



Universidad de Navarra

Facultad de Farmacia

DOCTORADO EN MEDICAMENTOS Y SALUD

**Angiogenic molecular signature in colorectal cancer:
Pharmacogenomic implications for the use
of antiangiogenic therapies**

Ana María Abajo Guijarro

Tesis doctoral

2012



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Pharmacogenomic implications for the use of antiangiogenic
therapies**

Memoria presentada por Ana María Abajo Guijarro para aspirar al grado de Doctor en Farmacia por la Universidad de Navarra

(firma del doctorando)

El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Oncología (CUN) y en el laboratorio de Farmacogenómica (CIMA) autorizo su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, 16 de Febrero de 2012

(firma de los Directores de la Tesis Doctoral)

Dr. Jesús García-Foncillas López

Dra. Eva Bandrés Elizalde

Me gustaría expresar mi más sincero agradecimiento:

A la Dra. Eva Bandrés, Investigadora del área de Farmacogenómica de CIMA de la Universidad de Navarra, por la dirección de este trabajo. Por su apoyo tanto personal como científico durante todos estos años, que ha hecho posible sacar adelante este proyecto.

Al Dr. Jesús García-Foncillas, director del área de Farmacogenómica de CIMA de la Universidad de Navarra, por haberme permitido realizar la Tesis Doctoral en su grupo de investigación y por la dirección de este trabajo. Por la confianza que ha depositado en mí.

A mis compañeras: Raquel, Elena, Inés, Marisol, Ruth, Natalia, Nerea, Valentina. Por vuestras enseñanzas, inestimable colaboración y hacer muy especiales mis días en el laboratorio.

Al Dr. Javier Rodríguez, Dra. Ana Chopitea y Dr. Antonio Viudez del Departamento de Oncología Médica de la Clínica Universitaria de Navarra. Por contribuir enormemente a hacer posible este proyecto y por toda la ayuda recibida.

A mis compañeros de la planta de Oncología de CIMA: Lola, Nerea, Iria, Carmen, Ana, Elena, Lucía, etc.

A mis amigos. Especialmente a Ele y Emi, por ser mi familia en los inicios en Pamplona.

A mis padres. Por vuestro apoyo durante estos años que ha hecho posible que hoy presente esta Tesis. A Pablo.

A Javi. Por tantas cosas... y especialmente por tu comprensión y tu empeño en que esta Tesis saliera adelante.

Gracias a todos.

ABBREVIATIONS

5-FU	5-Fluorouracil
AJCC	American Joint Committee on Cancer
ALP	Alkaline phosphatase
Ang	Angiopoietin
APC	Adenomatous polyposis coli
BSC	Best supportive care
CEA	Carcinoembryonic antigen
CEC	Circulating endothelial cell
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CTACK	Cutaneous T-cell attracting chemokine
DCC	Deleted in colorectal carcinoma
DCE-MRI,-CT	Dynamic contrast enhanced-magnetic resonance imaging,- computed tomography
DFS	Disease free survival
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIA	Enzyme immunoassay
ENA78	Epithelial cell-derived neutrophil-activating peptide 78
EPC	Endothelial precursor cells
FGF	Fibroblast growth factor
FLT-1	Fms-like tyrosine kinase
FLT3L	FMS-like tyrosine kinase 3 ligand
GBM	Glioblastoma multiforme
G-CSF	Granulocyte colony-stimulating factor
Gd	Gadolinium
GMB	Gemcitabine

GM-CSF	Granulocyte macrophage colony-stimulating factor
GRO	Growth regulated oncogene
Hb	Haemoglobin
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
HIF-1	Hypoxia-inducible factor-1
HSC	Hematopoietic stem cells
I-309	Monocyte chemoattractant protein I-309
ICAM-1	Platelet-endothelial cell adhesion molecule 1
IFN- γ	Interferon-gamma
IGF	Insulin-like growth factor
IgG1	Immunoglobulin G1
IHC	Immunohistochemistry
IL	Interleukin
IP-10	Interferon -gamma-induced protein-10
I-TAC	Interferon gamma inducible T cell alpha chemoattractant protein
KDR	Kinase domain region
LDH	Lactate dehydrogenase
LNM	Lymph node metastases
LV	Leucovorin
MBA	Multiplex-bead assay
MCP	Macrophage chemoattractant protein
mCRC	Metastatic colorectal cancer
MDC	Macrophage-derived chemokine
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MMR	Mismatch repair
MoAB	Monoclonal antibody
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSS	Microsatellite stable
MSI	Microsatellite instability
MVD	Microvessel density
NIH	National Institutes of Health
NOS	Nitric oxide synthase

NRP-1	Neuropilin-1
NSCLC	Non-small cell lung cancer
OS	Overall survival
PAI-1	Plasminogen activator inhibitor-1
PDGF-BB	Platelet-derived growth factor-beta polypeptide
PECAM	Platelet-endothelial cell-adhesion molecule
PET	Positron emission tomography
PFS	Progression free survival
PI3K	Phosphoinositide 3-kinase
PIGF	Placental growth factor
PS	Performance status
PTEN	Phosphatase and tensin homolog
Q-RT-PCR	Quantitative reverse-transcription PCR
RANTES	Regulated on activation, normal T cell expressed and secreted
RCC	Renal cell carcinoma
RKIP	Raf-1 kinase inhibitor protein
RNRM1	Ribonucleotide reductase M1
RTK	Tyrosine kinase receptor
SDF	Stromal-derived factor
SNP	Single-nucleotide polymorphism
TARC	Thymus and activation-regulated chemokine
TGF- β	Transforming growth factor-beta
Tie2	Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2
TIMP	Tissue inhibitor of metalloproteinases
TKI	Tyrosine kinase inhibitor
TNF	Tumour necrosis factor
TNM	Tumour, node, metastases system
TS	Thymidylate synthase
TSP-1,-2	Thrombospondin-1,-2
TTP	Time to progression
uPAR	Urokinase-type plasminogen activator-receptor
UTRs	Untranslated regions
VCAM-1	Vascular cell adhesion protein-1
VEGF	Vascular endothelial growth factor

VEGFR	Vascular endothelial growth factor receptor
VEGI	Vascular endothelial growth inhibitor
WB	Western blot
WBC	White blood cell

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INTRODUCTION AND BACKGROUND

1. PERSONALISED MEDICINE AND TRANSLATIONAL RESEARCH IN CANCER

The relatively high availability of drugs, the complexities of combination therapy and the management of patients with sequential lines of treatment in cancer, particularly since the introduction of biologic drugs, has created new challenges in determining the actual magnitude of benefit for new agents under clinical investigation.

Translational research refers to the bench-to-bedside activity of connecting basic science knowledge with new treatment options for patients, translating research results into real clinical practice ¹. In light of great progress in our understanding of the biology of cancer and unprecedented development of therapies, all efforts are being made to move all the scientific findings into the clinic. Translational research is a powerful manner to drive clinical research by characterizing valid surrogates for patient outcomes, which will ultimately facilitate a more rational use of therapies ².

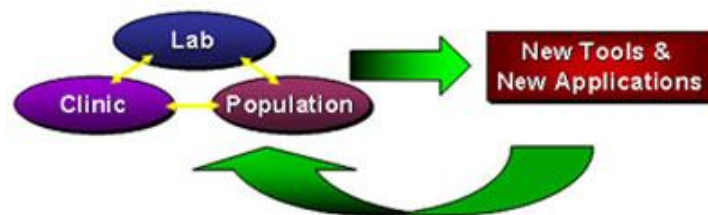


Figure 1. Translational research by USA NCI, Translational Research Working Group

Since there are inter-individual differences in the toxicity and response profile to the currently available treatments, personalized medicine has definitely developed with the molecular medicine era. Personalized medicine uses data on genes and proteins to prevent, diagnose and treat a specific disease pattern. Identifying those patients at greater risk of disease and more likely to respond to a treatment would provide more effective care and enable treatments to be more efficiently allocated ³.

1.1 BIOMARKERS IDENTIFICATION AND PHARMACOGENOMICS

Recognizing the genetic characteristic to cancer development could lead to the discovery of diagnostic and prognostic markers as well as novel drug targets, helping understanding the mechanisms underlying tumour origin and driving resistance mechanisms. In recent years, extensive research has attempted to define molecular markers that may be useful predictors of treatment outcomes. Furthermore, it has aimed

to a more efficient use of available therapeutic options through the identification of differentially expressed molecular profiles^{4,5}.

The official NIH definition of biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Biomarkers are molecular, cellular or functional measurable parameters indicative of a particular genetic, epigenetic or functional status of a biological system². The value of a biomarker should be ideally tested and validated in controlled, phase III clinical trials- and correlated with relevant clinical endpoints. Biomarkers should be repeatable, reproducible and, ideally, measurable through minimally invasive procedures. Preferably, biomarkers should be measurable in an easily obtainable sample. Analytical tests for marker measurement should be reliable, reproducible, specific and widely available. Frequently, a biomarker is the result of a bioassay, laboratory technique for processing biological material from humans, expressed quantitatively or categorically⁶.

In cancer, biomarkers can be used for diagnosis, staging, prognosis and treatment selection. A biomarker has prognostic value when it informs of the natural course and outcome of the disease, identifying patients with differing risks for a specific outcome. A predictive marker anticipates the benefit derived from a specific therapy⁷.

Several mechanisms are suggested to contribute to drug resistance: amplification or overexpression of membrane transporters, changes in cellular proteins involved in detoxification or in DNA repair, apoptosis and activation of oncogenes or inactivation of tumour suppressor genes⁸. Pharmacogenomics deals with the influence of genetic variation on response to treatments by correlating gene expression or single-nucleotide polymorphisms (SNPs) with efficacy and toxicity outcomes⁹. Pharmacogenomics' tools are useful in biomarkers identification in order to optimise drug therapy. While current treatments' dosage schemes are based on individual's weight and age, the aim is to move towards dosage based on an individual's genetics, in order to maximize the therapeutic value.

2. ANGIOGENESIS

Angiogenesis is the development of new vascular vessels from endothelial cells (ECs) in the existing vascular network⁴. Further to the involvement in embryogenesis, angiogenesis is critical in physiological processes including skeletal growth, wound healing and reproductive functions. Angiogenesis is focal and self-limited in time, through days (ovulation), weeks (wound healing) or months (placentation)¹⁰. Furthermore, an excessive or abnormal formation of blood vessels has been revealed involved in different pathological processes, the *angiogenesis-dependent diseases*, a group of more than

seventy disorders, including cancer, age-related macular degeneration, and various ischemic, autoimmune and inflammatory diseases ^{11,12}.

Newly formed blood vessels consist of a tube of endothelial cells in a single layer, which will eventually form the deepest layer of the blood vessel in contact with the blood. Further layers add on as the blood vessels mature depending on their function. Different signalling pathways in endothelial cells coordinate angiogenesis through a wide range of proangiogenic factors and inhibitors ¹³ (Table 1). A balance between pro and antiangiogenic signals is required for maintaining the physiological function. In response to angiogenic signals, proangiogenic factors are released from surrounding cells and activate angiogenesis through downstream effects in endothelial cells, such as proliferation and migration, survival and induction of vascular permeability ¹⁴.

Activators	Function
VEGF family members ⁺	Stimulate angiogenesis, permeability, leukocyte adhesion
VEGFR, NRP-1	Integrate angiogenic and survival signals
Ang1 and Tie2 ⁺	Stabilize vessels, inhibit permeability
PDGF-BB and receptors	Recruit smooth muscle cells
TGF- β 1*, endoglin, TGF- β receptors	Stimulate extracellular matrix production
FGF, HGF, MCP-1	Stimulate angio/arteriogenesis
Integrins avb3, avb5, a5b1	Receptors for matrix macromolecules and proteinases
Plasminogen activators, MMPs	Remodel matrix, release and activate growth factors
VE-cadherin; PECAM (CD31)	Endothelial junctional molecules
Ephrins	Regulate arterial/venous specification
PAI-1	Stabilize nascent vessels
NOS; COX-2	Stimulate angiogenesis and vasodilation
Chemokines*	Pleiotropic role in angiogenesis
Id1/Id3	Determine endothelial plasticity
IL-8	Enhance endothelial cell proliferation
G-CSF	Promotes angiogenesis
Inhibitors	Function
VEGFR-1; soluble VEGFR-1; soluble NRP-1	Sink for VEGF, VEGF-B, PlGF
Ang2*	Antagonist of Ang1
Endostatin (collagen XVIII fragment)	Inhibit endothelial survival and migration
Vasostatin (calreticulin fragment)	Inhibit endothelial growth
Platelet factor-4	Inhibits binding of bFGF and VEGF
IFN- α , - β , - γ ; IP-10, IL-4, IL-12, IL-18	Inhibit endothelial migration; down regulate bFGF
Prolactin (Mr, 16K)	Inhibits bFGF/VEGF
TIMPs; MMP inhibitors; PEX	Suppress pathological angiogenesis
Meth-1; Meth-2	Inhibitors containing MMP, TSP and disintegrin domains
TSP-1,-2	Inhibit endothelial migration, growth, adhesion and survival
Angiostatin and related plasminogen kringle-1	Suppress tumour angiogenesis
Prothrombin kringle-2; antithrombin III fragment	Suppress endothelial growth
VEGI	Modulate cell growth
Fragment of SPARC	Inhibit endothelial binding and activity of VEGF

Table 1. Angiogenesis Activators and Inhibitors.*Opposite effect in some contexts. ⁺Present also in/affecting non-endothelial cells. Adapted from Carmeliet *et al*, and Ferrara ^{4,15}.

2.1 TUMOUR ANGIOGENESIS

Tumours can be observed as a *two compartment system* where tumour cells and endothelial cells co-exist, promoting mutual growth and survival via molecular signals¹⁶. The hypothesis that tumours produce a diffusible *angiogenic* substance was anticipated in 1968¹⁷. In 1971, Folkman proposed that tumour growth and metastasis are angiogenesis-dependent, and hence, blocking angiogenesis would be a strategy to arrest tumour growth^{11, 12}. Gullino showed how cells in pre-cancerous state would gain angiogenic capacity on transforming into cancerous¹⁸, and findings were later confirmed by Hanahan and Weinberg¹⁹.

Tumourigenesis models evaluating vascularisation evidenced neovascularisation developing well before the emergence of an invasive malignancy, discrete premalignant stages and a hyperplastic phase followed by a stochastic angiogenic stage²⁰. Sustained angiogenesis is one of the acquired functional capabilities of normal cells in order to become malignant, a *hallmark of cancer*, and the concept of the *angiogenic switch* is widely accepted. Arising tumour cells in a premalignant stage within an avascular tumour do not elicit angiogenic signals²¹. Factors driving the angiogenic switch induce a neovascularisation converging towards the tumour. The avascular phase characterized by a dormant tumour moves into the vascular one, where exponential tumour growth proceeds. The vascularised tumour grows through tumour cells' proliferation, inducing changes on angiogenic factors' gene expression^{19, 22}.

The *balance hypothesis* assumes that the level of angiogenesis activators and inhibitors rules cell differentiation states of quiescence or angiogenesis. The *angiogenic switch* is the result of significant progressive alterations within the tumour microenvironment²², including lymphangiogenesis²³ and mobilization of bone marrow-derived stem cells in the peripheral circulation favouring the differentiation of hematopoietic stem cells towards the EPC lineage, recruited for tumour-induced neovascularisation²⁴. The tumour cell's angiogenic phenotype is also driven by genetic instability. Oncogenes' activation and loss of tumour suppressor genes that underlie malignant transformation after dormancy are also involved in the *angiogenic switch*.

2.1.1 Tumour Angiogenesis: Molecular and Cellular Mechanisms

Different molecular and cellular mechanisms are involved in the formation of tumour vessels. Tumour vessels can grow by: sprouting (the vascular network expands by growth of endothelial sprouts or formation of bridges)⁴; intussusception (tumour vessels remodel and expand by the insertion of interstitial tissue columns into the lumen of pre-existing vessels); and by incorporation of bone marrow-derived EPC or angioblasts. Additionally, tumour cells may also co-opt existing vessels.

Of the identified angiogenic factors, vascular endothelial growth factor (VEGF; vascular permeability factor) is the most potent and specific angiogenic factor, during embryogenesis, physiological processes and in several pathologies^{25, 26}. The VEGF family consists of homodimeric glycoproteins structurally related to the platelet-derived

growth factor (PDGF), including different isoforms of the factors VEGF-A, PlGF (placental growth factor), VEGF-B, VEGF-C, VEGF-D (primarily lymphangiogenic factors through VEGFR-3 signalling). The human *VEGF-A* gene is assigned to chromosome 6p12-p21, organized into 8 exons separated by 7 introns. VEGF is a highly conserved, homodimeric, heparin-binding glycoprotein. Alternative exon splicing of *VEGF* gene results in different molecular species which transcripts encode polypeptides of different amino acids number after signal sequence cleavage: VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆.

VEGF distribution within the tumour and stromal cells was first shown by Dvorak *et al*²⁶. Tumour cells represent the major source of VEGF, together with the tumour-associated stroma, such as fibroblasts and immune/inflammatory cell infiltrates²⁷. The angiogenic effects of VEGF are mediated through binding and activation of receptors on the surface of endothelial cells: Flt-1 (fms-like tyrosine kinase) and KDR (kinase domain region). VEGF/VEGFR-2 signaling exerts signals of endothelial cell mitogenesis and migration (initiates sprout formation), induction of proteinases remodelling the ECM and vascular permeability in both physiological and pathological conditions²⁵.

The neuropilin receptor (NRP-1) has been demonstrated necessary for an effective VEGF/VEGFR signalling²⁵. NRP-1 does not contain a tyrosine-kinase domain and acts as a co-receptor, since ECs expressing NRP-1 but not VEGFR-2 do not respond to any VEGF isoform. Since VEGF binds to NRP-1 through amino acids residing at the carboxyl-terminal part of the exon 7-encoded peptide of VEGF₁₆₅, not all isoforms bind NRP-1, therefore differing in their functionality. The various VEGF isoforms vary in their heparin-binding ability, being the larger ones of greater capacity to bind NRP-1 as well as matrix and cell surface heparan sulfate proteoglycans. However, the ECM-bound isoforms may be released in diffusible forms following proteolytic cleavage. VEGF₁₆₅ is suggested the most biologically active isoform as both secreted by and bound to the cell surface and ECM^{28, 29}.

VEGFR-1 does not mediate a highly effective mitogenic signal in EC and has, especially during early embryonic development, an inhibitory role. This *decoy* role is also performed by the alternatively spliced soluble receptor form (sVEGFR-1) which can almost completely block VEGF in the medium. However, VEGFR-1 has an established signalling role in mediating monocyte chemotaxis. In addition, in hematopoietic stem cells (HSC) or leukemic cells, both VEGFR-1 and VEGFR-2 mediate chemotactic and survival signals³⁰. More recently, a VEGF and VEGFR co-expression suggestive of autocrine and paracrine VEGF stimulation of tumour growth has been demonstrated³¹⁻³³.

Tumours, especially in more advanced stages do not rely on a unique angiogenesis driver⁴. A network of multiple cytokines and growth factors create a crosstalk within the tumour microenvironment which ultimately drives tumour angiogenesis^{4, 5}. During *sprouting* angiogenesis, vessels initially dilate and become permeable in response to VEGF. The tightening effect of angiopoietin 1 (Ang1) that helps maintaining a normalized state in blood vessels by binding to the endothelial Tie2 (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2) together with the

adhesion molecules VE-cadherin and platelet-endothelial cell-adhesion molecule (PECAM) on vessels needs to be overcome.

Tie is a tyrosine kinases receptor (RTKs) that is predominantly expressed by vascular ECs. Ang1-4 factors bind to Tie1 and Tie2 and have different specificity and affinity behaviours. Ang1 activates Tie1 and Tie2, while Ang2 activates Tie1, but inhibits Tie2 activation. Ang3 may inhibit Tie2 activation, while Ang4 may activate Tie2. Tie receptor signalling can lead to activation of growth factor signalling kinases, such as ERK1/2 and Akt. Phosphorylation changes mediated by these kinases regulate proteins involved in cell proliferation, cell-cell interactions and cell migration during angiogenesis.

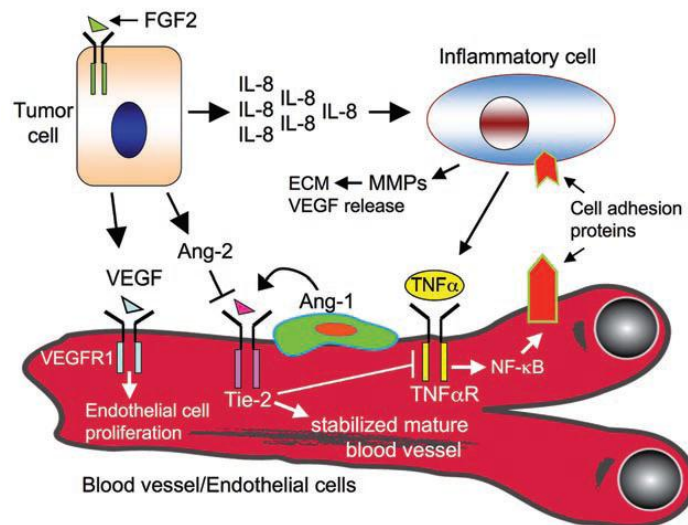


Figure 2. Angiogenesis: a multiple signalling process ⁵

Ang2 and proteinases are mediators of vessel wall remodelling and mediate the dissolution of the basal membrane and the interstitial matrix. Tumour cells secrete Ang2, which competes with Ang1 for binding to the endothelial Tie2 receptor, and increases the degradation of vascular basement membrane and migration of endothelial cells, therefore facilitating sprout formation. VEGF expression actually determines the activity of Ang-1/Ang-2 and the expression of MCPs (macrophage chemoattractant proteins) ^{34,35}. Matrix metalloproteinases (MMPs) are also released from both tumour cells and ECs via VEGF stimulation. MMP-1 and MMP-9 play an important role tumour invasion and metastasis ³⁶. MMPs mobilize proangiogenic proteins from stroma, but can also cleave endostatin from collagen-18 in the vessel wall and participate in the cleavage of angiostatin from circulating plasminogen.

Fibroblast growth factor (FGF) family constitutes another signalling pathway involved in angiogenesis. From the FGF super-family, which consists of twenty-three members, the original cytokine, FGF-2 or bFGF, is known to induce proliferation of ECs ²⁰. These cytokines interact with four tyrosine kinase FGF receptors, with different specificities

noted for almost all FGFs²⁶. The FGFs are considered to play important roles in development, angiogenesis and tumourigenesis⁵.

Chemokines such as the proinflammatory IL-2 intervenes in apoptosis mechanisms. IL-1 α , IL-6 and IL-8 play also an important role in angiogenesis, and interact with MMPs and other pathways³⁷⁻³⁹. Within the tumour stroma, tumour-associated macrophages have been shown to play a supportive role, promoting tumour cell survival, proliferation, and metastasis^{40, 41}. Interestingly, the macrophage-derived chemokine (MDC) has central roles in T helper-cell recruitment into the tissues and MDC increased gene expression in tumour tissues has been hypothesized to be predictive for better prognosis⁴², showing a “protective” effect. Myeloid cell recruitment within tumour bed also plays a role in angiogenesis and tumour progression. Some cytokines released by tumour cells like I-309, SDF-1, MCPs and eotaxin are potent chemoattractants for myeloid cells and hematopoietic progenitor cells and stimulate monocyte chemotaxis^{43, 44}.

The maturation of emerging vessels involves formation of a new basal membrane and investment of new vessels with pericytes and smooth muscle cells. PDGF-BB recruits smooth muscle cells, whereas signalling through TGF- β 1 and Ang1/Tie2 stabilizes the interaction between endothelial and smooth muscle cells. Proteinase inhibitors like PAI-1 prevent degradation of the provisional extracellular matrix for endothelial cells migration. Maintenance of new vessels depends on the survival of ECs.

Most angiogenesis inhibitors are pro-apoptotic for ECs. Many of those were identified as antiangiogenic effectors of tumour angiogenesis while their role in physiological vascular growth, where quiescent ECs can survive for several years, remains largely unknown. In fact, some inductors of angiogenesis may be subsequently processed proteolytically into angiogenesis inhibitors. The soluble receptors VEGFR-1 and neuropilin-1 bind VEGF hence reducing its angiogenic activity.

2.1.2 Vascular Endothelial Growth Factor Regulation in Tumour Angiogenesis

VEGF is physiologically expressed in different tissues and upregulated by tissue oxygen tension via molecular pathways similar to those that regulate erythropoietin gene expression. The mechanisms governing *VEGF* gene expression involve transcriptional to post-translational regulation⁴⁵. Transcriptional regulation is the main mechanism and analysis of the *VEGF* gene promoter region revealed a single major transcription start site that lays near a cluster of potential SP1 transcription factor binding sites⁴⁵.

Hypoxia is the primary stimulus to angiogenesis and common in solid tumours, as a result of increased tissue mass. The cellular response to hypoxia is driven largely by dynamic changes in gene expression. Oxygen sensing mechanisms comprise enhancer elements positioned in the 5' and 3' UTRs of the gene¹⁴ and exposure to hypoxia induces the expression of VEGF through both increased transcription and stabilization of its mRNA. The hypoxia-inducible factor (HIF-1) is the main transcription factor for *VEGF*. Several growth factors and cytokines associated with tissue damage in the tumour

microenvironment like EGF, FGF, IGF, HGF and TGF- β are transcription factors for VEGF. Furthermore, the *VEGF* gene 5' and 3' UTRs confer increased mRNA stability during hypoxia⁴⁶.

In addition, some single-nucleotide polymorphisms in the promoter, 5' and 3' UTRs have been described⁴⁷ associated with variability in *VEGF* expression⁴⁸. The variant allele T of the +936C>T polymorphism, located in the 3' UTR, has been associated with lower VEGF plasma levels. The -460T>C polymorphism is located in the promoter region and the T allele has been associated with a decreased *VEGF* promoter activity levels.

The uncontrolled growth that characterizes tumours arises from both, the activation of oncogenes and the loss of tumour suppressors in cell signalling pathways⁴⁹. The tumour suppressors p53 and p73 inhibit VEGF expression in tumour cell lines through the same general region of the *VEGF* promoter⁵⁰. The Ras/Raf/Mek/Erk pathway has been shown to increase VEGF expression and deregulate anti-angiogenic factors⁵¹.

VEGF expression is also regulated post-transcriptionally. During pre-mRNA splicing, varying amounts of each VEGF isoform mRNA can be generated so that cells can express certain subsets or all VEGF isoforms. Given the distinct functions of the various isoforms *in vivo* and differential effects on tumour growth, it is still unknown how VEGF isoform levels are regulated and whether the various upstream effectors of VEGF transcription also modulate the ratio of VEGF isoforms expressed by tumour cells²⁹.

VEGF is translationally regulated by mRNA capping proteins, including eukaryotic initiation factor-4E⁵². VEGF protein translation can also be regulated through utilization of different internal ribosomal entry sites or IRES within the *VEGF* 5' UTR⁵³. VEGF may suffer post-translational regulation by mechanisms such as glycosylation, reported to impact VEGF secretion⁵⁴. In addition, VEGF protein isoforms are post-translationally cleaved by urokinase and plasmin leading to more freely soluble forms.

3. ANGIOGENESIS AS A THERAPEUTIC TARGET IN CANCER

Antiangiogenesis is an approach to cancer therapy with the advantage of inhibiting a process with a minor physiological role in adults, being the target a normal host cell. The microvascular EC has a stable genome and an extremely low mutation rate, reducing the risk of drug-induced resistance. Angiogenesis inhibitors differ from conventional cytotoxic chemotherapy agents in their mechanism of action and cellular target. Angiogenesis inhibitors target receptors on endothelial cells or clear angiogenic growth factors from the circulation preventing their binding to the receptors and therefore the activation of the signalling pathways of these growth factors. Another group of angiogenesis inhibitors are

the angiostatic agents with a direct effect on the endothelium, independently of the tumour cells⁵⁵.

A large body of evidence has experimentally confirmed that tumour progression can be arrested by antiangiogenesis¹¹ leading to several angiogenesis inhibitors approved for its use in cancer. In addition, extensive clinical development programs are currently underway⁵⁶. The clinical development strategy for some of the angiogenesis inhibitors is the combination with cytotoxic chemotherapy or immunotherapy. The rationale for this strategy comes from the different effects and targets: a synergistic antitumour activity not leading to additive toxicities excluding overlapping patterns of resistance.⁵⁵ Anti-angiogenic agents, in which the mechanism of action is cytostatic, brought in the field a revision of the traditional strategy in clinical development. Antiangiogenic therapy prunes and *normalizes* the tumour vasculature leading to substantial systemic effects such as modulation of circulating proangiogenic and proinflammatory cells and cytokines. Effects that might not shrink but rather stabilize the tumour size, particularly when used as monotherapy with agents such as sunitinib (Sutent[®], Pfizer) or sorafenib (Nexavar[®], Bayer) in renal cell carcinoma (RCC) and hepatocellular carcinoma (HCC)^{57, 58}. Clinical studies have evaluated these agents following the *cytostatic paradigm* in which the time to progression (TTP) becomes the decision-making endpoint in early phases of clinical development, rather than the traditionally used objective response rate (ORR).

However, contrary to initially expected, experience is showing the importance of physiological angiogenesis maintaining homeostasis in adults. The role of angiogenesis-related signalling pathways in haematopoiesis, myelopoiesis and EC survival leads to a number of side effects in patients treated with antiangiogenics for which there are a number of underlying responsible mechanisms⁵⁹.

3.1 VEGF INHIBITION: RATIONALE AND MECHANISM OF ACTION

Strategies for inhibiting VEGF and its receptors are those interacting with VEGF ligand and those that impair VEGF receptor signalling by interacting with the receptor or its intracellular catalytic domain. Therapeutic agents developed include the specific monoclonal antibodies (MoAB) against VEGF ligand or VEGF receptors; multitargeted agents, small molecule tyrosine kinase inhibitors (TKI) of VEGF receptors; soluble VEGF receptors or traps; and ribozymes that specifically target VEGF mRNA⁶⁰.

VEGF inhibition blocks the recruitment and migration of EPC preventing vessel regrowth and tumour neovascularisation⁶¹. VEGF inhibition has rapid and substantial effects on tumour microvasculature. The suppression of endothelial cells proliferation, migration and vascular *sprouting* begins within 24 hours of VEGF inhibition⁶². These findings were first confirmed in rectal cancer patients, where a single infusion of anti-VEGF therapy resulted in a significant reduction of the tumour microvasculature^{63, 64}.

Anti-VEGF therapy reverses structural and functional abnormalities in tumour vasculature through a *normalization window*, which ultimately determines the tumour response. It decreases vessel diameter, vascular basement membrane thickness, vascular permeability, interstitial fluid pressure and increases pericyte coverage^{65, 66}. The remaining tumour vasculature is more regularly shaped and has less intercellular gaps, fewer sprouts and more tightly associated pericytes, restoring the vessel permeability. Such vascular changes enable blood supply to be transiently established and improves tissue oxygen tension, increasing the access of chemotherapeutic drugs and reducing resistance to radiotherapy⁶⁷.

Relatively slight increases in the number of cells undergoing apoptosis in tumours can cause a dramatic tumour regression. VEGF is a survival factor for ECs, but it has been postulated that it may be also a survival factor for tumour cells. VEGF was shown to induce expression of the anti-apoptotic Bcl-2 and directly inhibit apoptosis in breast cancer cell lines⁶⁸. Anti-VEGF therapies could therefore increase tumour cell apoptosis by removing the protection that VEGF confers. VEGF involvement in suppression of dendritic cell maturation may also help tumours to evade the host immune system, allowing tumour progression^{28, 61}. VEGF inhibition would additionally help the host immune system to attack tumours more effectively.

These strategies are demonstrating therapeutic efficacy in an increasing number of human cancers, though, remarkably, they have in general not produced a durable efficacy in terms of tumour shrinkage and long-term survival^{69, 70}. Conversely, the benefit mainly lays on a period of clinical benefit and delayed time to disease progression⁷¹. If antiangiogenesis is persistent, it may totally destroy the vessel network, impeding delivery of oxygen and nutrients, and ultimately starving the tumour. In preclinical models, the sustained inhibition progresses towards the *normalization window* close. Though, it has been shown for certain antiangiogenic agents in patients that the inhibition eventually reverts⁷². Emerging data support two modes of unconventional resistance: evasive resistance or escape -adaptation to circumvent the specific angiogenic blockade, by activation of alternative pathways- and intrinsic or pre-existing resistance -mutation of the target leading to unresponsiveness to therapy-. Tumours that have escaped the effect of treatment remain sensitive to the original therapy; though they have found a mechanism to circumvent its effects^{71, 73, 74}.

One of the first antiangiogenics developed was the humanised monoclonal IgG1 antibody bevacizumab (Avastin[®], Roche) which has demonstrated a reasonable safety profile and efficacy in several indications⁷⁵. Bevacizumab potently binds to all isoforms of VEGF and neutralises its binding to VEGF receptors by a steric blocking which leads to the inhibition of the VEGF/VEGFRs signal transduction pathways. Bevacizumab elicits its actions direct antivasular effect in human tumours in the lumen of vascular vessels^{64, 76}. In 1993, Kim *et al.* reported the inhibition of human xenograft tumours growth in a nude mice model by an anti-human VEGF antibody, A4.6.1. This finding provided the first evidence that inhibition of an endogenous angiogenic factor may result in suppression of tumour growth

and subsequent experiments confirmed the results in a variety of human xenograft tumours including carcinomas of colorectal, prostate and ovarian origin ⁶⁰.

3.2 COLORECTAL CANCER AS A MODEL TO ANTIANGIOGENESIS

Colorectal cancer (CRC) is one of the most frequent malignancies, second to breast cancer in women and third to lung cancer and prostate cancer in men ⁷⁷. In recent years, CRC mortality has decreased due to advances in early diagnosis programs and new therapeutics. However, it is the third cause of cancer death worldwide with almost 500,000 related deaths every year ⁷⁷. Colorectal cancers are usually staged upon histopathological examination of a surgical specimen and classified following the AJCC TNM Staging System for Colorectal Cancer -tumour, node, metastases system- developed by the American Joint Committee on Cancer Staging and End Result Reporting ⁷⁸. CRC prognosis is dependent upon the extent of the disease. Approximately 60% of patients diagnosed with CRC develop metastases. Patients with stage IV disease -any T, any N, M1- can present with liver, lung or abdominal peritoneal metastases, though the liver is the most common site for metastatic spread. The 5-year survival rate ranges between 60% to 70% in individuals with lymph node involvement but less than 10% in those with distant metastatic disease ⁷⁷.

Surgery remains the most effective primary treatment offering a potential cure. However, about 40% of patients present with local regionally advanced or metastatic disease which cannot be cured by surgery alone ⁷⁹. Despite apparently curative resection, a significant number of patients develop secondary disease due to growth of undetected micrometastases ⁸⁰. Therefore, systemic therapy is still the basis of the strategy for management of metastatic CRC (mCRC). While the Dukes and TNM staging systems identify broad patients groups that vary in their prognosis, considerable heterogeneity exists in response to treatment within the different chemotherapy agents ^{79, 80}.

3.2.1 Current Therapies in Colorectal Cancer

Several treatment options have emerged since the discovery of the antimetabolite 5-fluorouracil (5-FU) 40 years ago. 5-FU activity lays mainly on thymidylate synthase (TS) inhibition and incorporation of 5-FU into RNA and DNA ⁸¹. Despite improvements in response rates not always translate into significant survival benefit ^{82, 83}, 5-FU in combination with leucovorin (5-FU/LV) became the standard chemotherapy for CRC in the 1980's. Phase III clinical trials demonstrated that infusional 5-FU/LV was associated with a longer median overall survival (OS) as compared with bolus 5-FU/LV (Mayo Clinic regimen) ⁸⁴.

Treatment options expanded with the introduction of the topoisomerase I inhibitor CPT-11 (irinotecan, Camptosar®, Pfizer), a semi-synthetic derivative of camptothecin. Camptothecins interact specifically with the enzyme topoisomerase I that relieves

torsional strain in DNA by inducing reversible single-strand breaks. Irinotecan and its active metabolite SN-38 bind to the topoisomerase I-DNA complex and prevent religation of these single-strand breaks. This cytotoxic activity was found to be time-dependent and specific to the S phase. The combination of irinotecan with infusional 5-FU/LV led to a greater improvement in OS (14.1 vs. 17.4 months, $p < 0.05$)⁸⁴. The addition of irinotecan to 5-FU/LV was associated with a higher incidence on grade 3/4 diarrhoea, grade 3/4 vomiting, grade 4 neutropenia and asthenia. Irinotecan in combination with bolus 5-FU/LV (IFL) became the standard of care in the first line treatment of mCRC in 1996 for patients able to tolerate intensive chemotherapy⁸⁴⁻⁸⁶.

Oxaliplatin is a third-generation platinum analogue, which was first approved in 2002 as second line therapy for the treatment of mCRC IFL-refractory patients. The addition of oxaliplatin to infusional 5-FU/LV (FOLFOX) in the first line treatment of mCRC patients has shown improvement in survival in different trials⁸⁷. Goldberg *et al.* demonstrated a greater clinical benefit for first line FOLFOX compared with IFL which led to the approval of FOLFOX regimen as first line therapy for mCRC. Capecitabine is an oral fluoropyrimidine analogue indicated in the first line treatment of mCRC. Two phase III trials compared capecitabine with bolus 5-FU/LV (Mayo Clinic regimen) in patients with previously untreated mCRC. While TTP and OS were similar in both treatment groups, RR was significantly higher in capecitabine treated patients, with a better tolerability profile⁸⁸.

The relatively recent introduction of targeted agents such as bevacizumab or cetuximab (Erbix[®], Merck) in the management of CRC has modified the previous chemotherapeutic standard of treatment for patients with advanced CRC (Figure 3). However, still with the current outcomes in first line, patients receive second and further lines of systemic therapy at relapse⁸⁹, for which overall RR between 15-25% and OS around 12 months have been reported^{90,91}.

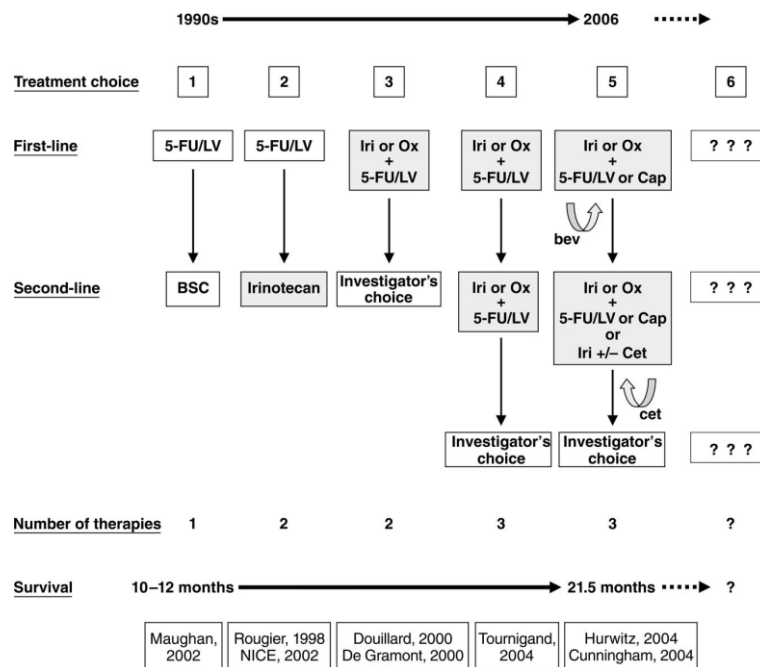


Figure 3. Evolution of treatment paradigms in advanced CRC. 5-FU/LV: 5-FU in combination with leucovorin; Iri: irinotecan; Ox: oxaliplatin; Cap: capecitabine; BSC: best supportive care; bev: bevacizumab; cet: cetuximab

Bevacizumab has also shown activity in pretreated mCRC patients. Despite inconsistent results⁹², outcomes have proved a benefit after failure to chemotherapy or cetuximab in combination with regimens including irinotecan and oxaliplatin⁹³⁻⁹⁵. Although these results show a great improvement, there is still a medical need that justifies the continuing search for alternative combinations.

Two phase I studies evaluating bevacizumab as a single agent and in combination with cytotoxic chemotherapy regimens in patients with advanced malignancies showed a well-tolerated drug. On the other hand, phase II studies outlined some safety issues with haemorrhagic events, in the non-small cell lung cancer (NSCLC) AVF0757g, and venous and arterial thrombosis, in the mCRC AVF0780g study⁷⁵. The phase II AVF2192g study evaluated bevacizumab in combination with 5-FU/FA (Roswell Park regimen) in the first line treatment for mCRC patients who were not optimal candidates for irinotecan treatment. The phase III AVF2107g study, combining bevacizumab with bolus- IFL (Saltz regimen), the standard treatment of mCRC at the time that phase III trial with bevacizumab was initiated, evidenced a clinically meaningful improvement in progression free survival (PFS) and OS becoming the standard of care treatment⁹⁶. Two additional studies were conducted in the first (NO16966 trial) and second line (E3200 trial) treatment of mCRC, combining bevacizumab with FOLFOX-4 (5-FU/LV/oxaliplatin) and XELOX (capecitabine/oxaliplatin), respectively.

Gemcitabine (GMB, Gemzar®, Lilly), a difluorinated analogue of deoxycytidine, exerts its antitumour activity through inhibition of ribonucleotide reductase (RNRM1) and DNA synthesis⁹⁷, and represents an alternative treatment option in CRC. Several phase I/II

trials of single agent gemcitabine have demonstrated minimal activity in mCRC patients⁹⁸. However, clinical outcomes remarkably improve when gemcitabine is used in combination regimens since GMB synergistically interacts with some of the most widely used agents in mCRC⁹⁹. *In vitro* blockade of VEGF-receptor activation has proved to enhance the efficacy of GMB¹⁰⁰. In addition, a synergistic sequence-dependent interaction of GMB and SN-38 has been found in preclinical models, since the incorporation of GMB into DNA enhances camptothecin-induced topo-I cleavage complexes and the capacity to overcome S-phase checkpoint-mediated resistance¹⁰¹. In CRC cell lines, GMB was shown to induce the expression of all topoisomerase enzymes when combined with topoisomerase I poisons¹⁰².

This preclinical background prompted the design of clinical studies with GMB-based combinations, mainly oxaliplatin and fluoropyrimidines, in pretreated mCRC patients¹⁰³, with interesting tumour control rates and a favourable toxicity profile. Gemcitabine represents a strong though not much explored therapeutic option for the salvage treatment of patients with advanced CRC after failure to standard regimens¹⁰⁴.

3.2.2 Prognostic and Predictive Markers in Colorectal Cancer

Patients with mCRC undergoing similar protocols of standard antineoplastic treatment are very heterogeneous with respect to survival outcomes. Several clinical and molecular prognostic factors have been hypothesized including performance status (PS), elevated lactate dehydrogenase (LDH), white blood cells (WBC) count, serum albumin, elevated liver transaminases, Hb, platelets, pathological grading and localisation of the primary lesion, as well as tumour markers such as the carcinoembryonic antigen (CEA)¹⁰⁵. The traditional predictor of clinical outcomes in CRC by Dukes' staging and the UICC TNM system is based on the pathological staging. However, the staging may not be able to predict the recurrence or the development of metastasis in some cases^{106, 107}. CEA remains the most widely used serum marker in CRC. In stage II/III CRC patients CEA measurement is recommended every 2–3 months for at least 3 years after treatment completion¹⁰⁵.

Efforts are being made in order to integrate the knowledge of molecular pathways involved in CRC development with epidemiology data, to help defining individual risk profiles. Köhne *et al.* performed the largest multivariate analysis evaluating clinical prognostic factors to classify mCRC patients treated with 5-FU-based chemotherapy into different risk groups¹⁰⁸. According to their results, patients can be divided into at least three risk groups depending on four baseline clinical parameters: PS, WBC count, alkaline phosphatase (ALP) and number of metastatic sites. Any raising molecular or biological marker shall be normally validated against these clinical parameters.

Chromosomal loss at 18q has been reported in up to 70% of CRCs¹⁰⁹. The gene deleted in colorectal carcinoma (*DCC*), which maps to 18q21, is recognised as a key player in colorectal carcinogenesis and is the primary candidate for the biological effect of chromosome 18q allelic imbalance. Patients with locally advanced stage II and stage III

disease with loss of 18q present a significantly poorer prognosis ¹¹⁰. Approximately 75% of CRCs arise through the chromosomal instability (microsatellite stable, MSS) pathway characterised by aneuploidy, allelic losses, amplifications, translocations and mutation of adenomatous polyposis coli (*APC*), *KRAS* and *TP53* ¹¹¹. The remaining cases have high frequency of microsatellite instability (MSI-H) characterised by inactivation of mismatch repair (*MMR*) genes. A recent meta-analysis found significant improved overall survival time for MSI tumours compared to MSS CRCs ¹¹².

The WNT signalling pathway involving β -catenin, *APC*, E-cadherin ¹¹³; TGF- β pathway including SMAD proteins ¹¹³ and the RAS-MAP kinase signalling pathway involving Raf-1 kinase inhibitor protein (RKIP) ¹¹⁴ are targets of IHC prognostic studies. In addition, IL-6 ¹¹⁵ and IL-8 have been associated with tumour size, depth of tumour infiltration, disease stage, and liver metastasis ¹¹⁶. Furthermore, stem cell markers like CD133 are being studied for association with CRC features ¹¹⁷. However, the lack of specificity and sensitivity precludes the use of identified serum markers for the early detection of CRC as diagnostic indicators ¹¹⁸.

To date, the only molecular marker with clinical application is the *KRAS* mutational status, prognostic and predictive for the anti-EGFR MoAB therapy outcomes. Data showing *KRAS* mutational status that predicts resistance to anti-EGFR MoAB therapy have modified treatment algorithms ¹¹⁹ and other modulators of anti-EGFR activity have been recently raised as potential markers including epiregulin, amphiregulin, PTEN expression, *PI3K* status and *EGFR* gene copy number ¹²⁰.

4. BIOMARKERS OF ANGIOGENESIS IN CANCER

4.1 CURRENT STRATEGIES AND TECHNICAL ADVANCES IN BIOMARKERS DISCOVERY

Supporting evidence for the evaluation of tumour angiogenesis in cancer patients has increased after reports showing that different types of tumours and patients respond differently to antiangiogenic agents ¹²¹⁻¹²³. Promising candidates have been identified, however important challenges limit their translation into practice ^{122, 123}. First, difficulties arise with establishing adequate response criteria to agents that target the tumour stroma in the currently used clinical trials design and methodology. Second, the heterogeneous and dynamic nature of cancer represents another issue since the biology of metastases might be different from the primary tumour and it might also change with tumour progression and treatment exposure. Finally, standardizing the various biomarker assays results in another challenge. Different approaches are being used to measure vascular imaging parameters or circulating markers, which make it difficult to compare different

trials' results. This is further confounded by the inability of the imaging techniques to distinguish anti-vascular effects from antitumour effects of antiangiogenic agents ¹²³.

A number of potential biomarkers have emerged from recently completed phase I-III studies, providing the current bulk of biomarker data ¹²⁴ (Table 2). Tumour tissue markers of angiogenesis like microvessel density ¹²⁵ require of highly invasive sampling and are subject to heterogeneity within the tumour for sample preparation. The need to assess dynamic biomarkers together with the inability to perform repeated biopsies is being addressed by genotypic analyses (VEGF or IL-8 polymorphisms), circulating markers, imaging parameters [K^{trans} , constant of volume transfer of gadolinium (Gd) between blood plasma and the extravascular extracellular space, measured by magnetic resonance imaging (MRI)] ¹²⁶⁻¹²⁹ as well as systemic effects derived from antiangiogenic therapy. Notably, one of the most widely used pharmacodynamic biomarkers is the most prevalent side effect of antiangiogenics, hypertension, associated with a better outcome in patients with metastatic breast cancer ¹³⁰.

Since the majority of angiogenic cytokines and growth factors are soluble and diffusible peptides secreted by tumours, the circulating level of angiogenic factors could in theory reflect the overall angiogenic activity of the tumour. This approach has the advantages of being a more precise (by quantitative immunoassay), non-invasive (no need of biopsy material), and less expensive and time-consuming measurement than tissue ones. The fact of the non-invasiveness for such screening techniques is crucial in order to develop standard of care routine testing for patients. Nevertheless, this approach presents the difficulty in accurately determining the role of tumour-secreted circulating factors, because both host and tumour cells are producing pro and antiangiogenic factors. However, since tumours are truly a *two compartment system* in which tumour cells and endothelial cells mutually co-exist promoting growth and survival, the angiogenic status will be resulting of the coordination between both of them ¹³¹.

An additional conflicting matter is whether to measure VEGF and other angiogenic factors in plasma or serum. Plasma VEGF levels are close to the lower limits of detection of the currently available enzyme immunoassay (EIA) and, subsequently, serum assessments may provide a greater sensitivity ¹³². Several studies demonstrated that paired serum and plasma VEGF levels correlated in mCRC, and both of them increase with advanced disease stage ¹³³. In fact, plasma VEGF levels have only recently been shown predictive for outcome with elevations of proangiogenic cytokines, notably bFGF, PlGF, and HGF, observed in patients treated with FOLFIRI and bevacizumab before radiographic disease progression, using a multiplex-bead assay (MBA) ¹³⁴.

Biomarkers	Technique	Examples
Molecular		
Circulating angiogenic factors	EIA, WB, proteomics, multiplex assays, cytokine antibody arrays	VEGF, FGF-2, MMP-9, IL-8, IL-6, HGF
EC-derived molecules	EIA, WB, proteomics, cytokine antibody arrays	sVEGFR-1, sVEGFR-2, sVEGFR-3, sTie-2, VCAM-1
Circulating proteins or peptides	EIA, WB, proteomics, cytokine antibody arrays	Endostatin
Signalling events	IHC, Immunofluorescence	Phospo-Erk, Phospo-Akt
Biological		
MVD, EC proliferation/death	IHC, Immunofluorescence	CD31+, CD34+, VEGFR2+, Ki67/CD31
CEC or CECP	Flow cytometry, Veridex technology	EC: CD45-, CD31+, CD146+, CD144+, VEGFR-2+ CECP: CD133 CD34+, CD144+, VEGFR-2
Functional		
Functional imaging	DCE MRI, DCE-CT, PET, ultrasound Doppler, Contrast-enhanced ultrasound	Gd chelate tracers, Iodine-based tracers
Molecular imaging	Tracer coupled to mAb or peptide against a vascular target, detected by PET or ultrasound	Targeting EDB+-fibronectin, targeting $\alpha V\beta 3$ integrin

Table 2. Candidate biomarkers and techniques for monitoring angiogenesis. Modified from Jain et al. ¹²³

Enzyme immunoassay has been the most common method used to measure cytokines expression with high specificity and sensitivity. However, novel high-throughput biological applications in the drug discovery process and disease diagnosis require a parallel, miniaturised device technology applied to proteins and their biochemical pathways.

Cytokine antibody arrays represent an alternative to simultaneously detect the expression of multiple cytokines in minute amounts of sample in a single experiment ¹³⁵. Still, the major limitation is the detection sensitivity, where using high-quantity samples and performing replicate experiments is recommended to generate reliable data.

Cytokine antibody arrays were initially developed based on the sandwich EIA principle, with a capture and a detection antibody, where, however, the requirement for a detection antibody increases the complexity and restrains the likelihood of development of high-density cytokine antibody arrays. Several platforms of cytokine antibody arrays have been developed. Membrane-based cytokine antibody arrays allow signals measured by chemiluminescence, fluorescence or colour detection. A glass slide format has also been developed, following DNA arrays, where the experiment can be performed using DNA array instruments such as laser scanner ¹³⁵. Cytokine expression can also be determined by label-based antibody arrays where samples are labelled with a detection molecule such as biotin and fluorophore, and incubated with the antibody array chip. Captured proteins are detected based on the labelled molecule, using fluorescence-labelled streptavidin.

Multiplex bead assays are the most recent technology used in biomarkers research, where the core technology is based on microspheres (beads) coded with different ratios of two fluorescent dyes¹³⁶. The specific ratio of each microsphere creates a unique spectral signature, which can be determined by its fluorescent ratio. In addition, each set of beads is coated with a specific capture antibody. The reaction is carried out in a solution by adding the corresponding quality-controlled beads in the system to detect a particular group of cytokines. The cytokines, which are bound to the antibody-coated beads, are then detected with fluorescently labelled detection antibodies. The signal intensities are measured by flow cytometry, through a quantitative determination of the amount of captured targets on each individual bead.

4.2 CLINICAL IMPLICATIONS OF IDENTIFIED ANGIOGENESIS BIOMARKERS

The benefit derived from antiangiogenic agents has been shown to be transitory in many cases⁷¹. The basement membrane may be responsible for the potential vessel re-growth when VEGF inhibition is not maintained^{72,137} as part of an adaptive-evasive response of tumours to antiangiogenic therapies. Reports support the prognostic value of other circulating angiogenic factors such as bFGF, PIGF, TGF- β and angiogenin¹³⁸⁻¹⁴¹. Extensive preclinical work has suggested these alternative proangiogenic factors to modulate sensitivity to anti-VEGF therapy allowing re-growth of tumour-associated vasculature⁷⁴. Furthermore, VEGF and PIGF expression levels have been shown to increase in response to antiangiogenic treatment¹⁴² and targeting PIGF is being considered to prevent tumour escape from anti-VEGF therapy. Notably, the extent of the increase in PIGF levels in plasma was associated with a better outcome in rectal cancer patients treated with bevacizumab and chemo radiation, and in patients with recurrent glioblastoma multiforme (GBM) treated with cediranib monotherapy⁵⁷.

Most of the evidence on resistance biomarkers comes from circulating factors, as tumour tissue is difficult to obtain at recurrence after therapy. Elevated plasma bFGF and SDF1- α in patients with recurrent GBM receiving cediranib and elevated plasma SDF1- α and IL-6 and circulating progenitor cells in patients with advanced HCC treated with sunitinib, were associated with a poor outcome^{57, 143}. These proangiogenic and proinflammatory biomarkers of resistance, not directly helping in the clinical management of patients, may aid in the identification of new targets. Ultimately, the hope for biomarkers research is to guide the use of combination of single-targeted and multi-targeted agents to substantially improve patients' outcomes allowing for individualized antiangiogenic therapy.

Given the central role for VEGF in tumour angiogenesis, the VEGF/VEGFR pathway is the most extensively explored source for biomarkers and VEGF originally considered as the most valuable potential biomarker in many tumour types. Studies reported have normally shown an association between tumour VEGF expression upregulation and clinical outcome. VEGF is an indicator of prognosis in breast cancer, NSCLC, RCC, CRC

and gastric carcinoma, among others⁶⁰. Weidner *et al* reported the association of neovascular formation in primary breast tumours with a worse prognosis¹⁴⁴ and intratumour microvessel density (MVD) was shown as an independent prognostic factor for a range of solid malignancies¹³¹. Circulating VEGF is positively correlated with tumour volume in soft tissue sarcoma, tumour growth in CRC and tumour degree, stage or grade in breast, CRC, hepatic and renal cancer¹³¹. While circulating VEGF may also predict and help monitoring tumour response to anticancer therapies^{134, 145, 146}, there are conflicting results reported. Baseline plasma VEGF levels have been correlated with time to progression in patients with metastatic breast cancer¹⁴⁷, but not in mCRC^{148, 149}.

Furthermore, there is evidence for the implication of VEGF SNPs in the risk of cancer and other diseases with a putative angiogenic basis^{150, 151}. The evaluation of the *VEGF* genotype definitely emerged as a predictive biomarker candidate from the phase III study of bevacizumab with chemotherapy in patients with metastatic breast cancer ECOG2100. In that study, the VEGF-2578AA genotype was associated with a superior OS and should be tested in future trials of bevacizumab and other anti-VEGF agents¹³⁰.

4.2.1 Biomarkers of Angiogenesis in Colorectal Cancer

The main argument in support of evaluating biomarkers of angiogenesis in CRC patients is to inform therapeutic decisions with prognostic information not provided by clinico-pathologic indicators¹³¹. More than 60% of CRC patients show high VEGF expression levels and there is evidence for different markers of angiogenesis to have a prognostic value. Hanrahan showed VEGF mRNA levels correlated significantly with tumour grade and size. VEGFR-1 mRNA levels also significantly correlated with tumour grade, Duke's stage and lymph node involvement and VEGFR-2 mRNA levels with lymph node involvement¹⁵².

Increased VEGF expression detected by IHC has been correlated with tumour invasiveness, vascular density, metastases, recurrence and prognosis in terms of poor disease-free and overall survival, distant metastatic spread and decreased response to preoperative radiotherapy¹⁵³⁻¹⁵⁶. In addition, it has been shown that the levels of VEGF in the ascites of patients with mCRC are markedly elevated¹⁵⁷. Upregulation of VEGF has also been linked with uPA and MMPs, which function to degrade the basement membrane and ECM providing a scaffold for migrating endothelial cells¹⁵⁸ and with the Tie-2 receptor and development of metastases¹⁵⁹. Increased MVD has independently predicted tumour recurrence and was associated with vascular invasion of cancer cells in two studies, also significantly predicting poor survival¹⁶⁰. Ang-2, TNF- α , IL-6, IL-8 and ICAM-1 expression correlated with disease severity and shorter survival, having a potential prognostic value¹¹⁵.

Mutations or misregulation of Wnt signalling pathway members have a causative role in the development and progression of colon cancer. In both pre-malignant colorectal adenomas and invasive colorectal adenomas, VEGF is upregulated by the Wnt signalling pathway⁵¹. P53 dysfunction and the Ras/Raf/Mek/Erk signalling pathway have also been

associated with increased expression of VEGF and decreased expression of angiogenesis inhibitors⁵⁰ suggesting that gene mutations may influence the response to antiangiogenic therapy^{161, 162}.

However, in general, VEGF expression has not been shown predictive of the benefit provided by antiangiogenic therapy^{148, 149, 160}. To date, only one study has shown greater risk of post-operative recurrence in patients with VEGF positive tumours after adjuvant fluoropyrimidines¹⁶³. Furthermore, the retrospective analysis of the AVF2107g Avastin study did not show a statistically significant relationship between *P53*, *KRAS* and *BRAF* mutations and survival with the addition of bevacizumab to IFL in mCRC¹⁴⁹. Neither did another study determining the association between VEGF, TSP2, MVD and survival¹⁴⁸.

Several studies have shown diverse evidence on the association and value of VEGF circulating levels and tumour expression in the different settings of CRC^{134, 147}. Serum VEGF levels were not correlated to angiogenic activity in a study¹²², while higher serum VEGF predicted poorer DFS and earlier metastases¹⁵⁹ in another. A recent report shows a biomarkers analysis in patients treated with FOLFIRI and bevacizumab where prior to radiographic progression, there is a shift in balance of circulating angiogenic factors, with a rise in alternate proangiogenic cytokines and myeloid recruitment factors that may represent mechanisms of resistance¹³⁴.

Among other circulating angiogenic factors which expression may have a prognostic value in CRC, increased ICAM-1 levels were correlated with more advanced staging, and lymph node metastases (LNM)¹⁶⁴. IGF-1 and IGF-2 have been also associated with poorer prognosis, but have limited prognostic value¹⁶⁵. Expression of bFGF correlated positively with tumour grade, stage and LNM¹⁶⁶. Higher serum Tie-2 receptor levels are associated with poorer DFS and earlier metastases and independently predicted poorer outcome¹⁵⁹. Over-expression of Ang-1 correlated with tumour MVD, but did not predict survival while over-expression of Ang-2 correlated with LNM, venous invasion and high MVD, and independently predicted poorer DFS and OS¹¹⁵. Plasma concentrations of MMP-2 and MMP-9 have been correlated with clinical staging¹⁶⁷.

VEGF polymorphisms in CRC are thought to play a role in the degree of regulation of angiogenesis and, furthermore, in the response to targeted therapy. Numerous *VEGF* SNPs in the promoter, 5' and 3' UTRs have been described in CRC which might play a role in the risk of development of this cancer. A protective haplotype -2578A, -460T, and +405G and two different high-risk haplotypes -2578A, -460C, and +405G and -2578C, -460C, and +405C were identified¹⁶⁸. The C+936T VEGF and VEGFR-2 (+4422 AC-repeat and T+1416A) polymorphisms were associated with risk of tumour recurrence in stage III colon cancer patients. VEGFR-2 AC-repeat polymorphisms were also associated with risk of recurrence in stage II colon cancer patients^{151, 169}. The VEGF-2578 CC genotype has also been associated with an inferior median OS compared to alternative genotypes in mCRC patients treated with irinotecan-based chemotherapy and bevacizumab in the first-line setting¹⁷⁰. However, the SNPs' definite predictive value for antiangiogenics outcomes in mCRC remains to be determined¹⁵¹. Moreover, the

correlation between those polymorphisms and therapy-derived toxicity should be studied and accounted in order to reach a more favourable benefit/risk ratio.

HYPOTHESIS

The progressive growth of colon cancer is dependent on the tumour angiogenic network, and antiangiogenic strategies have emerged as effective therapies. Differences in the magnitude of benefit achieved with current treatment options point to the underlying tumour microenvironment as responsible for inconsistent clinical outcomes.

Different tumours, and especially in more advanced stages, do not rely on a unique angiogenesis driver and the activation of alternative pathways is one of the described mechanisms of resistance to antiangiogenic agents.

Serum circulating cytokines, growth factors and angiogenesis-related molecules, are hypothesized to be valid markers of the tumour microenvironment angiogenic profile which may offer prognostic and predictive information beyond conventional clinico-pathological indicators. Identifying the proteins responsible for the diverse behaviour of metastatic colorectal tumours seems warranted in order to more effectively use available therapies.

We hypothesized that the “secretome” of CRC patients shall provide predictive markers for the currently accepted clinical endpoints, guiding the use of drugs to substantially improve patients’ outcomes, allowing for individualized antiangiogenic therapy. Furthermore, defining molecular profiles related to patients’ risk and/or outcome could ultimately help defining valid surrogate endpoints. Finally, filling the current gap on biomarkers of CRC might also help defining novel intervention targets and new treatment options.

Moreover, the work hypothesis includes the use of analyses methods which require non-invasive sampling, allowing for greater feasibility when aiming incorporation into clinical practice.

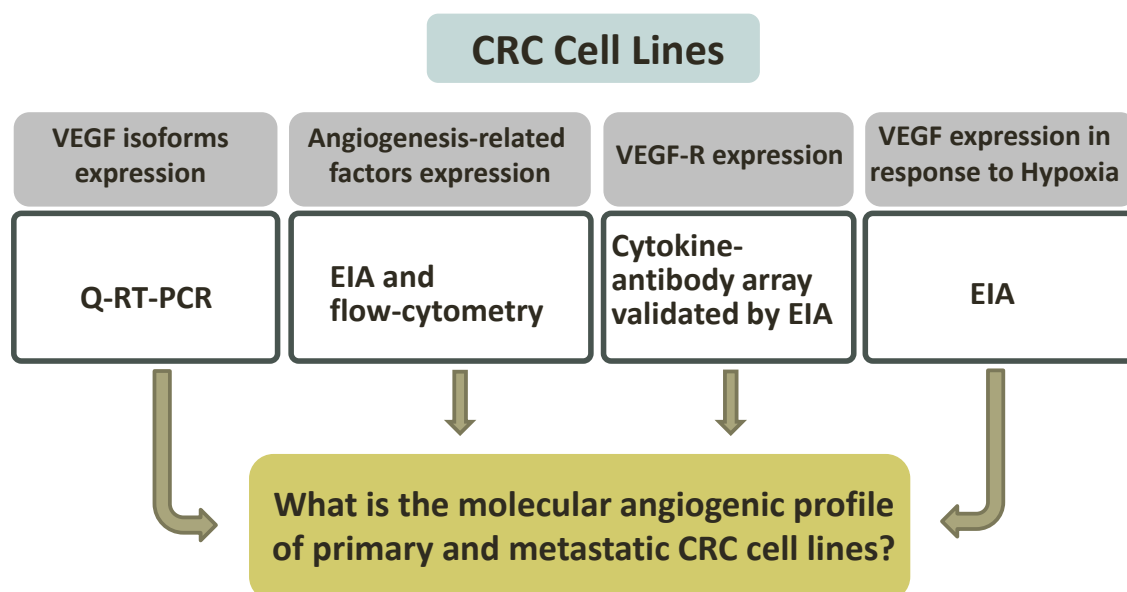
OBJECTIVES

The general objective of this thesis is to determine the angiogenic molecular signature in colorectal cancer. In the framework of biomarkers research, the ultimate objective is to add to the body of knowledge on the biological and molecular implications for the use of anti-angiogenic therapies in colorectal cancer patients.

1. IN VITRO

The aim is to characterize the angiogenic molecular signature in CRC using a set of CRC cell lines. This model is considered valid to study this tumour type with enhanced feasibility when compared to patients' tumour samples. Therefore, we aim to:

- Evaluate the differential expression of angiogenesis-related cytokines and growth factors by a novel protein antibody-array in colorectal cancer cell lines of metastatic and primary origin.
- Characterize the expression of the vascular endothelial growth factor (VEGF), pattern of VEGF isoforms and VEGF receptors 1 and 2.
- Determine the VEGF/VEGFR pathway in response to hypoxia in colorectal cancer cell lines of metastatic and primary origin.

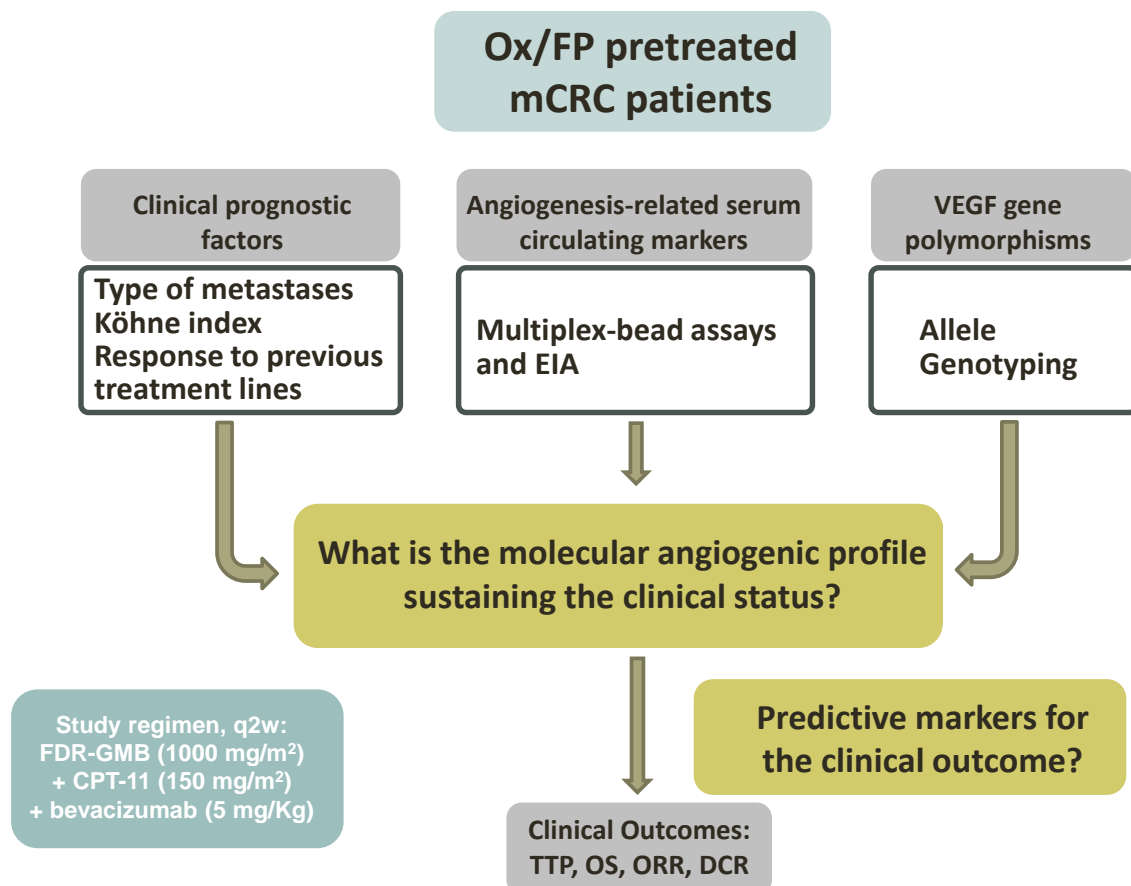


EIA= enzyme immunoassay, Q-RT-PCR= Quantitative reverse-transcription PCR

2. CLINICAL STUDY

The aim is to evaluate the pharmacogenomic implications of the angiogenic molecular signature for the use of anti-angiogenic therapies in colorectal cancer patients by using a non-invasive approach for biomarker identification: cytokine expression and gene polymorphisms in peripheral blood. The clinical setting for this study consists of oxaliplatin/fluoropyrimidines-pretreated mCRC patients treated with irinotecan in combination with fixed-rate infusion of gemcitabine and bevacizumab. Therefore, the aim is to:

- Explore the predictive value of *VEGF* gene polymorphisms for the patients' clinical outcomes.
- Explore the association between serum-circulating angiogenesis-related cytokines and growth factors and patients' clinical outcomes.



FDR-GMB: fixed dose rate gemcitabine; CPT-11: irinotecan; Ox/FP=Oxaliplatin/fluoropyrimidines; TTP: time to progression; OS: overall survival; ORR: objective response rate; DCR: disease control rate

CHAPTERS

IDENTIFICATION OF COLORECTAL CANCER
METASTASIS MARKERS BY AN ANGIOGENESIS-
RELATED CYTOKINE-ANTIBODY ARRAY

**Abajo A, Bitarte N, Zarate R, Boni V, Lopez I, Gonzalez-Huarriz
M, Rodriguez J, Bandres E, Garcia-Foncillas J**

Full Paper. *World J Gastroenterol* 2012; 18(7): 637-645

Identification of colorectal cancer metastasis markers by an angiogenesis-related cytokine-antibody array

Ana Abajo, Nerea Bitarte, Ruth Zarate, Valentina Boni, Ines Lopez, Marisol Gonzalez-Huarriz, Javier Rodriguez, Eva Bandres, Jesus Garcia-Foncillas

Ana Abajo, Nerea Bitarte, Ruth Zarate, Ines Lopez, Marisol Gonzalez-Huarriz, Eva Bandres, Laboratory of Pharmacogenomics, Division of Oncology, Center for Applied Medical Research, University of Navarra, Avda Pio XII 55, 31008 Pamplona, Spain

Valentina Boni, Javier Rodriguez, Jesus Garcia-Foncillas, Department of Oncology, University Clinic of Navarra, University of Navarra, Avda Pio XII 55, 31008 Pamplona, Spain

Author contributions: Abajo A, Bitarte N, Bandres E and Garcia-Foncillas J designed the research; Abajo A, Bitarte N, Lopez I and Gonzalez-Huarriz M performed the research; Abajo A, Zarate R, Boni V, Bandres E and Rodriguez J analyzed the data; Abajo A, Bandres E and Garcia-Foncillas J wrote the paper.

Supported by A grant of "Department of Health, Government of Navarra, Spain (23/2009)"

Correspondence to: Jesus Garcia-Foncillas, MD, PhD, Department of Oncology, University Clinic of Navarra, University of Navarra, Avda Pio XII 55, 31008 Pamplona, Spain. jgfoncillas@fjd.es

Telephone: +34-948-194700 Fax: +34-948-194714

Received: May 16, 2011 Revised: July 4, 2011

Accepted: July 11, 2011

Published online: February 21, 2012

Abstract

AIM: To investigate the angiogenesis-related protein expression profile characterizing metastatic colorectal cancer (mCRC) with the aim of identifying prognostic markers.

METHODS: The expression of 44 angiogenesis-secreted factors was measured by a novel cytokine antibody array methodology. We evaluated vascular endothelial growth factor (VEGF) and its soluble receptor sVEGFR-1 protein levels by enzyme immunoassay (EIA) in a panel of 16 CRC cell lines. mRNA VEGF and VEGF-A isoforms were quantified by quantitative reverse-transcription polymerase chain reaction (Q-RT-PCR) and vascular endothelial growth factor receptor (VEGFR)-2 expression

was analyzed by flow cytometry.

RESULTS: Metastasis-derived CRC cell lines expressed a distinctive molecular profile as compared with those isolated from a primary tumor site. Metastatic CRC cell lines were characterized by higher expression of angiogenin-2 (Ang-2), macrophage chemoattractant proteins-3/4 (MCP-3/4), matrix metalloproteinase-1 (MMP-1), and the chemokines interferon γ inducible T cell α chemoattractant protein (I-TAC), monocyte chemoattractant protein I-309, and interleukins interleukin (IL)-2 and IL-1 α , as compared to primary tumor cell lines. In contrast, primary CRC cell lines expressed higher levels of interferon γ (IFN- γ), insulin-like growth factor-1 (IGF-1), IL-6, leptin, epidermal growth factor (EGF), placental growth factor (PIGF), thrombopoietin, transforming growth factor β 1 (TGF- β 1) and VEGF-D, as compared with the metastatic cell lines. VEGF expression does not significantly differ according to the CRC cellular origin in normoxia. Severe hypoxia induced VEGF expression up-regulation but contrary to expectations, metastatic CRC cell lines did not respond as much as primary cell lines to the hypoxic stimulus. In CRC primary-derived cell lines, we observed a two-fold increase in VEGF expression between normoxia and hypoxia as compared to metastatic cell lines. CRC cell lines express a similar pattern of VEGF isoforms (VEGF₁₂₁, ₁₆₅ and ₁₈₉) despite variability in VEGF expression, where the major transcript was VEGF₁₂₁. No relevant expression of VEGFR-2 was found in CRC cell lines, as compared to that of human umbilical vein endothelial cells and sVEGFR-1 expression did not depend on the CRC cellular origin.

CONCLUSION: A distinct angiogenesis-related expression pattern characterizes metastatic CRC cell lines. Factors other than VEGF appear as prognostic markers and intervention targets in the metastatic CRC setting.

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Key words: Colorectal cancer metastasis; Cytokine-antibody array; Angiogenesis; Vascular endothelial growth factor; Biomarkers

Peer reviewer: Marek Bebenek, MD, PhD, Department of Surgical Oncology, Regional Comprehensive Cancer Center, pl. Hirszfelda 12, 53-413 Wrocław, Poland

Abajo A, Bitarte N, Zarate R, Boni V, Lopez I, Gonzalez-Huarriz M, Rodriguez J, Bandres E, Garcia-Foncillas J. Identification of colorectal cancer metastasis markers by an angiogenesis-related cytokine-antibody array. *World J Gastroenterol* 2012; 18(7): 637-645 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i7/637.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i7.637>

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths. The prognosis of CRC is dependent upon the extent of disease and approximately 60% of patients develop metastases after surgical resection. With a 5-year survival rate of less than 10% in patients with distant metastatic disease, targeting the metastatic process and sites should provide an effective treatment^[1]. The progressive growth of colon cancer and subsequent metastatic process is dependent on an angiogenic network^[2,3]. Thus, anti-angiogenic strategies have emerged as effective therapies in patients with colon cancer, especially in the metastatic setting of the disease^[4-6]. Yet, differences in the magnitude of survival benefit point to alternative pathways in the tumor microenvironment as responsible for inconsistent outcomes^[7].

Angiogenesis is a complex process dependent on the angiogenic factors secreted by the tumor and stroma cells^[8]. Vascular endothelial growth factor is considered the major pro-angiogenic factor^[9]. The vascular endothelial growth factor (VEGF) gene encodes for six alternatively spliced isoforms^[10] with differential diffusion potential and binding to receptors^[11]. The question currently consists of understanding the significance of VEGF/vascular endothelial growth factor receptor (VEGFR) signaling in cancer cells^[12,13]. The VEGF isoforms and VEGF receptor expression pattern would drive the activity and functionality of the VEGF/VEGFR pathway in both tumor and endothelial cells. The multistep process of angiogenesis accompanies the multistage development of a tumor^[14]. The switch into the metastatic phenotype brings a number of changes within the tumor microenvironment, including acquisition of hypoxia-tolerance mechanisms^[15]. While up-regulation of VEGF expression is activated mainly under hypoxia^[9], recent reports reflect on the question of whether metastatic tumors rely as much on angiogenesis and VEGF as primary tumors^[15].

Other studies report that tumors in more advanced stages do not rely on a unique angiogenesis driver^[2]. A network of multiple cytokines and growth factors create a crosstalk within the tumor microenvironment which

Table 1 Colorectal cancer cell lines origin

Cell line	Type/Origin
SW620	Colon adenocarcinoma. Derived from: metastasis to lymph node
T84	Colon carcinoma. Derived from metastasis to lung
LoVo	Derived from metastatic site: left supraclavicular region
SW480	Colon adenocarcinoma
WiDr	Colon adenocarcinoma
RKO	Colon carcinoma
HT29	Colon adenocarcinoma
HCT15	Colon adenocarcinoma
HCT116	Colon carcinoma
SW1116	Colon adenocarcinoma
SW1417	Colon adenocarcinoma
LS174T	Colon adenocarcinoma
LS513	Colon carcinoma
Caco2	Colon adenocarcinoma
DLD-1	Colon adenocarcinoma
LS411N	Colon adenocarcinoma
Colo320	Colon adenocarcinoma

ultimately drives tumor angiogenesis^[2,16]. The mediators of vessel wall remodeling matrix metalloproteinases, macrophage chemoattractant proteins and angiogenin, involved in invasion and metastasis processes, exert pro-angiogenic signals^[8,17]. Chemokines such as interleukin (IL)-1 α and IL-8 play an important role in colon cancer progression and angiogenesis^[18], and IL-8 up-regulates MMPs^[19]. VEGF expression actually determines the activity of Ang-1/Ang-2 and the expression of MCPs^[20,21].

Great efforts have been made to characterize biomarkers in CRC^[22]. However, the question of biomarkers of CRC metastasis remains currently unresolved. On this basis, the aim of this study was to characterize the protein factors behind the angiogenic potential of CRC cell lines of metastatic origin.

MATERIALS AND METHODS

Cell cultures and conditioned media

We used 16 CRC cell lines: HT29, WiDr, HCT116, RKO, SW480, Colo320, Caco2, SW1116, LS174T, SW1417, DLD-1, LS513, HCT15, SW620, LoVo and T84 (all from American Type Culture Collection, Manassas, VA) (Table 1). The cell lines were maintained in the recommended growth media supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO). For harvesting conditioned media, CRC lines cells were grown approximately to 70% confluence in serum free media. The conditioned media were collected after 24 h of incubation, centrifuged and kept frozen.

VEGF and sVEGFR1 protein detection by quantitative immunoassay

VEGF-A in supernatant was determined using the Human VEGF Quantikine[®] EIA kit (R and D Systems) and sVEGFR-1 was quantified by EIA (Human sVEGF R1/Flt-1 Quantikine[®], R and D Systems), according to the manufacturer's instructions. We normalized VEGF and sVEGFR-1 protein levels per number of cells. Results are

Table 2 Primer and probe sequences for vascular endothelial growth factor isoforms quantitative reverse-transcription polymerase chain reaction

	Sense primer	Antisense primer	Taqman probe	Amplicon size (bp)
VEGF end-point and cloning	ACTGCCATCCAATCGAGACC	GATGGCTTGAAGATGTACTCGATCT		
GAPDH end-point and cloning	TGGTATCGTGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC		189
VEGF ₁₂₁ mRNA	CAAGGCCAGCACATAGGAGA	CTCGGCTGTGCACATTTTTC	CTTCCTACAGCACAACAAATGT-GAATGCAGA	101
VEGF ₁₆₅ mRNA	TGTGAATGCAGACCAAAGAAAGA	TGCTTTCCTCCGCTCTGAGC	AGAGCAAGACAAGAAAATCCCT-GTGGGC	74
VEGF ₁₈₉ mRNA	CGCAAGAAATCCCGTATAAGT	TGCTTTCCTCCGCTCTGAGC	AGGCCACAGGGAACGCTCCAG	65
GAPDH	TGGTATCGTGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC	CCCAGAGACTGTGGATGGCCCC	189

VEGF: Vascular endothelial growth factor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

the average of replicates.

Total VEGF and isoforms mRNA determination by quantitative reverse-transcription polymerase chain reaction

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). Single strand DNA was synthesized from 1 µg total RNA using the cDNA Archive kit (Applied Biosystems). Quantitative reverse-transcription polymerase chain reaction (Q-RT-PCR) for total VEGF was performed using primers and probes purchased from Applied Biosystems (Hs00900054_m1). RNA18s (Hs99999901_s1) was used as an endogenous control and data obtained was represented as $2^{-\Delta Ct}$.

VEGF isoforms were determined by Q-RT-PCR using primers designed specifically for VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control (Table 2). The relative quantification of samples was performed using a standard curve by dilution of a specific plasmid for each isoform (ranging from 10 pg to 1 fg). Human VEGF cDNA for each isoform and GAPDH were cloned from total RNA isolated from lung cancer resection as follows. PCR products were run through a 1% agar gel and bands of the size expected for VEGF₁₂₁, VEGF₁₆₅ and VEGF₁₈₉ were isolated and purified. Each VEGF isoform was cloned into the pCRII vector (Invitrogen) and sequenced (ABI PRISM Big Dye Terminator Cycle Sequencing reaction kit; ABI Protocol, Gene Amp 9600, Applied Biosystems) to verify its identity.

Time course hypoxia-normoxia

The cell lines were maintained in the recommended growth media supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO). After washing with phosphate buffered saline (PBS), serum-free medium was added and the cells exposed to normoxic or hypoxic conditions for 6 h, 12 h, 24 h, 36 h, 48 h and 72 h. Hypoxic conditions were achieved by culturing cells in a modulator incubation chamber (Sanyo MCO-18 M) gassed with 1% O₂, 50 mL/L CO₂, and 94% N₂. VEGF protein secretion was measured in the

supernatant by enzyme immune-assay (EIA) and VEGF mRNA levels by Q-RT-PCR. Cell proliferation was evaluated by the Trypan Blue exclusion method.

VEGFR-2 expression in colorectal cancer cell lines by flow cytometry

The expression of VEGFR-2 (KDR) in CRC cell lines was determined by flow cytometry (FacScan, Becton-Dickinson). After trypsinization, cells were incubated in medium for 12 h on a rocker platform to enable regeneration of the receptors. Cells were Fc-blocked by treatment with 100 µL of AB human serum for 15 min at room temperature prior to staining with 10 µL of PE-conjugated anti-VEGFR-2 antibody (Becton Dickinson Biosystems) for 30 min at 4 °C. Following the incubation, unbound anti-VEGFR-2 antibody was removed by washing the cells twice in 4 mL PBS buffer. The human umbilical vein endothelial cells (HUVEC) cell line was used as a positive control.

Secreted angiogenic profile by cytokine antibody-array

The secretion of angiogenic factors by CRC cell lines was evaluated in duplicate using a protein array method (RayBio® Human Angiogenesis Antibody Array, RayBiotech C Series 1000, RayBiotech, Inc Norgross, GA). This assay is capable of simultaneously detecting 44 different angiogenic factors (spotted in sub-arrays I and II) with high specificity. The sensitivity of the antibodies present in the arrays ranged from 1-2000 pg/mL. Conditioned media was obtained after the incubation of 2×10^5 cells in serum-free medium for 20 h at 37 °C and 5% CO₂. Each array was incubated with 1.2 mL of medium at 4 °C overnight, and bound antigens were detected according to the manufacturer's instructions. To determine the relative concentrations of angiogenic factors in the media, the densities of individual spots were measured using Imagen 4.1 software (Biodiscovery Inc., Marina Del Rey, United States) for image capture and analysis.

Statistical analysis

Statistical analysis was carried out with SPSS 13.0 software (SPSS Inc.). Associations between VEGF expres-

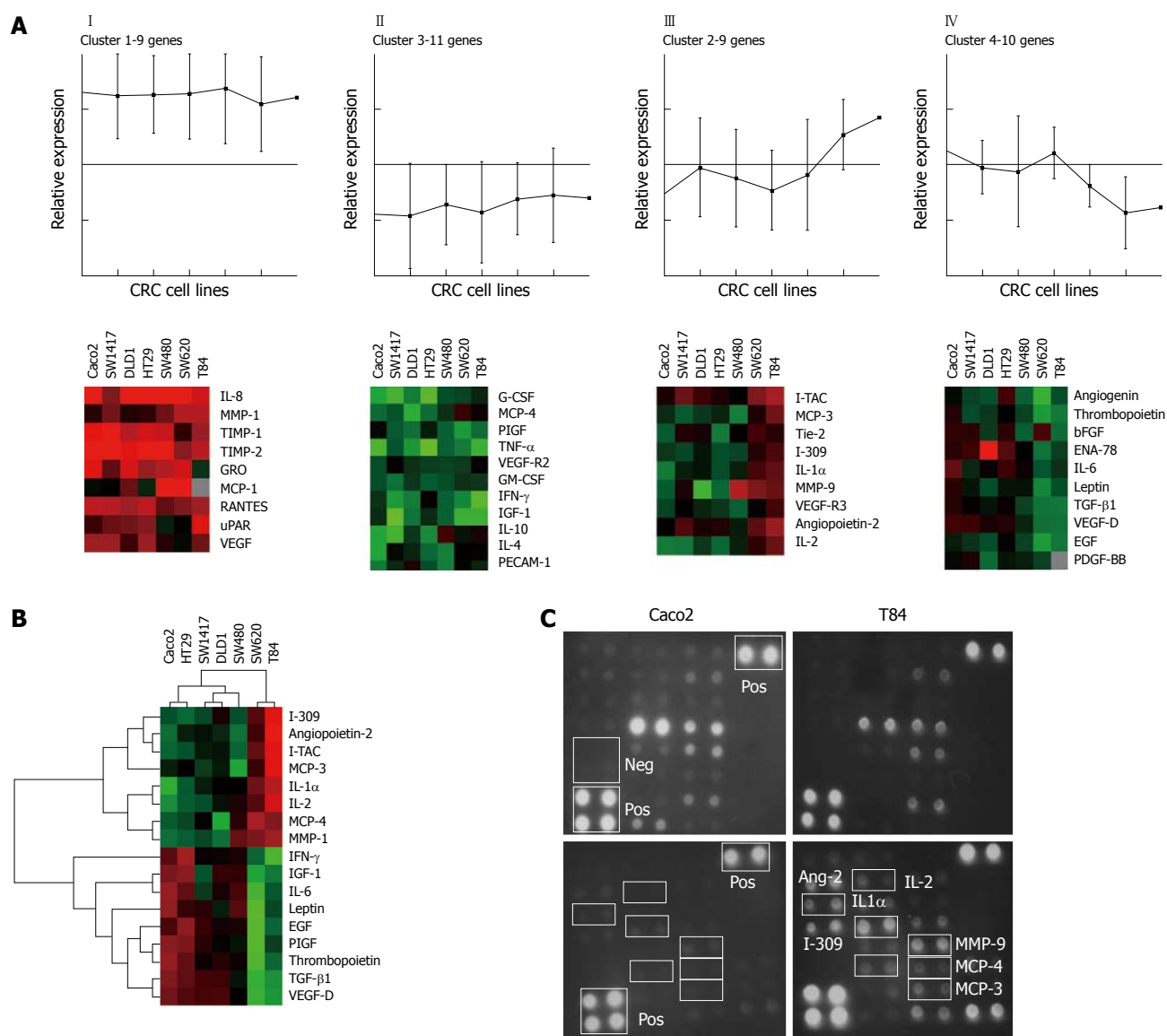


Figure 1 Angiogenesis-related factors expression profile in colorectal cancer cell lines as determined by cytokine antibody-array. A: K-means ($n = 4$) clustering grouped the angiogenesis-related proteins according to level of expression; B: Unsupervised-hierarchical clustering of the factors with a significantly different expression in primary and metastatic colorectal cancer (CRC) cell lines; C: Images of subarrays I and II of the primary Caco2 and the metastatic T84 CRC cell lines after detection and processing. IL: Interleukin; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinases; GRO: Growth related oncogene; MCP: Macrophage chemoattractant proteins; RANTES: Regulated upon activation normally T-expressed and secreted; uPAR: Urokinase-type plasminogen activator-receptor; G-CSF: Granulocyte colony-stimulating factor; PIGF: Phosphatidylinositol glycan, class F; TNF- α : Tumor necrosis factor- α ; GM-CSF: Granulocyte macrophage colony-stimulating factor; IFN- γ : Interferon γ ; IGF: Insulin-like growth factor; PECAM: Platelet-endothelial cell adhesion molecule; I-TAC: Inducible T cell α chemoattractant protein; ENA: Epithelial neutrophil activating protein; EGF: Epidermal growth factor; PDGF-BB: Platelet-derived growth factor, β polypeptide; TGF- β 1: Transforming growth factor β 1; Neg: Negative control; Pos: Positive control.

sion and VEGF isoforms pattern were determined with the Spearman correlation. Differences between groups were determined by the Mann-Whitney U test. The level of two-tailed statistical significance was 0.05.

CRC cell line angiogenesis cytokine antibody-arrays raw data were normalized to the global median [BRB Array Tools 3.6.0 (NCI)] of signals detected as per manufacturer's instructions. GENESIS Software (Institute for biomedical engineering, Graz University of Technology, Graz, Austria) was used for the analyses of clustering of samples and genes and K-means and hierarchical unsupervised clustering analyses were performed to determine cytokine profiles.

RESULTS

Distinct angiogenesis-related expression pattern in primary and metastatic colorectal cancer cell lines

To identify the angiogenesis-related "secretome" of CRC cell lines in normoxia, we analyzed 44 angiogenesis-related cytokines and growth factors by an antibody-array in primary (Caco2, SW1417, DLD1, HT29 and SW480) and metastatic (SW620 and T84) CRC cell lines. K-means analysis classified CRC cell line angiogenesis-related secreted factors according to their level of secretion (Figure 1A). Cluster I showed a homogeneous high expression of the pro-angiogenic IL-8, MMP-1, MCP-1, growth

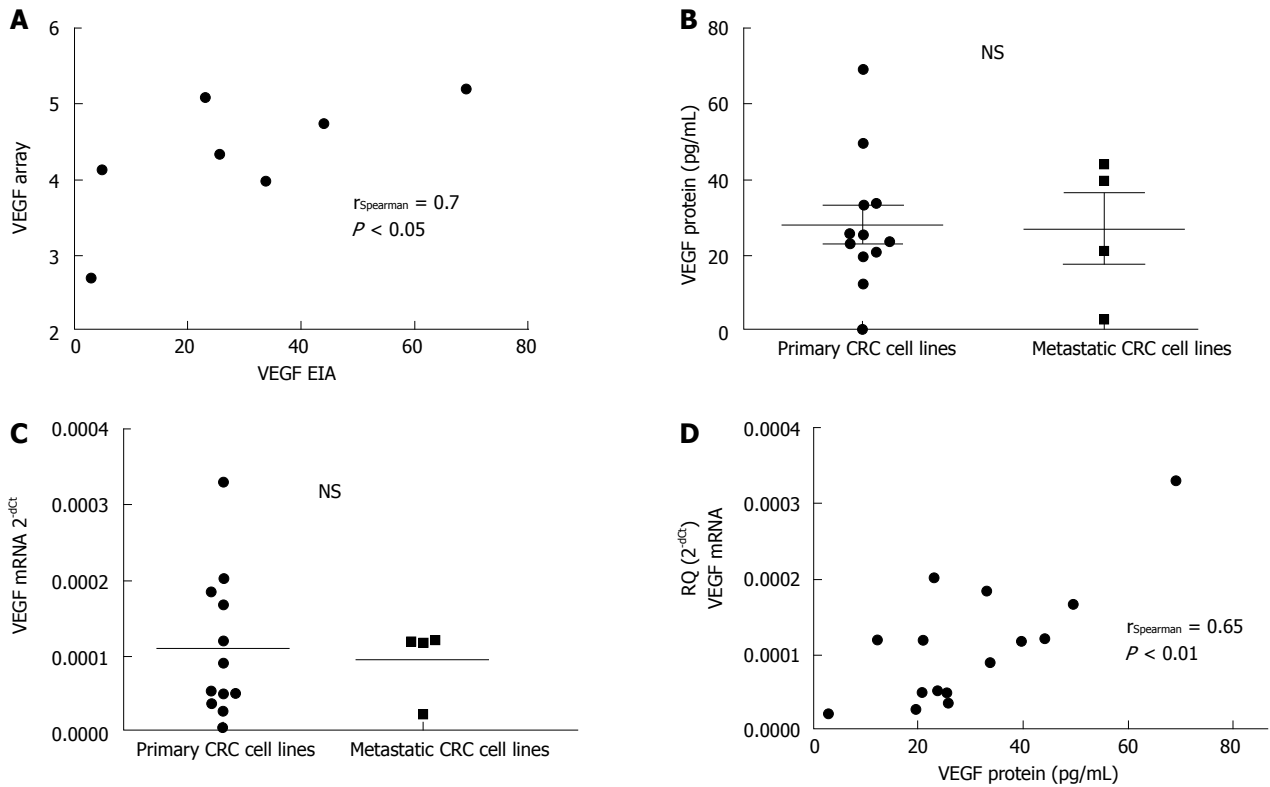


Figure 2 Vascular endothelial growth factor expression in colorectal cancer cell lines. A: A statistically significant positive correlation is found between vascular endothelial growth factor (VEGF) protein as determined by antibody-array and by enzyme immunoassay (EIA), validating the array method; B and C: Colorectal cancer (CRC) cell lines exhibit variability in VEGF protein (B) and mRNA (C) expression according to their primary or metastatic origin (not statistically significant); D: A statistically significant positive correlation is found between VEGF protein by EIA and VEGF mRNA, suggesting the major role of transcriptional mechanisms in the regulation of VEGF expression. NS: Not significant.

related oncogene (GRO)- α , regulated upon activation, normal T-cell expressed, and secreted protein (RANTES), urokinase-type plasminogen activator-receptor (uPAR) and VEGF; and the anti-angiogenic tissue inhibitor of metalloproteinases tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2 (Figure 1A, cluste I). Cluster II integrated angiogenic factors not secreted by CRC cell lines in normoxia, including VEGF family proteins placental growth factor (PlGF) and sVEGFR-2 and inflammatory cytokines with pro-angiogenic properties granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IFN- γ , tumor necrosis factor- α (TNF- α) (Figure 1A, cluster II). Primary tumor- and metastasis-derived CRC cell lines were characterized by a distinct angiogenesis-related molecular pattern in normoxia (Figure 1A, cluster III and IV). Figure 1B shows the unsupervised hierarchical clustering of the antibody-array proteins significantly differing in expression according to their cellular origin. One-way ANOVA ($P < 0.05$) grouped primary and metastatic cell lines according to their differential molecular expression pattern. Metastasis-derived cell lines were characterized by higher expression of Ang-2, MCP-3, MCP-4, MMP-1 and the chemokines I-TAC, I-309, IL-2 and IL-1 α ($P < 0.05$), and a trend was found for MMP-9, as compared to primary tumor cell lines (Figure 1B). On the other hand, CRC cell lines isolated from a primary tumor site were

clustered together according to the higher expression of IFN- γ , IGF-1, IL-6, leptin, EGF, PlGF, thrombopoietin, TGF- β 1 and VEGF-D ($P < 0.05$), as compared with the metastatic ones (Figure 1B). Interestingly, VEGF-A (VEGF) was not found among the proteins differentially expressed according to the cellular source of isolation. Figure 1C illustrates processed antibody-arrays and the images captured of Caco2 (primary CRC cell line) and T84 (metastatic CRC cell line).

VEGF expression in primary and metastatic colorectal cancer cell lines

The antibody array data showed no significant changes in VEGF secretion between primary and metastasis-derived CRC cell lines (Figure 1B). To validate the antibody array results, we analyzed VEGF levels by EIA. The results were confirmed by a statistically significant positive correlation between VEGF protein as determined by the antibody-array and by EIA (r Spearman = 0.7, $P < 0.05$) (Figure 2A).

In a second step, VEGF secretion by EIA and VEGF mRNA expression was analyzed in a larger panel of 16 CRC cell lines. As shown in Figures 2B and 2C, we did not detect any significant difference in VEGF expression according to the primary or metastatic CRC cell lines (mean of 28.9 pg/mL and 22.7 pg/mL VEGF protein; 0.011 and 0.009 (relative quantification) VEGF mRNA,

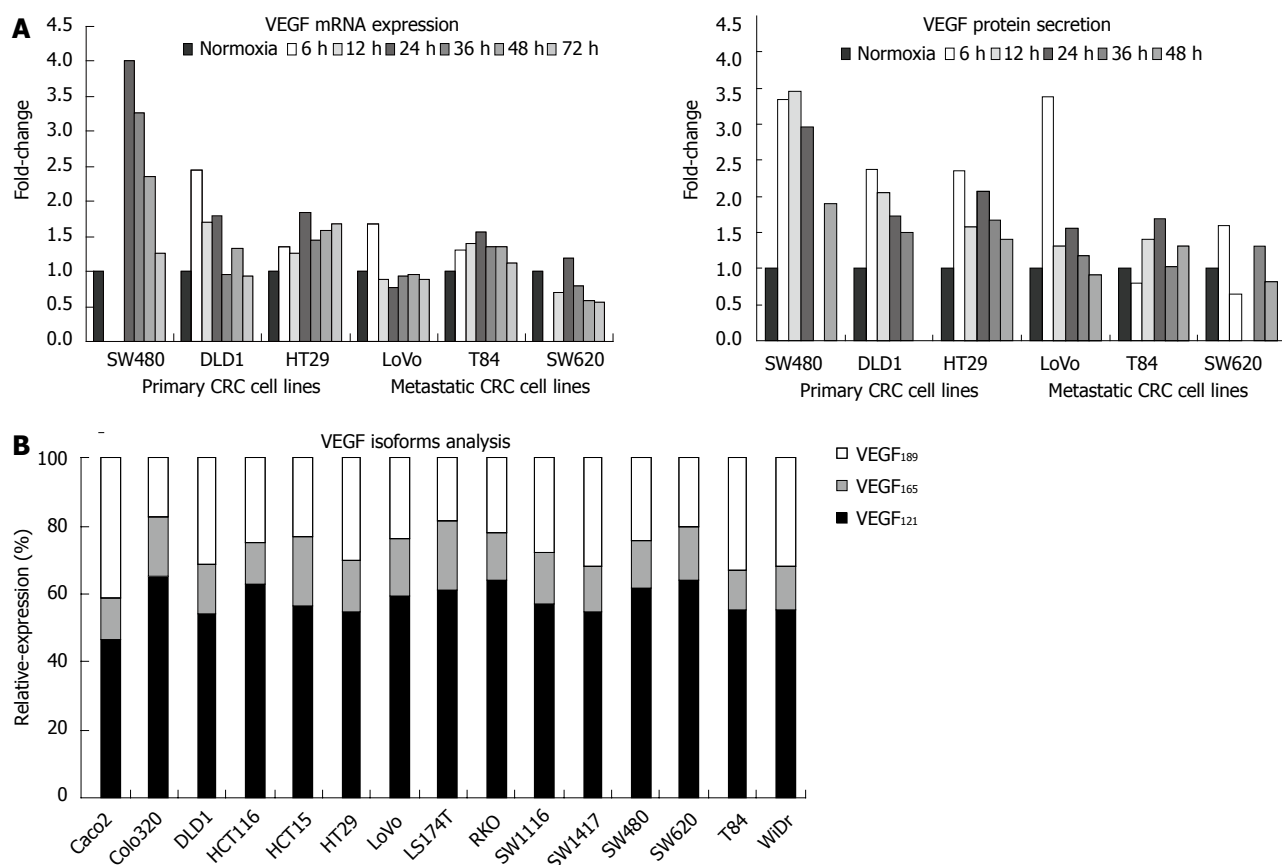


Figure 3 Vascular endothelial growth factor expression regulation. A: Modulation of vascular endothelial growth factor (VEGF) expression (mRNA and protein) in response to severe hypoxia in primary and metastatic colorectal cancer (CRC) cell lines; B: Expression of VEGF isoforms 121, 189 and 165 by CRC cells in normoxia.

Table 3 Association between vascular endothelial growth factor mRNA isoforms and vascular endothelial growth factor protein secretion

	VEGF protein	VEGF ₁₂₁ mRNA	VEGF ₁₆₅ mRNA
VEGF ₁₂₁ mRNA	r = 0.55 P = 0.034		
VEGF ₁₆₅ mRNA	r = 0.67 P = 0.007	r = 0.93 P = 0.000	
VEGF ₁₈₉ mRNA	r = 0.69 P = 0.005	r = 0.95 P = 0.000	r = 0.92 P = 0.000

VEGF: Vascular endothelial growth factor.

respectively). Further, a strong correlation ($r = 0.65$, $P < 0.01$) was detected between VEGF protein (by EIA) and VEGF mRNA expression (Figure 2D) in CRC cell lines, indicative of the major role of transcriptional mechanisms in the regulation of VEGF expression^[23]. A similar correlation was observed in hypoxia between VEGF protein (by EIA) and VEGF mRNA expression (Figure 3A). Severe hypoxia induced different levels of VEGF expression up-regulation depending on the CRC cellular origin. Surprisingly, the fold change normoxia-hypoxia in VEGF expression of metastatic CRC cell lines was ≤ 1.5 in the majority of time points tested, as compared with the > 1.5 -4.0 fold change in primary cell lines for both protein and mRNA VEGF (Figure 3A).

VEGF isoforms have differential angiogenic and tumorigenic activities and their expression pattern may also define the CRC cell angiogenic capacity^[24]. Primary and metastatic CRC cell lines had a similar expression pattern of the three major isoforms VEGF₁₂₁, VEGF₁₆₅ and VEGF₁₈₉, despite variability in VEGF expression (Figure 3B), implying a similar mechanism of regulation. VEGF₁₂₁ was the predominant isoform expressed by CRC cell lines ($58.23\% \pm 5.05\%$ of total VEGF mRNA), as compared to VEGF₁₆₅ and VEGF₁₈₉ ($15.13\% \pm 2.71\%$ and $26.6\% \pm 6.5\%$ of VEGF transcripts, respectively). In line with a previous study on tumor tissue^[25], the expression of the three isoforms was significantly associated with total VEGF protein; $r = 0.55$, $P < 0.05$ for VEGF₁₂₁ and furthermore, VEGF₁₆₅ and VEGF₁₈₉ showed higher correlation ($r = 0.67$ and $r = 0.69$, $P < 0.01$, respectively) (Table 3).

VEGFR expression in colorectal cancer cell lines

While the role of the VEGF/VEGFR pathway in endothelial cells is well characterized, its functionality and expression by tumor cells is still controversial^[13]. Soluble VEGFR-1 was quantified in CRC cell line supernatants at a lower range than VEGF (mean 8.3 and 27.8 pg/mL respectively) and no differences were found according to the cellular origin (7.57 ± 2.12 and 10.67 ± 3.1 , in primary and metastatic CRC cell lines, respectively) (Figure 4A). In

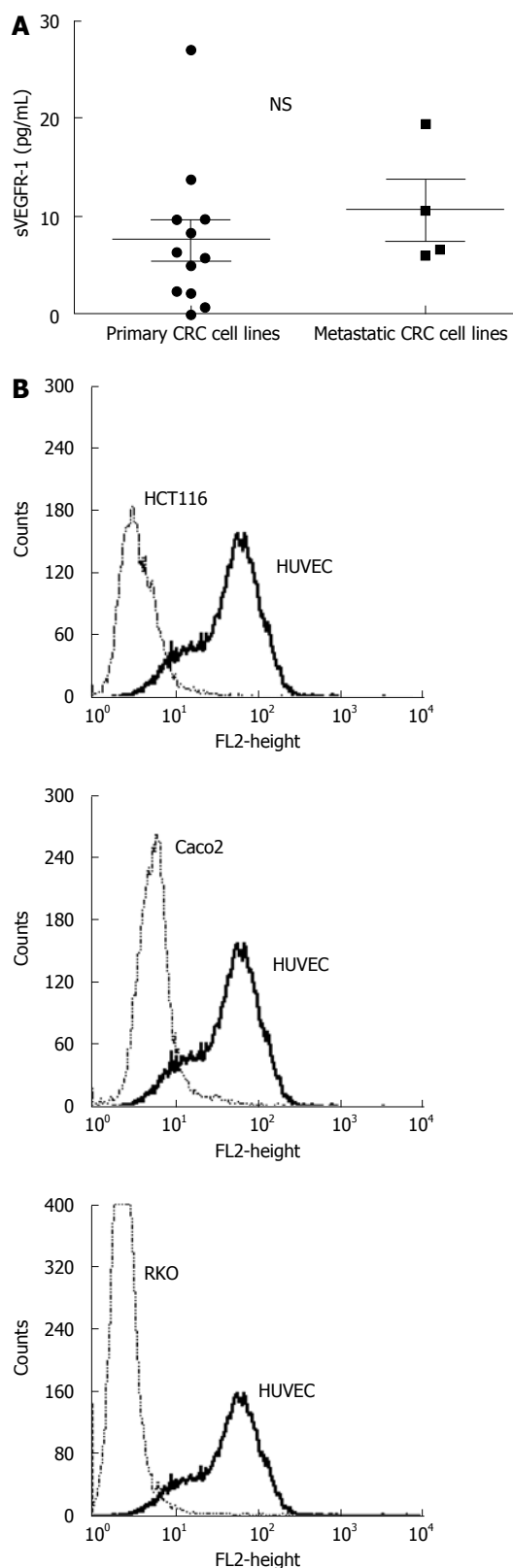


Figure 4 Vascular endothelial growth factor receptors expression in colorectal cancer cell lines. A: Soluble vascular endothelial growth factor receptor (sVEGFR)-1 expression measured by EIA is not significantly different between primary and metastatic colorectal cancer (CRC) cell lines; B: Flow cytometry of the surface expression of vascular endothelial growth factor receptor (VEGFR)-2 in human umbilical vein endothelial cells (HUVEC) and the primary CRC cell lines HCT116, Caco2 and RKO under normoxic conditions reveals a general lack of VEGFR-2 expression on the surface of CRC cells as compared to HUVEC. NS: Not significant.

agreement with other studies^[26], a trend was observed for an inverse correlation between sVEGFR-1 and VEGF expression (data not shown), indicative of the angiogenesis inhibiting role of sVEGFR-1^[13].

In our CRC cell lines panel, the antibody array data showed a lack of expression of sVEGFR-2 (Figure 1A). Given the hypothesis that earlier tumor stages are more dependent on the VEGF/VEGFR signaling pathway^[15], we analyzed surface VEGFR-2 expression in CRC cells of primary origin. Flow cytometry revealed a general lack of surface VEGFR-2 expression in CRC cells of medium to high VEGF expression, as compared to HUVEC cell line (Figure 4B). These findings add to the stock of controversial results to date^[27,28].

DISCUSSION

Identifying the proteins responsible for the different behavior of more advanced CRC tumors seems warranted in order to more effectively use current treatment options. Furthermore, there is a need to characterize definite biomarkers of CRC metastasis to serve as prognostic indicators and novel interventional targets. As derived from our findings in vitro, the tumor microenvironment of CRC metastases would be different to that of primary tumors, because of the effect of the CRC cells secreted factors. Metastatic CRC cell lines are characterized by a greater expression of cytokines majorly involved in metastasis, migration and invasion, while being proven pro-angiogenic effectors. MMP-1 plays an important role in CRC tumor invasion and metastasis^[29] and MMP-9 has proved to be of prognostic value in stage II colon cancer patients, where tumors with higher protein expression had a higher recurrence rate^[30]. The monocyte attractant chemokine I-309 has been shown to stimulate chemotaxis and invasion of endothelial cells and the roles of IL-1 α in colon cancer angiogenesis and of IL-2 in inflammation and apoptosis, seem also consistent with the metastatic phenotype^[18,31,32].

Hypoxia is widely recognized as the major transcription effector for VEGF expression^[9]. However, the greater (two-fold increase) induction of VEGF expression in hypoxia observed in primary CRC as compared to metastatic cell lines is an interesting finding which agrees with recent hypotheses. Tolerance to hypoxia is frequently acquired by tumor cells progressing towards more advanced phenotypes^[15]. Our finding suggests the metastatic CRC molecular phenotype provides some intrinsic resistance to the hypoxic induction of VEGF expression. Some authors have shown that hypoxia would select more malignant metastatic cells, less sensitive to anti-angiogenic treatment^[33], to yield poorer patients outcomes^[34,35]. The community still agrees that angiogenesis is a hallmark of cancer in metastatic stages^[36]. However, given the broad angiogenic network in the tumor microenvironment, research should move in the direction of investigating the mechanisms by which metastatic tumors depend on VEGF, since they seem to be different to those exploited by primary tumors^[15]. Furthermore, with the objective of

individualized care in mCRC, the distinct metastatic “secretome” proteins emerge as alternative targets to consider in the management of advanced disease.

Further to the VEGF expression profile, the pattern of VEGF isoforms represents the next step to identifying intrinsic differences to guide treatment choice. However, the similar expression of VEGF isoforms across cell lines does not offer clarification. Further to this finding, it would be of interest to explore how VEGF transcription factors modulate the ratio of VEGF isoforms as disease progresses, given the changes on VEGF dependence. Interestingly, a novel class of VEGF isoforms, VEGF_{xxx}b, generated through alternative splicing of exon 8, has been recently described^[37]. Studies suggest anti-angiogenic or weak angiogenic properties for these isoforms^[38,39]. Not exempt from controversy, this discovery will help in further defining the role of VEGF/VEGFR signaling in CRC, yet still the testing techniques need refinement in specificity between the two classes.

Emerging data suggest VEGF to be a growth factor also for tumor cells and VEGF/VEGFR signaling to regulate their expression. However, this hypothesis remains unproven until consolidated results on VEGF receptor expression on tumor cells become available^[12,28]. Extensive work has been done on the activity of VEGF/VEGFR-1 signaling in CRC cells showing that it mediates cell motility and invasiveness but not cell proliferation^[13]. While this would involve VEGF/VEGFR-1 in CRC progression and metastatic processes, sVEGFR-1 secretion was not found of significant relevance in metastasis-derived CRC cells. In contrast, not so much is known about the activity of VEGF/VEGFR-2 in cancer cells. Reports suggest an involvement in the sensitivity of CRC cells to inhibition of VEGF-related survival pathways^[40]. However, controversial results on the VEGFR-2 expression on tumor cells to date^[27,28], to which our results add, do not help to resolve this question. Definite confirmation of the expression and functionality of this pathway is necessary in order to shed more light on the mechanism of action of anti-VEGF therapies^[40].

Consistent with the key role of VEGF in the “angiogenic switch” and the hypoxia-resistance mechanisms in metastatic stages, CRC cell dependence on VEGF in more advanced settings seems attenuated in favor of other cytokines in the progression of metastasis. Further investigation of these findings and testing the significance of the distinct “secretome” of CRC metastases at the clinic side seems warranted given the implications for patient outcomes.

ACKNOWLEDGMENTS

We thank Dr. Paul Miller for English editorial work.

COMMENTS

Background

Identifying the proteins responsible for the different behavior of more advanced colorectal cancer is necessary in order to more effectively use current treatment

options. The progressive growth of colon cancer depends on the blood vessels (angiogenesis) network within the tumor. Therapies targeting angiogenesis have emerged in the field; however, variances in the magnitude benefit lead to great amount of research to explain inter-individual differences. It is thought that different proteins or biomarkers in the tumor microenvironment are responsible for these facts.

Research frontiers

The lack in understanding of biomarkers of colorectal cancer metastasis led the authors to set up this work. Using a novel cytokine antibody array technique, this work identifies the differences in angiogenesis-related protein expression of colorectal cancer cell lines of primary and metastatic origin. This is the first step prior to translation into a clinic setting, where these differences are to be corroborated in patients with colorectal cancer.

Innovations and breakthroughs

The distinct profile of metastatic cell lines comprises eight proteins with different cellular properties, including favoring the growth of those tumor blood vessels. Interestingly, the classical angiogenesis marker vascular endothelial growth factor is not in such a profile, indicating that tumors in more advanced phases tend to rely on different mechanisms for their growth.

Applications

The findings of this work show that a number of markers might be of value when determining the course of disease in colorectal cancer. Furthermore, these proteins arise as novel intervention targets in the metastatic colorectal cancer setting.

Peer review

The researchers intent was to investigate the angiogenesis-related protein expression profile characterizing metastatic colorectal cancer with the aim of identifying prognostic markers. The subject of biomarkers of colorectal cancer (CRC) metastasis is not well understood up to this time. Because of that, efforts of authors to characterize the protein factors behind the angiogenic potential of CRC cell lines of metastatic origin is of great importance. This work is a next step forward to identify the proteins responsible for the different behavior of metastatic colorectal cancers and for developing new treatment options.

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S- Editor Lv S L- Editor O'Neill M E- Editor Zhang DN

Abajo A, Rodriguez J, Garcia-Foncillas J, et al. Dose-finding study and pharmacogenomic analysis of fixed-rate infusion of gemcitabine, irinotecan and bevacizumab in pretreated metastatic colorectal cancer patients. *British Journal Of Cancer* [serial online]. November 9, 2010;103(10):1529-1535 Doi:10.1038/sj.bjc.6605908

IDENTIFICATION OF PREDICTIVE CIRCULATING
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CANCER PATIENTS

**A Abajo, V Boni, I Lopez, M Gonzalez-Huarriz, J Rodriguez, R
Zarate, E Bandres, J Garcia-Foncillas**

Short communication submitted

Short communication

Identification of predictive circulating biomarkers of bevacizumab efficacy in pretreated metastatic colorectal cancer patients.

A Abajo¹, V Boni^{1, 2}, I Lopez¹, M Gonzalez-Huarriz¹, J Rodriguez², R Zarate¹, E Bandres^{2*}, J Garcia-Foncillas^{1, 2*}.

¹ Laboratory of Pharmacogenomics, Division of Oncology, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain; ²Department of Oncology, University Clinic of Navarra, University of Navarra, Pamplona, Spain.

*Correspondence: E Bandres and J Garcia-Foncillas;

E-mail: ebandres@hotmail.es or jgfoncillas@fjd.es

Full postal address: Laboratory of Pharmacogenomics, Division of Oncology, Center for Applied Medical Research (CIMA), Avda Pio XII 55, 31008 Pamplona, Spain

Running title: Predictive circulating markers of bevacizumab efficacy in colorectal cancer

Keywords: Bevacizumab, serum biomarkers, colorectal cancer, multiplex-bead assays

ABSTRACT

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 twenty-four cytokines were measured using a multiplex bead assay (MBA) in thirty-two pretreated mCRC patients treated with irinotecan plus bevacizumab-based salvage therapy. MDC (macrophage-derived chemokine), interleukins 8 and 6 levels were also validated by enzyme-linked immunosorbent assay (EIA) at different time points during therapy.

Results

Higher 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 EIA 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 regimens.

INTRODUCTION

Anti-VEGF therapies have proven efficacy in metastatic colorectal cancer (mCRC) ¹⁻³, although varying degrees of benefit in patients outcome have been reported ^{4,5}. Several mechanisms of resistance to the angiogenesis blockade, either by target mutation or by activation of alternative pathways ⁶⁻⁸ have been described. Nevertheless, further insight into the biological mechanisms responsible for the observed differences in outcome seem warranted ⁹.

Serum circulating cytokines, growth factors and angiogenesis-related molecules, are hypothesized to be valid markers of the tumour microenvironment angiogenic profile ¹⁰, and may offer prognostic and predictive information beyond conventional clinico-pathological indicators ¹⁰. Extensive research suggests the prognostic value of angiogenesis-related factors for tumour stage ^{11,12} and plasma concentrations of some of these molecules are markedly increased in mCRC patients compared to healthy individuals ¹³. 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 ¹⁴. The intrinsic complexity of the tumour microenvironment requires a parallel, miniaturized device technology to be applied to proteins and their biochemical pathways ¹⁵. Prior studies have reported promising results with the use of multiplex bead assays as an alternative to simultaneously detect the expression of multiple cytokines in minute amounts of sample ¹⁵.

On this basis, in the present study we performed a MBA-based exploratory analysis of twenty-four serum cytokines from mCRC patients treated with a bevacizumab-containing regimen. Levels of five serum cytokines at baseline were identified as individually associated with objective response to treatment. An even stronger association was observed when combined together into a *risk signature*. Although no association was found when cytokines levels were considered separately, the *risk signature* significantly correlated with both time to progression and overall survival.

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 and with available baseline serum samples were analysed. Patients' characteristics, staging work-up, clinical management, treatment administration and long-term outcomes have been reported elsewhere ¹⁶. Briefly, the median age of the patients was 58 years (range, 43 to 77), and 26% were female. Overall response rate was 46.9%, with a disease control rate of 50.0%. Median TTP and OS were 5.2 (95% CI: 3.6–6.7) and 10.4 months (95% CI: 4.1–16.7), respectively. No differences were noted between this subset of patients and the whole cohort.

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°C until analysis. Twenty-four angiogenic growth factors and cytokines (Supplementary Table 1) were measured using multiplex bead suspension bead arrays (Millipore; Bedford, MA); each sample was analysed in duplicate following manufacturer's instructions. EIA of MDC, IL-8 and IL-6 (R&D Systems, Minneapolis, MN) were analysed as per manufacturers' directions.

Statistical methods

The relationship between continuous variables was assessed by non-parametric Spearman correlation. Association between angiogenesis-related cytokines levels and patients clinical outcomes was assessed using the Mann-Whitney U test. Clinical outcomes refers to ORR and DCR (disease control rate; CR, PR and SD lasting >6 months). Since determination of an optimal cut-off 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 summarized by Kaplan-Meier methods and compared using log-

rank or Breslow tests. Differences between baseline and on-treatment cytokines' levels were assessed using Wilcoxon tests. All P values are two sided.

RESULTS

Baseline (pretreatment) serum cytokines and growth factors levels

Baseline levels of 24 angiogenesis-related molecules, inflammatory cytokines and growth factors were first analysed by a MBA. This technique allows the simultaneous screening of multiple angiogenesis related molecules using a small serum volume through an antibody suspension bead arrays system. These baseline levels are shown in Supplementary Table 1. A subset of proangiogenic cytokines with known roles in the inflammatory responses within tumour stroma was found significantly associated with ORR and DCR outcomes. Higher baseline levels of EGF and MDC were seen in patients responding to therapy compared to non-responders ($p<0.05$). As shown in Figures 1 A and B, higher median EGF and MDC levels (282.8 vs. 138.9 pg/mL and 838.6 vs. 696.9 pg/mL, respectively) were observed in patients who achieved clinical benefit from bevacizumab-based therapy. In addition, lower levels of IL-10 (median 0.0 vs. 35.7 pg/mL), IL-6 (median 0.0 vs. 37.1 pg/mL), and IL-8 (median 30.3 vs. 33.6 pg/mL), all of them chemoattractant for inflammatory cells into the tumour stroma, were observed in responding patients (Figure 1C-E).

A statistically significant correlation was found between these interleukins ($p<0.01$), 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 2).

We next assessed whether the combination of the five differentially expressed at baseline serum-factors would further increase the predictive ability of each individual factor (Supplementary Table 1). 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 to 22% in those with ≥ 3 high-risk factors ($p<0.001$). Finally, median TTP (8.1 vs. 2.8, $p<0.05$,

Breslow 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 EIA

To validate the MBA results, the levels of IL-6, IL-8 and MDC were analysed by EIA. As previously reported¹⁷, MBA detection levels were 1.5-2-fold higher than those attained by EIA. In fact, only eight out of thirty-two samples had detectable levels of IL-8 by EIA (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 EIA data for the three analysed cytokines: IL-8 ($r_{\text{Spearman}} 0.67$, $P < 0.01$), IL-6 ($r_{\text{Spearman}} 0.67$, $P < 0.01$) and MDC ($r_{\text{Spearman}} 0.53$, $P < 0.01$). In agreement with the MBA results, when IL-6 and/or IL-8 EIA-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 EIA-measured MDC levels compared to the low MDC group (66.7 vs. 26.7%, $p < 0.05$).

Treatment modulation of serum markers

Monitoring the effect of treatment by analysing dynamic changes in circulating factors has become of great interest to understand treatment failure^{18,19}. We measured cytokines' levels at different time points in order to evaluate the treatment modulation. Measurement of cytokines concentrations after between two and five treatment cycles compared to baseline levels revealed that exposure to bevacizumab-based therapy correlated with a global decrease in the proinflammatory and proangiogenic IL-8 ($p < 0.05$). Given the low detection ability of IL-8 levels by EIA, only six patients are analysed having baseline-detectable samples, with the proportion 5 non-responders/1 responding patient (Figure 3A). In addition, exposure to treatment revealed an increase in the chemokine MDC ($p < 0.05$) (Figure 3B). No relevant changes were observed for IL-6.

DISCUSSION

The addition of bevacizumab to cytotoxic chemotherapy has demonstrated a survival benefit in the first and second line treatment of mCRC, although no impact on response rate has consistently been demonstrated. Furthermore, the identification of biomarkers that may influence response to antiangiogenic therapy is of considerable interest. In recent years intensive research efforts have been aimed to a more efficient use of available therapeutic options through the identification of differentially expressed molecular profiles by means of simple and reliable screening tests^{11,20}. For this reason, the implementation of non-invasive techniques is appealing. In the present work, we have simultaneously analysed a subset of circulating cytokines with proangiogenic and inflammatory functions within the tumour microenvironment, and a known prognostic value in mCRC patients^{21,22} by a MBA technique, with results being subsequently validated by EIA.

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. Macrophage-derived chemokine 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 MDC²³. According to our results, high baseline levels of MDC in 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 growth²⁴. Moreover, an MDC increased gene expression in tumour tissue turned out to be a favourable prognostic factor in lung cancer²⁵. MDC concentration strongly

correlates with the frequency of FOXP3-positive cells²⁶. A high density of intratumour FOXP3-positive T regulatory cells has been associated with poor outcomes in a wide variety of solid tumours^{27,28}. However, an opposite effect has been observed in colorectal cancer, with intratumour regulatory T cells being associated with improved prognosis^{29,30}. 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^{21,22}. IL-8 has been reported to mediate angiogenesis by stimulating endothelial cell proliferation in response to hypoxia^{31,32}, and escape to antiangiogenic therapy has been correlated with increased secretion of IL-8³³. Furthermore, the predictive role of low baseline IL-8 levels and their bevacizumab-induced decrease are in agreement with recently reported clinical data¹⁹.

Given the biological complexity of tumour angiogenesis our results should be viewed with caution. Although there is a biological rationale to support the present observations, the data are exploratory and independent and prospective validation is required, since it is plausible that other inflammatory mediators may arise^{34,35} as 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.

FIGURE LEGENDS

Figure 1: MBA measured cytokine levels at baseline according to ORR clinical outcome.

EGF (A) and MDC (B) are higher in responders to treatment and IL-10 (C), IL-6 (D) and IL-8 (E) are lower in responders to treatment.

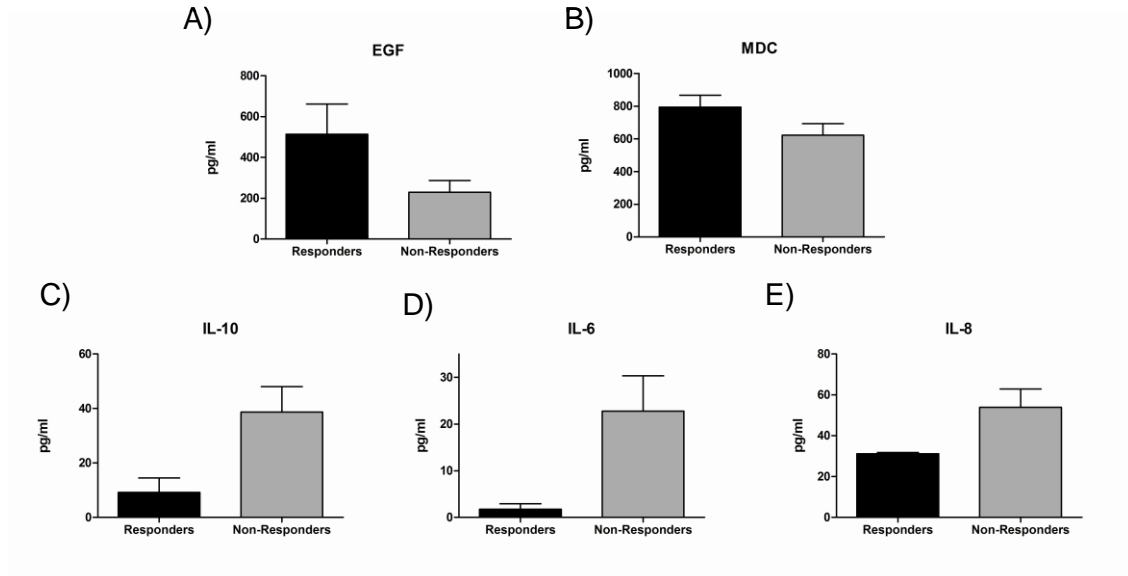


Figure 2: Kaplan-Meier plots. (A) Time to progression and (B) overall survival according to the “high-risk signature” calculated by baseline levels of EGF, MDC, IL-10, IL-6 and IL-8.

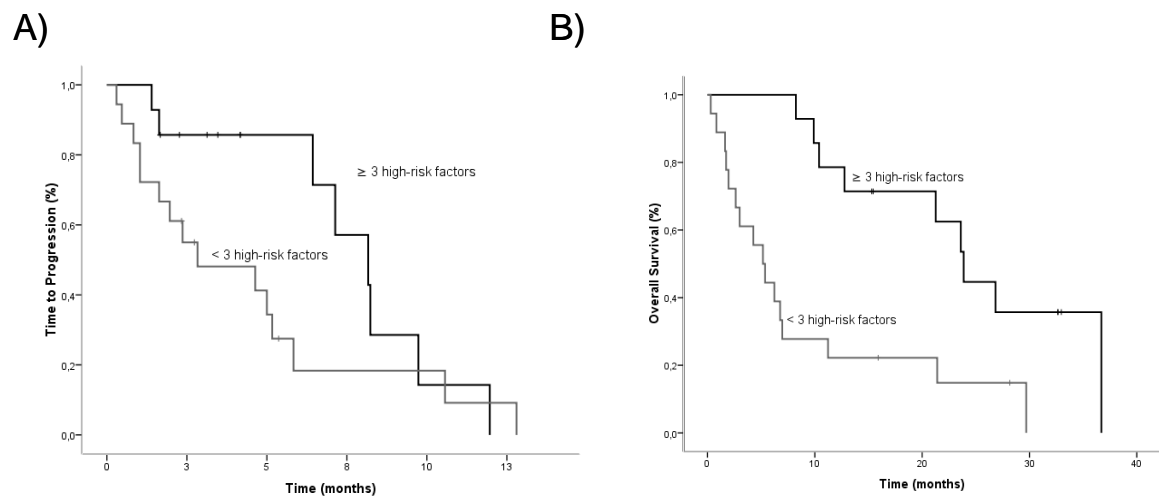
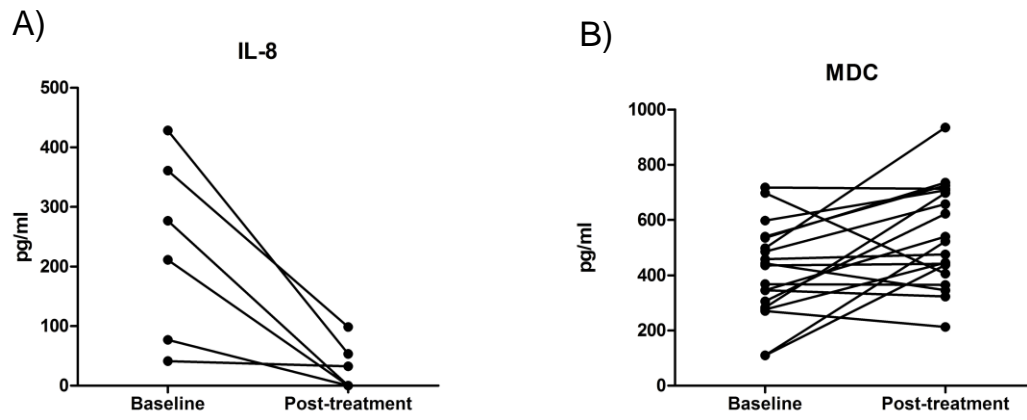


Figure 3: Treatment modulation of cytokines. (A) IL-8 is reduced with exposure to treatment
(B) MDC increases with exposure to treatment



TABLES

Supplementary Table 1: Level of angiogenic factors and cytokines measured at baseline by MBA.

Function	Sample	Mean (pg/mL)	Median (pg/mL)
Proangiogenic	EGF	362.5	226.2
	FLT-3L	60.2	53.2
	GRO- α	681.0	435.7
	TNF- α	35.6	37.5
	VEGF	449.2	355.9
	IL-6	23.5	36.5
	IL-8	43.3	33.0
	MDC	703.3	740.8
Antiangiogenic	IP10	421.9	295.9
Hematopoietic growth factors	G-CSF	73.5	64.2
Other interleukins	IL-10	24.8	0.0
	IL-12P40	16.0	0.0
	IL-12P70	34.1	26.3
	IL-16	90.1	75.7
Monocyte chemotaxis	MCP-1	487.3	212.2
	MCP-4	122.9	112.7
	MIP1 α	40.0	44.9
	MIP1 β	85.2	84.0
	CTACK	914.3	947.9
	ENA-78	756.8	656.8
	EOTAXIN	54.1	50.6
	EOTAXIN-2	2345.5	1418.9
	SDF-1 α + β	4608.4	4400.3
TARC	139.0	113.5	

Abbreviations: CTACK, cutaneous T-cell attracting chemokine (CCL 27); G-CSF, granulocyte colony stimulating factor; GRO- α , growth regulated oncogene alpha (CXCL1); EGF, epithelial growth factor; TNF, tumour necrosis factor; IL, interleukin; IP-10, IFN-gamma-induced protein-10; MCP, macrophage chemoattractant protein; MIP, macrophage inflammatory protein; VEGF, vascular endothelial growth factor; SDF, stromal-derived factor (CXCL12); ENA78, epithelial cell-derived neutrophil-activating peptide 78; MDC, Macrophage-Derived Chemokine; FLT3L, FMS-like tyrosine kinase 3 ligand; TARC, thymus and activation-regulated chemokine (CCL17)

Supplementary Table 2: Correlation between levels of cytokines detected by MBA and significantly associated with response outcome measures.

r Spearman (p)	IL-6	IL-8	MDC
IL-8	0.67 (p<0.01)		
MDC	-0.24 (p=0.18)	-0.56 (p<0.01)	
IL-10	0.56 (p<0.01)	0.5 (p<0.01)	-0.4 (p<0.01)

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DISCUSSION

In an attempt to add to the body of knowledge in the framework of personalized medicine in antiangiogenesis, in this multi-step project we took a translational research approach¹⁷¹ to explore the angiogenic molecular signature of advanced CRC patients. Identifying the proteins responsible for the different behaviour of more advanced CRC tumours seems warranted in order to more effectively use current treatment options. Therefore, there is a need to characterize definite biomarkers of mCRC to serve as prognostic and predictive indicators and furthermore, identify novel interventional targets. Using pharmacogenomics methodology, we hypothesized that the identification of predictive biomarkers of response or resistance to an anti-VEGF bevacizumab-based combination therapy would allow a more informed therapeutic decision for patients with mCRC, investigating the clinical and the molecular implications.

1. PHARMACOGENOMIC ANALYSES: TECHNIQUES USED

One of the major technical difficulties in biomarkers research arises with the selection of techniques to screen patients. Aiming incorporation into clinical practice and being aware of previous works' conclusions performed after surgical tumour resection, one of the drivers of our pharmacogenomic analysis was the convenience of the sampling method. Most commonly, methods used to measure tumour angiogenesis are microvessel density counts, immuno-staining and RT-PCR for angiogenic cytokines¹²⁵.

Tumour angiogenesis can also be assessed by non-invasive methods, which represent an advantage towards implementation into screening protocols in routine patients' care¹²⁹. Imaging studies and pharmacogenomic studies of circulating angiogenic cytokines and growth factors are more convenient methods and likely to offer prognostic and predictive information beyond conventional clinico-pathological indicators¹³¹.

Studies of cytokines expression and gene polymorphisms measured in peripheral blood reduce patient's burden while being less expensive, as compared to novel imaging methods. The non-invasive approach and methodology used in our study is valuable also for monitoring the effect of treatment on dynamic changes in circulating factors, which has become of great importance in order to understand treatment failure and therapeutic resistance^{67, 134}.

The circulating molecular signature approach followed in this study presents the advantages of being more precise, requiring non-invasive sampling and a less time-consuming measurement, compared to the evaluation in tumour tissue¹²³. Yet, the

debate of whether to use plasma or serum samples for the measurement of circulating angiogenic factors seems still unsolved according to the varied results reported. We considered a technical aspect in order to decide in favour of testing serum samples. Since the VEGF plasma levels are closer to the lower limits of detection of the currently available EIA assay, they provide lower sensitivity than the serum ones ¹³².

The circulating molecular signature approach yet carries the difficulty of accurately determining the role of tumour-secreted circulating factors, since both host and tumour cells are producing pro and antiangiogenic factors. On the other hand, such rationale is contravened by the fact that tumours are believed to be a two compartment system in which tumour cells and endothelial cells co-exist, mutually promoting growth and survival ¹⁶. If this was true thus, the circulating molecular signature would, in theory, reflect the tumour (and host) expression, and consequently circulating angiogenic factors would be valid and clinically relevant indicators.

In addition, when trying to proof valid a set of *in vitro* findings by moving to explore the molecular implications for the use of a certain intervention in patients, the selection of the methodology to translate the results is crucial ¹²³. Several techniques and different methodologies were applied because they were considered, after literature review, the most relevant and reliable ones. Some were already consolidated as standard and inexpensive (EIA, pharmacogenomic analysis of *VEGF* SNPs) approaches, and others were starting to become widely accessible (cytokine protein arrays, multiplex-bead assays) at the time this study was initiated ¹³⁵.

Still nowadays EIA is the most common method used to measure cytokine expression levels due to its high specificity and sensitivity ¹⁷². However, current research requires of more advanced and faster tools to simultaneously measure multiple cytokines. Cytokine antibody arrays and multiplex-bead assays represent an alternative to simultaneously detect the expression of multiple cytokines in minute amounts of sample in a single experiment ¹³⁵.

Certainly, technical difficulties also arise with novel and rapidly marketed methods, which are to be used with vigilance. The multiplexed tools are generally used as a screening to identify a number of candidate markers with potential for later validation by an independent method. Cytokine-antibody arrays have been referred to as a form of *low-cost proteomics*; however, the lower detection sensitivity as compared to EIA is a major limitation ¹⁷³. Great attention must be paid to cut-off levels and the low expression cytokines if close to the background levels, when working with this technique. In addition, the normalization step must be managed carefully ¹³⁵. In all our experiments, normalization of the cytokine-antibody array data was performed following manufacturer's recommendation.

With the results derived from cytokine-antibody arrays data at the *in vitro* setting, which were validated by EIA, the approach to be used in the patients' setting was shifted to multiplex-bead assay, due to its various advantages. The use of multiplex-bead assay has been reported already with promising results, emerging as the alternative to

cytokine-antibody arrays, due to higher reliability^{134, 174}. Further, the normalization step required with cytokine antibody arrays is not necessary with the multiplex-bead assay. The standard controls are used to plot a standard curve that allows a quantitative and not semi quantitative calculation of cytokine concentrations, similarly to the EIA methodology.

Multiplex-bead assays seem to be widely accepted as the next generation of screening methods in pharmacogenomic studies, with already some robust results reported, including the only study to date that has shown alternate proangiogenic cytokines in plasma to be modulated by a bevacizumab-containing regimen¹³⁴. In fact, multiplex-bead assays will probably not require of the validation step with EIA once they start to be more widely used. As referred in some reports¹⁷⁵, its detection levels are somewhat higher than EIA ones. Our results agree with this, with an almost twice-higher detection level by multiplex-bead assay using Luminex[®]¹³⁶, where in some cases cytokines levels detected by multiplex-bead assay were not detected by EIA. Given the differences inherent to the two techniques, the reliability of the results we report for the molecular signature in serum of mCRC patients, is based on the statistically significant correlation between both techniques.

2. BIOMARKERS IDENTIFICATION *IN VITRO*

In the era of translational research, the prediction of outcomes in the clinical setting is tested in the preclinical one, aiming an earlier source of conclusions¹. Firstly, we hypothesized that the variability in mCRC patients' outcomes would come from a heterogeneous angiogenic potential of tumours and that CRC cell lines could serve as a study model. As derived from our findings *in vitro*, the tumour microenvironment of CRC metastases would be different to that of primary tumours. Metastatic CRC cell lines are characterized by a greater expression of cytokines majorly involved in metastasis, migration and invasion, which are proven pro-angiogenic effectors. Of those, MMP-1 plays an important role in CRC tumour invasion and metastasis³⁶ and MMP-9 has proved to be of prognostic value in stage II colon cancer patients, where tumours with higher protein expression had a higher recurrence rate¹⁷⁶. The monocyte attractant chemokine I-309 has been shown to stimulate chemotaxis and invasion of endothelial cells and the roles of IL-1 α in colon cancer angiogenesis and of IL-2 in inflammation and apoptosis, seem also consistent with the metastatic phenotype^{37, 38, 44}.

In addition, we evaluated how the major proangiogenic effector, hypoxia, would be a differential factor depending on the metastatic or primary phenotype. It is known that tolerance to hypoxia is frequently acquired by tumour cells progressing towards more advanced phenotypes¹⁷⁷ and the greater induction of VEGF expression under hypoxia observed in our primary CRC lines as compared to metastatic cell lines agrees with these recent hypotheses. The mCRC molecular phenotype with a more aggressive phenotype and tumorigenic potential provides some intrinsic resistance to the hypoxic

induction of *VEGF* expression. Some authors have shown that hypoxia would select more malignant metastatic cells, less sensitive to anti-angiogenic treatment¹⁷⁸, to yield poorer patients' outcomes^{94, 95}. Research needs to move into the direction of investigating the mechanisms by which metastatic tumours depend on *VEGF*, since they seem to be different to those exploited by primary tumours¹⁷⁷.

Furthermore, it would be of interest to explore how *VEGF* transcription factors modulate the ratio of *VEGF* isoforms as disease progresses, given the changes on *VEGF* dependence. With the objective of individualized care in mCRC, the distinct metastatic "secretome" proteins emerge as alternative targets to consider in the management of advanced disease. Interestingly, a novel class of *VEGF* isoforms, *VEGF*_{xxx}*b*, generated through alternative splicing of exon 8, has been recently described¹⁷⁹. Studies suggest anti-angiogenic or weak angiogenic properties for these isoforms^{180, 181}. The balance between pro- and antiangiogenic *VEGF*-A isoforms may have implications for therapy, after an initial report showing *VEGF* 165 b to bind and inhibit bevacizumab treatment in an experimental colorectal carcinoma model¹⁸².

The findings *in vitro* showed that factors other than *VEGF* come to scene as potential prognostic markers and intervention targets in the mCRC setting. Furthermore, reports suggest *VEGF*/*VEGFR*-2 to be involved in the sensitivity of CRC cells to inhibition of *VEGF*-related survival pathways¹⁸³. *VEGF* has been shown to induce expression of the anti-apoptotic *Bcl*-2 and directly inhibit apoptosis in breast cancer cell lines whereas *VEGF* blocking decreased *Bcl*-2 expression eliciting pro-apoptotic effects⁶⁸. Anti-*VEGF* therapies could therefore increase tumour cell apoptosis by removing the protection that *VEGF* confers. However, the controversial results on the *VEGFR*-2 expression on tumour cells^{184, 185}, to which our *in vitro* results add, do not help to solve this question.

A definitive confirmation functionality of this pathway is necessary in order to shed light on the mechanism of action of anti-*VEGF* therapies¹⁸³. On this basis, next step in the project was to test whether or not the differential angiogenic niche behind earlier and more advanced disease settings might drive inter-individual differences in mCRC patients' outcomes to the antiangiogenic treatment regimen tested^{73, 74}.

3. BIOMARKERS IDENTIFICATION IN THE CLINICAL SETTING

In the set-up of this translational research project, the applicability was carefully considered at the level of the target population as well as at the unfilled gap for biomarkers research in anti-angiogenesis. The clinical study design is decisive when setting up translational research projects¹²³. The selection of the target population was based on the unmet medical need of the mCRC patients subset requiring further treatment after being managed with sequential standard regimens⁸⁹. In a heterogeneous study population, considering the previous systemic therapies and the high percentage of patients bearing a resistant and refractory disease, the clinical

outcomes are sufficiently robust to open this salvage treatment option as an alternative to other irinotecan-based regimens^{91, 186} in the mCRC setting resistant to standard chemotherapy backbones.

On the other hand, a subset of patients in this study did not reach stabilization of disease. A better comprehension of angiogenesis regulation is necessary to guide the use of therapeutic interventions targeting the VEGF/VEGFR signalling pathway. Anti-VEGF agents have not consistently produced remarkable survival benefit and emerging data support different mechanisms of resistance^{69, 70}. The evaluation of compensatory pathways other than VEGF/VEGFR interacting in the tumour microenvironment is warranted in order to design effective pharmacological strategies^{73, 74}.

With the referred exceptions, to date, most of the tested biomarkers proposed from preclinical studies have failed to discriminate risk profiles and also clinical outcomes in patients^{145, 146}. The contradictory results achieved in the studies measuring soluble angiogenesis factors for predicting the prognosis of the patients with CRC^{122, 155, 159, 187}, including baseline VEGF circulating levels¹³⁴, implies that the biological heterogeneity of tumours requires a multifaceted approach. Such comprehensive approach was followed in this project, evaluating the circulating molecular signature and analysing VEGF gene SNPs associated with variability in VEGF expression⁴⁸.

In our mCRC cohort, the platelet-normalized serum circulating VEGF baseline levels were significantly lower in VEGF-2578AA and VEGF-460CC carriers. Findings which seemed consistent with *in vitro* reports that have linked the VEGF-2578AA genotype with a decreased VEGF secretion in peripheral blood mononuclear cells¹⁸⁸ and a lower immunohistochemical VEGF expression in cancer specimens¹³⁰. Moving forward in the investigation, the biological and clinical significance was to be identified, and those low-VEGF levels-associated SNPs were found to correlate with a better clinical outcome in terms of TTP. This seemed consistent with data associating VEGF-2578CC genotype with inferior median OS compared to alternative genotypes in mCRC patients treated with irinotecan-based chemotherapy and bevacizumab in the first-line setting¹⁷⁰. Furthermore, the subset of patients in the cohort showing low baseline VEGF levels presented longer survival times, similarly to studies showing an increase in VEGF levels with advanced disease stage, for both serum and plasma paired analysis¹³³.

The predictive-risk score calculated considering the Köhne low-risk category and achievement of DCR as the favourable clinical factors and any favourable VEGF genotype as the molecular indicator was believed to be relevant, given the weight of both clinical and molecular or biological markers. While Köhne low and intermediate risk patients were more likely to have low VEGF baseline levels compared to the high-risk group, which comprises a potential confounding interaction between these variables, this finding would also suggest a potential link between the clinical and molecular indicator.

The pharmacogenomic evaluation was completed by analysing the circulating molecular signature in serum. This final step was going to test the significance of factors other than

VEGF in the clinical setting for their potential as markers of response to the bevacizumab-containing regimen. With this aim, we simultaneously analysed twenty-four cytokines with known proangiogenic and inflammatory functions within the tumour microenvironment, which have been reported to be potential prognostic factors for mCRC^{39, 174}, in a subset of thirty-two patients (for which a baseline sample was available) of the mCRC cohort.

Our findings suggest that in this subset of patients, a baseline circulating molecular signature correlated with clinical outcome. Given the intrinsic connection between inflammation, angiogenesis and cancer, accepted now as the seventh hallmark of cancer^{189, 190}, it is not surprising that other inflammatory mediators arise as potential predictive markers for antiangiogenics' outcomes. 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 summing-up the number of factors below and above the median for EGF and MDC, and IL-10, IL-6 and IL-8 significantly correlated with progression and survival outcomes, improving single factor's predictive ability. A DCR of 86% was achieved among patients with <3 high-risk factors compared to 22% in those with ≥ 3 high-risk factors ($p < 0.001$). Finally, median TTP (8.1 vs. 2.8, $p < 0.05$, Breslow test) and median OS (23.8 vs. 5.1, $P < 0.01$, log-rank test) were significantly longer in patients with <3 high-risk factors.

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. Macrophage-derived chemokine (MDC) attracts and activates a variety of cell types and enhances the immune response. Dendritic cells and IL-2-activated natural killer cells have demonstrated chemotactic response to MDC¹⁹¹. According to our results, high baseline levels of MDC in 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 growth¹⁹². Moreover, a MDC increased gene expression in tumour tissue turned out to be a favourable prognostic factor in lung cancer⁴². MDC concentration strongly correlates with the frequency of FOXP3-positive cells¹⁹³. A high density of intratumour FOXP3-positive T regulatory cells has been associated with poor outcomes in a wide variety of solid tumours^{194, 195}. However, an opposite effect has been observed in colorectal cancer, with intratumour regulatory T cells being associated with improved prognosis^{196, 197}. Subsequently, whether MDC has a prognostic or a predictive value for mCRC patients' outcomes deserves further research.

The protective effect suggested for MDC from all reported findings seems justified by the fact that this chemokine raises its levels by exposure to chemotherapy and bevacizumab. Furthermore, other well-known chemoattractant chemokines like MCPs or SDF were not found associated with outcomes in our study, as opposed to other reports¹⁹⁸, where we would reflect on the limited sample size.

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^{39, 174}. In addition, the dynamic analysis revealed that IL-8 is also modulated by bevacizumab and chemotherapy. Given the low detection ability of IL-8 levels by EIA, only six patients are analysed having baseline-detectable samples, with the proportion 5 non-responders/1 responding patient, which prevents from finding any differences associated with response outcome. Anti-VEGF therapies are known to revert immunosuppression, suppressing certain features of the tumour associated immune system, by inhibition of VEGF effects on dendritic cell maturation and various other immunosuppressive networks^{28, 61}. However, VEGF might not be the only target to revert immunosuppression, given the crosstalk between different signalling pathways. IL-8 has been reported to mediate angiogenesis by stimulating endothelial cell proliferation in response to hypoxia^{199, 200}, and escape to antiangiogenic therapy has been correlated with increased secretion of IL-8²⁰¹. Furthermore, the predictive role of low baseline IL-8 levels and their bevacizumab-induced decrease are in agreement with recently reported clinical data, using the same MBA methodology for cytokine measurement¹³⁴.

The limited sample size of this work is another difficulty to consider. Most published studies are still not large enough to draw sufficiently valid conclusions and all state an exploratory nature. Thus, the clinical significance of these findings remains inconclusive since the limited availability of comprehensive sets of validated data from large clinical studies^{138-141, 202}. In order to reach not only valid, but also validated results, research should be done in a cooperative manner in order to count with larger patients' subsets where clinical studies are not restricted by the difficulties to obtain a sufficient number of samples.

The markers identified as the molecular signature influencing our mCRC patients' outcomes had been previously reported as being prognostic, rather than predictive, for colon cancer progression and survival¹⁷⁴. However, the non-comparative study design does not allow a definitive answer to this question. Similarly, it does not allow a distinction between bevacizumab- and chemotherapy-induced cytokine changes. Furthermore, it is plausible that other cytokines and growth factors may arise as potential predictive markers of outcomes to antiangiogenic agents.

In conclusion, the present study suggests that pharmacogenomic analyses are valid tools to become readily available at the bedside for patients' screening; however, they are to be used and implemented in patients care with vigilance. There is a biological rationale to support the results presented in this study and the predictive value of the molecular signature identified is supported by the statistical and clinical significance of the observations discussed. However, the results are exploratory in nature and the hypotheses generated require prospective and independent evaluation in larger prospectively designed clinical trials.

Considering the current drug development processes, commercial interests and Regulatory Authorities driven constraints, the quest for biomarkers of angiogenesis and their successful use in the development of effective antiangiogenic therapies are challenges in clinical oncology and translational cancer research likely to remain a subject of significant clinical research in the forthcoming years.

CONCLUSIONS

1. A distinct angiogenesis-related expression pattern characterizes metastatic CRC cell lines. Metastatic CRC cell lines are characterized by higher expression of Ang-2, MCP-3, MCP-4, MMP-1 and the chemokines I-TAC, I-309, IL-2 and IL-1 α , as compared to primary tumour cell lines. Factors other than VEGF appear as prognostic markers and intervention targets in the metastatic CRC setting.
2. VEGF expression does not significantly differ according to the CRC cellular origin in normoxia. Severe hypoxia induces VEGF expression up-regulation but contrary to expected, metastatic CRC cell lines do not respond as much as primary cell lines to the hypoxic stimulus.
3. Colorectal cancer cell lines express a similar pattern of the three major VEGF isoforms (VEGF₁₂₁, 165 and 189) despite variability in VEGF expression, where the major transcript is VEGF₁₂₁.
4. Colorectal cancer cell lines do not express surface VEGFR-2 in a relevant manner as compared to HUVE cell line. The expression of the soluble form of VEGFR-1, natural antagonist of VEGF, does not depend on the cellular origin in colorectal cancer cell lines.
5. The baseline (pre-treatment) VEGF level in patients' serum is significantly correlated with VEGF-2578AA and VEGF-460CC genotypes and a trend toward statistical significance is observed with VEGF+405GG genotype.
6. A longer median TTP in oxaliplatin/fluoropyrimidines-pretreated metastatic colorectal cancer patients is associated with the presence of any of the favourable genotypes VEGF-2578AA, VEGF-460CC, or VEGF+405GG.
7. Favourable clinical and molecular factors of Köhne low-risk category, DCR achievement and any favourable VEGF genotype enable the calculation of a predictive risk score, which discriminates TTP outcomes in four different risk groups.
8. Serum high baseline levels of EGF and MDC and lower IL-10, IL-6 and IL-8 are associated with response to the bevacizumab-based regimen in pre-treated mCRC patients.
9. A risk score comprising the number of factors below and above the median for EGF and MDC, and IL-10, IL-6 and IL-8, respectively improves single factor's predictive ability. Patients with <3 high-risk factors have a greater DCR and a significantly longer median TTP and median OS than patients with \geq 3 high-risk factors.
10. Exposure to chemotherapy and bevacizumab increases levels of MDC and decreases levels of IL-8, confirming the modulation of serum-circulating cytokines mediated by the effect of the therapy.

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