to the tumour cells when Ang-2 serum levels are high. From a mechanistic point of view, the observation that patients with low serum Ang-2 were most likely to benefit from treatment with respect to major clinical end points supports such a biological role of Ang-2. From a clinical perspective, our observations suggest that serum Ang-2 could hold promise as a predictive biomarker allowing bevacizumab-containing treatment to be customised in CRC patients.

Having analysed a heterogeneous patient cohort of moderate size, our study is not without limitations. Nevertheless, subgroup analyses of known prognostic factors in CRC (for example, age, ECOG) showed no evidence that outcome by Ang-2 was biased by those factors. The major and novel finding of this study is that pre-therapeutic serum Ang-2 not only predicted OS in the study
population but was also predictive of therapeutic end points (PFS, response rate), suggesting that Ang-2 is a stromal determinant of both resistance and response to bevacizumab-containing therapy. As this is a single-arm study, it remains undecided whether serum Ang-2 is a specific outcome predictor for bevacizumab or chemotherapy or for the combination of both. Irrespective of its specific predictive properties, however, measurements of pre-therapeutic serum Ang-2 could be valuable. For example, CRC patients awaiting secondary resection of metastases could be stratified by serum Ang-2 levels into risk groups. Whereas patients with low serum Ang-2 are likely to benefit from neoadjuvant bevacizumab-containing treatment, patients with high serum Ang-2 could require escalation of chemotherapy or use of other biologicals such as EGFR antibodies in patients with wild type k-ras oncogene or Ang-2 inhibitors that are currently in clinical development (Hu and Cheng, 2009). Although definitive judgement on the role of Ang-2 as a specific biomarker of outcome to bevacizumab in CRC or other cancers will require analysis of large numbers of blood samples from phase III clinical trials comparing bevacizumab-containing therapy with chemotherapy alone, the promising results of this study should encourage researchers to further investigate the predictive value of Ang-2 in cancer treatment.

**ACKNOWLEDGEMENTS**

Dr U Fiedler (Heidelberg) is acknowledged for providing the human Ang-2 expression plasmid. VG and OC were funded by the Centre of Molecular Medicine Cologne (CMMC). Part of this work was funded by Mrs R Maassen (Erkelenz), the Moritz-Stiftung the Hilde-Kopp-Stiftung (Cologne) and the Marga and Walter Boll Stiftung (Kerpen).

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)
REFERENCES

Augustin HG, Koh GY, Thurston G, Altitalo K (2009) Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nat Rev Mol Cell Biol* 10: 165 – 177

Cercek A, Saltz LB (2008) First-line treatment of patients with metastatic colorectal cancer: an overview of recent data on chemotherapy plus targeted agents. *Clin Colorectal Cancer* 7(Suppl 2): S47 – S51

Chung YC, Hou YC, Chang CN, Hseu TH (2006) Expression and prognostic significance of angiopoietin in colorectal carcinoma. *J Surg Oncol* 94: 631 – 638

De Roock W, Biessmans B, De Schutter J, Teijpar S (2009) Clinical biomarkers in oncology: focus on colorectal cancer. *Mol Diagn Ther* 13: 103 – 114

Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate KH, Augustin HG (2000) Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. *Cancer Res* 60: 1388 – 1393

Falcon BL, Hashizume H, Koumoutsakos P, Chou J, Bready JV, Coxon A, Oliner JD, McDonald DM (2009) Contrasting actions of selective inhibitors of angiopoietin-1 and angiopoietin-2 on the normalization of tumor blood vessels. *Am J Pathol* 175: 2159 – 2170

Fiedler U, Scharpfenecker M, Koidl S, Hegen A, Grunow V, Schmidt JM, Augustin HG, Koh GY, Thurston G, Alitalo K (2009) Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Science* 327: 48 – 50

Helfrich I, Edler L, Sucker A, Thomas M, Christian S, Schadendorf D, Augustin HG (2009) Angiopoietin-2 levels are associated with disease progression in metastatic malignant melanoma. *Clin Cancer Res* 15: 1384 – 1392

Hu B, Cheng SY (2009) Angiopoietin-2: development of inhibitors for cancer therapy. *Curr Oncol Rep* 11: 111 – 116

Imanishi Y, Hu S, Jarzynka MJ, Guo P, Elishaev E, Bar-Joseph I, Cheng SY (2007) Angiopoietin-2 stimulates breast cancer metastasis through the alpha5(betal) integrin-mediated pathway. *Cancer Res* 67: 4254 – 4263

Jain RK (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 307: 58 – 62

Jain RK, Duda DG, Clark JW, Loeffler JS (2006) Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nat Clin Pract Oncol* 3: 24 – 40

Jubb AM, Hurwitz H, Bai W, Holmgren EB, Tobin P, Guerrero AS, Kabbinavar F, Holden SN, Novotny WF, Frantz GD, Hillian KJ, Koeppen H (2006a) Impact of vascular endothelial growth factor-A expression, thrombospondin-2 expression, and microvessel density on the treatment effect of bevacizumab in metastatic colorectal cancer. *J Clin Oncol* 24: 217 – 227

Jubb AM, Oates AJ, Holden S, Koeppen H (2006b) Predicting benefit from angiopoietin-2 in colorectal cancer. *N Engl J Med* 359: 1757 – 1765

Kashkar H, Kronke M, Jurgensmeier JM (2002) Defective Basa activation in Hodgkin B-cell lines confers resistance to staurosporine-induced apoptosis. *Cell Death Differ* 9: 750 – 757

Lievre A, Bachel JB, Le Corre D, Boige V, Landi B, Emile JF, Cote JF, Tomaic G, Penna C, Ducrueux M, Rougier P, Penault-Llorca F, Laurent-Puig P (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* 66: 3992 – 3995

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wieand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Vancopoulou GD (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277: 56 – 59

Mallett S, Timmer A, Saurerwein B, Altman DG (2010) Reporting of prognostic studies of tumour markers: a review of published articles in relation to REMARK guidelines. *Br J Cancer* 102: 173 – 180

McCormack PL, Keam SJ (2008) Bevacizumab: a review of its use in metastatic colorectal cancer. *Drugs* 68: 487 – 506

Murukesh N, Dive C, Jayson GC (2010) Biomarkers of angiogenesis and their role in the development of VEGF inhibitors. *Br J Cancer* 102: 8 – 18

Ochiium T, Tanaka S, Oka S, Hiyama T, Ito M, Kitadai Y, Haruka K, Chayama K (2004) Clinical significance of angiopoietin-2 expression at the deepest invasive tumor site of advanced colorectal carcinoma. *Int J Oncol* 24: 539 – 547

Ogawa M, Yamamoto H, Nagano H, Miyake Y, Sugita Y, Hata T, Kim BN, Ngan CY, Damdinsuren B, Ikemoto A, Ohue M, Nakamori S, Sekimoto M, Sakon M, Matsura N, Monden M (2004) Hepatic expression of ANG2 RNA in metastatic colorectal cancer. *Hepatology* 39: 525 – 539

Park JH, Park JK, Kim YS, Sheen SS, Lee KS, Lee HN, Oh YJ, Hwang SC (2007) Serum angiopoietin-2 as a clinical marker for lung cancer. *Chest* 132: 200 – 206

Reinacher-Schick AC, Kubicka S, Freier W, Arnold D, Dietrich G, Geissler M, Hegewisch-Becker S, Graeven U, Schmoll H, Schmiegl W (2008) Activity of the combination of bevacizumab (Bev) with capcitabine/irinotecan (Cap/Bev) or capcitabine/oxaliplatin (Cap/Ox/Bev) in advanced colorectal cancer (ACRC): a randomized phase II study of the AIO Colorectal Study Group (AIO trial 0604). *J Clin Oncol* 26: 4030

Reiss Y, Kneida A, Tal AO, Schmidt MH, Jugold M, Kessling F, Burger AM, Wolburg H, Deutsch U, Plate KH (2009) Switching of vascular phenotypes within a murine breast cancer model induced by angiopoietin-2. *J Pathol* 217: 571 – 580

Scharpfenecker M, Fiedler U, Reiss Y, Augustin HG (2005) The Tie-2 ligand angiopoietin-2 destabilizes quiescent endothelium through an internal autocrine loop mechanism. *J Cell Sci* 118: 771 – 780

Sessa C, Guibal A, Del Conte G, Ruegg C (2008) Biomarkers of angiogenesis for the development of antiangiogenic therapies in oncology: tools or decorations? *Nat Clin Pract Oncol* 5: 378 – 391

Sorensen AG, Batchelor TT, Zhang WT, Chen PJ, Yeo P, Wang M, Jennings D, Wen PY, Lahrendentra J, Ancukiewicz M, di Tomaso E, Duda DG, Jain RK (2009) A 'vascular normalization index' as potential mechanistic biomarker to predict survival after a single dose of cetuximab in recurrent glioblastoma patients. *Cancer Res* 69: 5296 – 5300

Thijssen VL, Brandweijr RJ, Dings RP, Griffioen AW (2004) Angiogenesis gene expression profiling in xenograft models to study cellular interactions. *Exp Cell Res* 299: 286 – 293

Willett CG, Boucher Y, di Tomaso E, Duda DG, Munn LL, Tong RT, Chung DC, Sahani DV, Kalva SP, Kozin SV, Mino M, Cohen KS, Scadden DT, Hartford AC, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, O'Brien-Jenkins A, Randall TC, Rubin SC, Coukos G (2003) Tumor-derived vascular endothelial growth factor up-regulates angiopoietin-2 in host endothelium and destabilizes host vasculature, supporting angiogenesis in ovarian cancer. *Cancer Res* 63: 3403 – 3412