British Journal of Cancer (2012) 107, 287–290

www.bjcancer.com

Short Communication

Identification of predictive circulating biomarkers of bevacizumab-containing regimen efficacy in pre-treated metastatic colorectal cancer patients

A Abajo[1], V Boni[1,2], I Lopez[1], M Gonzalez-Huarriz[1], N Bitarte[1], J Rodriguez[2], R Zarate[1], E Bandres[*,3] and J Garcia-Foncillas[*,1,2]

[1]Laboratory of Pharmacogenomics, Division of Oncology, Center for Applied Medical Research, University of Navarra, Pamplona, Spain; [2]Department of Medical Oncology, University Clinic of Navarra, University of Navarra, Pamplona, Spain; [3]Immunology Unit, Hematology Service, Complejo Hospitalario de Navarra, Pamplona, Spain

BACKGROUND: To identify whether circulating levels of angiogenesis-related factors may be predictive of bevacizumab efficacy in pre-treated metastatic colorectal cancer (mCRC) patients.

METHODS: Pre-treatment serum levels of 24 cytokines were measured using a multiplex bead assay (MBA) in 32 pre-treated mCRC patients treated with irinotecan plus bevacizumab-based salvage therapy. Macrophage-derived chemokine (MDC), interleukins (ILs) 8 and 6 levels were also validated by enzyme-linked immunosorbent assay (ELISA) at different time points during therapy.

RESULTS: Higher epidermal growth factor (EGF) and MDC baseline levels (2.2- and 1.4-fold, respectively) and lower IL-10, IL-6 and IL-8 levels (0.2-, 0.6-, and 0.6-fold, respectively, $P<0.05$) were observed in patients responding to therapy. Baseline levels of these five serum factors compose a risk signature that may define the subset of patients most likely to benefit from bevacizumab-based therapy in terms of response rate and survival times. A positive correlation was found between MBA and ELISA results ($P<0.01$). Treatment exposure increased MDC and had opposite effects on IL-8 levels, which were decreased ($P<0.05$).

CONCLUSION: This study suggests that a set of inflammatory and angiogenesis-related serum markers may be associated with the efficacy of bevacizumab-containing regimen.

British Journal of Cancer (2012) **107,** 287–290. doi:10.1038/bjc.2012.242 www.bjcancer.com
Published online 14 June 2012

Keywords: bevacizumab; serum biomarkers; colorectal cancer; multiplex bead assays

Anti-VEGF therapies have proven efficacy in metastatic colorectal cancer (mCRC) (Kabbinavar *et al*, 2003; Emmanoulide C, 2004; Hurwitz *et al*, 2004), although varying degrees of benefit in patients outcome have been reported (Giantonio *et al*, 2007; Lievre *et al*, 2009). Several mechanisms of resistance to the angiogenics blockade, either by target mutation or by activation of alternative pathways (Bergers and Hanahan, 2008; Ellis and Hicklin, 2008; Shojaei and Ferrara, 2008) have been described. Nevertheless, further insight into the biological mechanisms responsible for the observed differences in outcome seem warranted (Saito and Camilleri, 2006).

Serum circulating cytokines, growth factors and angiogenesis-related molecules, are hypothesised to be valid markers of the tumour microenvironment angiogenic profile and may offer prognostic and predictive information beyond conventional clinico-pathological indicators (Poon *et al*, 2001). Extensive research suggests the prognostic value of angiogenesis-related factors for tumour stage (Carmeliet and Jain, 2000; Ferrara, 2004) and plasma concentrations of some of these molecules are markedly increased in mCRC patients compared with healthy individuals (Sakamoto *et al*, 2012). The incorporation of non-invasive sampling techniques into clinical routine, avoiding the need for invasive biopsy procedures is of paramount importance in a palliative setting and allows a dynamic evaluation of putative candidate biomarkers (Jain *et al*, 2009). The intrinsic complexity of the tumour microenvironment requires a parallel, miniaturised device technology to be applied to proteins and their biochemical pathways (Huang *et al*, 2005). Prior studies have reported promising results with the use of multiplex bead assay (MBA) as an alternative to simultaneously detect the expression of multiple cytokines in minimal amounts of sample (Huang *et al*, 2005).

On this basis, in the present study we performed a MBA-based exploratory analysis of 24 serum cytokines from mCRC patients treated with a bevacizumab-containing regimen.

PATIENTS AND METHODS

Patients

Thirty-two histologically confirmed mCRC patients aged >18 years, ECOG 0–2, progressed after one prior oxaliplatin/fluoropyrimidine-based chemotherapy regimen for metastatic disease were selected from a larger cohort (Abajo *et al*, 2010) based on the availability of baseline serum samples. Patients' characteristics are shown in Supplementary Table 1. Complete cohort staging work-up, clinical management, treatment administration and

*Correspondence: Dr E Bandres; E-mail: ebandres@hotmail.es or Dr J Garcia-Foncillas; E-mail: jgfoncillas@fjd.es
Received 24 February 2012; revised 3 May 2012; accepted 3 May 2012; published online 14 June 2012

long-term outcomes have been reported elsewhere (Abajo *et al*, 2010). The local institutional review board approved the study and all patients provided written informed consent.

Serum sample collection and pharmacogenomic analysis

Serum samples were obtained at baseline in all patients and afterwards along the course of therapy after at least two treatment cycles (17 available samples). Venous blood was drawn and immediately processed for serum. Samples were stored at $-80\,^{\circ}C$ until analysis. Angiogenic growth factors and cytokines (Supplementary Table 2) were measured using MBA (Millipore, Bedford, MA, USA) and macrophage-derived chemokine (MDC), interleukins (IL-8 and IL-6) by ELISA (R&D Systems, Minneapolis, MN, USA) as per manufacturers' directions.

Statistical methods

The relationship between continuous variables was assessed by non-parametric Spearman's correlation. Association between angiogenesis-related cytokines levels and patients clinical outcomes was assessed using the Mann–Whitney *U* test. Clinical outcomes refers to objective response (ORR; CR and PR) and disease control rate (DCR; CR, PR and SD lasting >6 months). As determination of an optimal cutoff value was beyond the scope of the present work, patients were divided into two groups according to the median value of each cytokine. Time to progression (TTP) and overall survival (OS) distributions are summarised by Kaplan–Meier methods and compared using log-rank or Breslow's tests. Patients undergoing consolidative procedure are censored for TTP and patients receiving further lines of therapy are censored for OS. Differences between baseline and on-treatment cytokines' levels were assessed using Wilcoxon tests. All *P*-values are two sided.

RESULTS

Baseline (pre-treatment) serum cytokines and growth factors levels

The baseline levels of the 24 MBA-analysed angiogenesis-related molecules are shown in Supplementary Table 1, with some of them being significantly associated with ORR and DCR. As shown in Figures 1A and B, higher median epidermal growth factor (EGF) and MDC levels (282.8 *vs* 138.9 pg ml^{-1} and 838.6 *vs* 696.9 pg ml^{-1}, respectively, $P<0.05$) were observed in responding patients. In addition, lower levels of IL-10 (median 0.0 *vs* 35.7 pg ml^{-1}), IL-6 (median 0.0 *vs* 37.1 pg ml^{-1}), and IL-8 (median 30.3 *vs* 33.6 pg ml^{-1}) were also observed in responding patients (Figures 1C–E).

A statistically significant correlation was found between these ILs ($P<0.01$), with similar biological function and most probably regulated by same effectors; and a negative significant correlation was observed between MDC and IL-8 and IL-10, which seem to have opposite roles (Supplementary Table 3).

We next assessed whether the combination of the differentially expressed serum factors would further increase the predictive ability of each individual factor. A risk score was thus calculated for each patient, by summing-up the number of factors below and above the median for EGF and MDC, and IL-10, IL-6 and IL-8, respectively. A DCR of 86% was reported among patients with <3 high-risk factors compared with 22% in those with $\geqslant 3$ high-risk factors ($P<0.001$). Median TTP (8.1 *vs* 2.8, $P<0.05$, Breslow's test; Figure 2A) and median OS (23.8 *vs* 5.1, $P<0.01$, log-rank test; Figure 2B) were significantly longer in patients with <3 high-risk factors.

Technical validation of MBA results by ELISA

To validate the MBA results, the levels of IL-6, IL-8 and MDC were analysed by ELISA. As previously reported (Elshal and McCoy, 2006), MBA detection levels were 1.5–2-fold higher than those attained by ELISA. In fact, only 8 out of 32 samples had detectable levels of IL-8 by ELISA (seven out of those eight patients were non-responders), confirming the higher sensitivity of the MBA technique. Nevertheless, we found a statistically significant positive correlation between MBA and ELISA data for the three analysed cytokines: IL-8 ($r_{Spearman}$ 0.67, $P<0.01$), IL-6 ($r_{Spearman}$ 0.67, $P<0.01$) and MDC ($r_{Spearman}$ 0.53, $P<0.01$). In agreement with the MBA results, when IL-6 and/or IL-8 ELISA-based measurements were considered, DCR was 21.4% and 75% ($P<0.001$) for those patients with high and low levels, respectively. In addition, a statistically significant higher ORR was found for high ELISA-measured MDC levels compared with the low MDC group (66.7% *vs* 26.7%, $P<0.05$).

Treatment influence on serum markers levels

Monitoring the effect of treatment by analysing dynamic changes in circulating factors has become of great interest to understand treatment failure (Jain *et al*, 2006; Kopetz *et al*, 2010). In the subset of patients with available on-treatment samples, we observed that exposure to bevacizumab-based therapy translated

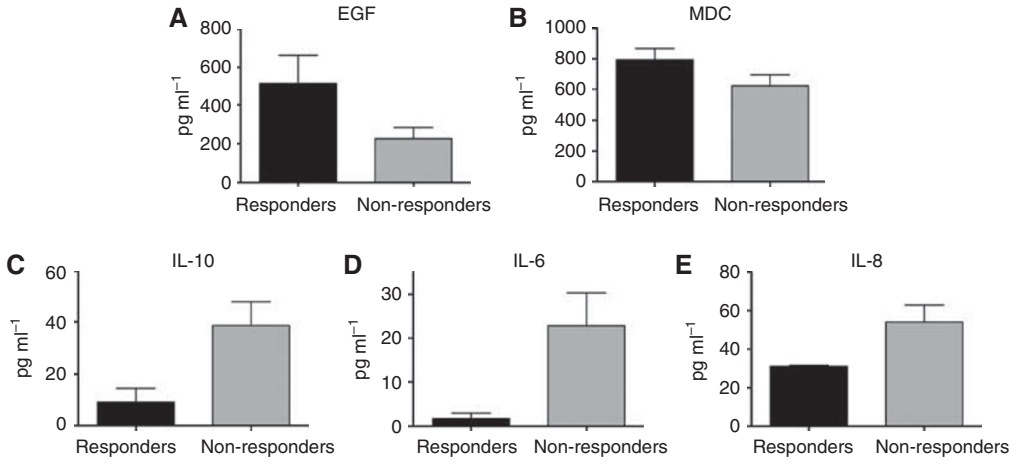

Figure 1 MBA measured cytokine levels at baseline according to ORR clinical outcome. EGF (**A**) and MDC (**B**) are higher in responders (CR + PR) to treatment and IL-10 (**C**), IL-6 (**D**) and IL-8 (**E**) are lower in responders to treatment.

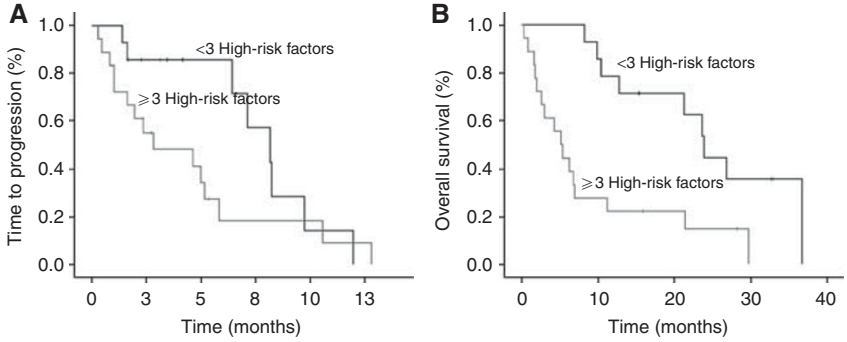

Figure 2 Kaplan–Meier plots. (**A**) Time to progression and (**B**) overall survival according to the 'high-risk signature' calculated by baseline levels of EGF, MDC, IL-10, IL-6 and IL-8.

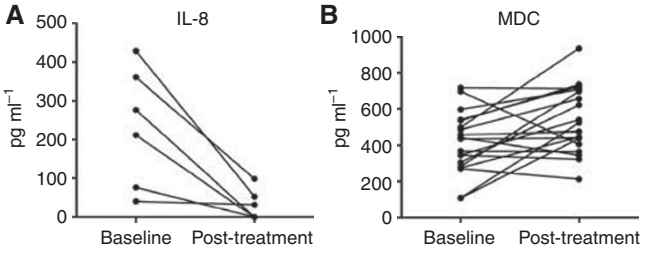

Figure 3 Treatment modulation of cytokines. (**A**) IL-8 is reduced with exposure to treatment and (**B**) MDC increases with exposure to treatment.

into decreased IL-8 levels and ($P<0.05$; Figure 3A) increased in the chemokine MDC ($P<0.05$; Figure 3B). No relevant changes were observed for IL-6.

DISCUSSION

Although the addition of bevacizumab to cytotoxic chemotherapy has demonstrated a survival benefit in the first- and second-line treatment of mCRC, the identification of predictive biomarkers of antiangiogenic therapy efficacy is of considerable interest. The detection of differentially expressed molecular profiles by means of simple, reliable, non-invasive screening tests (Carmeliet and Jain, 2000; Acevedo *et al*, 2009) is appealing. Our findings suggest that in this subset of patients, a baseline circulating molecular signature correlated with clinical outcome. High serum levels of EGF and MDC and low levels of IL-10, IL-6 and IL-8 were associated with a higher likelihood of response. Interestingly, a risk signature calculated by combining all of these five serum factors significantly correlated with TTP and OS, improving single factor's predictive ability.

Tumour-derived factors provide an essential support for the angiogenesis and the stroma remodelling required for tumour growth. Tumour-associated macrophages represent the major population of tumour-infiltrating inflammatory cells. MDC attracts and activates a variety of cell types and enhance the immune response. Dendritic cells and IL-2-activated natural killer cells have demonstrated chemotactic response to MDC (Godiska *et al*, 1997). Accordingly, high baseline levels of MDC in our responding patients support its role in promoting an immune response by T-helper cells recruitment. *In vivo* MDC has been shown to suppress lung and colon cancer growth (Cho *et al*, 2009). Moreover, an MDC-increased gene expression in tumour tissue turned out to be a favourable prognostic factor (Nakajima *et al*, 2006) and its concentration levels strongly correlates with the frequency of FOXP3-positive cells (Mizukami *et al*, 2008). A high density of intratumour FOXP3-positive T-regulatory cells has been associated with poor outcomes in a wide variety of solid tumours (Jang, 2008; Maruyama *et al*, 2010). However, an opposite effect has been observed in colorectal cancer, with intratumour regulatory T cells being associated with improved prognosis (Pages *et al*, 2005; Wagsater *et al*, 2008). Subsequently, whether MDC has a prognostic or a predictive value for mCRC patients' outcomes deserves further research.

The better outcome observed in the subset of patients with lower IL-6 and IL-8 baseline levels is in accordance with the role of these cytokines in colon cancer progression and angiogenesis (Li *et al*, 2003; Bunger *et al*, 2011). IL-8 has been reported to mediate angiogenesis by stimulating endothelial cell proliferation in response to hypoxia (Koch *et al*, 1992; Varney *et al*, 2002), and escape to antiangiogenic therapy has been correlated with increased secretion of IL-8 (Huang *et al*, 2010). Furthermore, the predictive role of low baseline IL-8 levels and their bevacizumab-induced decrease are in agreement with recently reported clinical data (Kopetz *et al*, 2010).

Given the biological complexity of tumour angiogenesis and the non-randomised study design, our results should be viewed with caution. Although there is a biological rationale to support the present observations, the present data are exploratory and retrospective. Prospective validation is required because it is plausible that other inflammatory mediators may arise (Cavallo *et al*, 2011; Hanahan and Weinberg, 2011) as a potential predictive markers of outcomes to angiogenesis blockade.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Department of Health, Government of Navarra, Spain.

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

REFERENCES

Abajo A, Rodriguez J, Bitarte N, Zarate R, Boni V, Ponz M, Chopitea A, Bandres E, Garcia-Foncillas J (2010) Dose-finding study and pharmacogenomic analysis of fixed-rate infusion of gemcitabine, irinotecan and bevacizumab in pretreated metastatic colorectal cancer patients. *Br J Cancer* **103**: 1529–1535

Acevedo VD, Ittmann M, Spencer DM (2009) Paths of FGFR-driven tumorigenesis. *Cell Cycle* **8**: 580–588

Bergers G, Hanahan D (2008) Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer* **8**: 592–603

Bunger S, Haug U, Kelly FM, Klempt-Giessing K, Cartwright A, Posorski N, Dibbelt L, Fitzgerald SP, Bruch HP, Roblick UJ, von Eggeling F, Brenner H, Habermann JK (2011) Toward standardized high-throughput serum diagnostics: multiplex-protein array identifies IL-8 and VEGF as serum markers for colon cancer. *J Biomol Screen* **16**: 1018–1026

Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. *Nature* **407**: 249–257

Cavallo F, De Giovanni C, Nanni P, Forni G, Lollini PL (2011) the immune hallmarks of cancer. *Cancer Immunol Immunother* **60**: 319–326

Cho S, Koizumi K, Takeno N, Kato S, Yamada M, Hashimoto I, Sakurai H, Tsukada K, Saiki I (2009) Anti-tumor effect of combining CC chemokine 22 and an anti-CD25 antibody on myeloma cells implanted subcutaneously into mice. *Mol Med Report* **2**: 773–777

Ellis LM, Hicklin DJ (2008) Pathways mediating resistance to vascular endothelial growth factor-targeted therapy. *Clin Cancer Res* **14**: 6371–6375

Elshal MF, McCoy JP (2006) Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. *Methods* **38**: 317–323

Emmanouilides C, Pegram M, Robinson R, Hecht R, Kabbinavar F, Isacoff W (2004) Anti-VEGF Anti bevacizumab (Avastin) with 5FU/LV as third line treatment for colorectal cancer. *Tech Coloproctol* **8**: 50–52

Ferrara N (2004) Vascular endothelial growth factor as a target for anticancer therapy. *Oncologist* **9**(Suppl 1): 2–10

Giantonio BJ, Catalano PJ, Meropol NJ, O'Dwyer PJ, Mitchell EP, Alberts SR, Schwartz MA, Benson 3rd AB (2007) Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. *J Clin Oncol* **25**: 1539–1544

Godiska R, Chantry D, Raport CJ, Sozzani S, Allavena P, Leviten D, Mantovani A, Gray PW (1997) Human macrophage-derived chemokine (MDC), a novel chemoattractant for monocytes, monocyte-derived dendritic cells, and natural killer cells. *J Exp Med* **185**: 1595–1604

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* **144**: 646–674

Huang D, Ding Y, Zhou M, Rini BI, Petillo D, Qian CN, Kahnoski R, Futreal PA, Furge KA, Teh BT (2010) Interleukin-8 mediates resistance to antiangiogenic agent sunitinib in renal cell carcinoma. *Cancer Res* **70**: 1063–1071

Huang RP, Yang W, Yang D, Flowers L, Horowitz IR, Cao X, Huang R (2005) The promise of cytokine antibody arrays in the drug discovery process. *Expert Opin Ther Targets* **9**: 601–615

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* **350**: 2335–2342

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

Jain RK, Duda DG, Willett CG, Sahani DV, Zhu AX, Loeffler JS, Batchelor TT, Sorensen AG (2009) Biomarkers of response and resistance to antiangiogenic therapy. *Nat Rev Clin Oncol* **6**: 327–338

Jang TJ (2008) Prevalence of Foxp3 positive T regulatory cells is increased during progression of cutaneous squamous tumors. *Yonsei Med J* **49**: 942–948

Kabbinavar F, Hurwitz HI, Fehrenbacher L, Meropol NJ, Novotny WF, Lieberman G, Griffing S, Bergsland E (2003) Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. *J Clin Oncol* **21**: 60–65

Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM (1992) Interleukin-8as a macrophage-derived mediator of angiogenesis. *Science* **258**: 1798–1801

Kopetz S, Hoff PM, Morris JS, Wolff RA, Eng C, Glover KY, Adinin R, Overman MJ, Valero V, Wen S, Lieu C, Yan S, Tran HT, Ellis LM, Abbruzzese JL, Heymach JV (2010) Phase II trial of infusional fluorouracil, irinotecan, and bevacizumab for metastatic colorectal cancer: efficacy and circulating angiogenic biomarkers associated with therapeutic resistance. *J Clin Oncol* **28**: 453–459

Li A, Dubey S, Varney ML, Dave BJ, Singh RK (2003) IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J Immunol* **170**: 3369–3376

Lievre A, Samalin E, Mitry E, Assenat E, Boyer-Gestin C, Lepere C, Bachet JB, Portales F, Vaillant JN, Ychou M, Rougier P (2009) Bevacizumab plus FOLFIRI or FOLFOX in chemotherapy-refractory patients with metastatic colorectal cancer: a retrospective study. *BMC Cancer* **9**: 347

Maruyama T, Kono K, Izawa S, Mizukami Y, Kawaguchi Y, Mimura K, Watanabe M, Fujii H (2010) CCL17 and CCL22 chemokines within tumor microenvironment are related to infiltration of regulatory T cells in esophageal squamous cell carcinoma. *Dis Esophagus* **23**: 422–429

Mizukami Y, Kono K, Kawaguchi Y, Akaike H, Kamimura K, Sugai H, Fujii H (2008) CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of Foxp3 + regulatory T cells in gastric cancer. *Int J Cancer* **122**: 2286–2293

Nakajima Y, Haraguchi M, Furukawa T, Yamamoto M, Nakanishi H, Tatematsu M, Akiyama S (2006) 2-Deoxy-L-ribose inhibits the invasion of thymidine phosphorylase-overexpressing tumors by suppressing matrix metalloproteinase-9. *Int J Cancer* **119**: 1710–1716

Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, Mlecnik B, Kirilovsky A, Nilsson M, Damotte D, Meatchi T, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Galon J (2005) Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med* **353**: 2654–2666

Poon RT, Fan ST, Wong J (2001) Clinical implications of circulating angiogenic factors in cancer patients. *J Clin Oncol* **19**: 1207–1225

Saito YA, Camilleri M (2006) Clinical application of pharmacogenetics in gastrointestinal diseases. *Expert Opin Pharmacother* **7**: 1857–1869

Sakamoto H, Kimura H, Sekijima M, Matsumoto K, Arao T, Chikugo T, Yamada Y, Kitano M, Ito A, Takeyama Y, Kudo M, Nishio K (2012) Plasma concentrations of angiogenesis-related molecules in patients with pancreatic cancer. *Jpn J Clin Oncol* **42**: 105–112

Shojaei F, Ferrara N (2008) Role of the microenvironment in tumor growth and in refractoriness/resistance to anti-angiogenic therapies. *Drug Resist Updat* **11**: 219–230

Varney ML, Olsen KJ, Mosley RL, Bucana CD, Talmadge JE, Singh RK (2002) Monocyte/macrophage recruitment, activation and differentiation modulate interleukin-8 production: a paracrine role of tumor-associated macrophages in tumor angiogenesis. *In Vivo* **16**: 471–477

Wagsater D, Dienus O, Lofgren S, Hugander A, Dimberg J (2008) Quantification of the chemokines CCL17 and CCL22 in human colorectal adenocarcinomas. *Mol Med Report* **1**: 211–217