Modern Pathology (2011) 24, 1404–1412

VEGFA gene locus (6p12) amplification identifies a small but highly aggressive subgroup of colorectal patients

Tatjana Vlajnic[1], Maria Carla Andreozzi[1], Sandra Schneider[1], Luigi Tornillo[1], Eva Karamitopoulou[2], Alessandro Lugli[1], Christian Ruiz[1], Inti Zlobec[1,*] and Luigi Terracciano[1,*]

[1]*Institute of Pathology, University Hospital Basel, Basel, Switzerland and* [2]*2nd Department of Pathology, University of Athens, Attikon University Hospital, Athens, Greece*

The aim of this study was to determine: (1) the frequency of *VEGFA* gene locus (6p12) amplification in colorectal cancers, (2) the effect of gene amplification on clinical outcome using two independent colorectal cancer patient cohorts and (3) the relationship between amplification and *KRAS* or *BRAF* gene mutation as well as with other RAS/MAPK signalling proteins. Single-punch ($n=1280$; cohort 1) and multiple-punch ($n=195$; cohort 2) tissue microarrays were used for dual-labelling fluorescence *in situ* hybridization (FISH). Amplification was defined as a ratio >2 times for 6p12/centromere 6 signals. Mutation analysis of *KRAS* (codons 12 and 13) and *BRAF* (codon V600E) and immunohistochemistry for p-MAPK3/MAPK1, PEBP1, HMMR, p-AKT, PLAU, PLAUR, TP53 and VEGFA were performed on cohort 1. In cohort 1, *VEGFA* amplification was found in 39/1280 (3%) cases and linked to higher pT stage ($P=0.022$), higher tumor grade ($P=0.024$) and vascular invasion ($P=0.003$). The 5-year disease-specific survival rates were 31% (95% CI 17–46) and 57% (95% CI 54–60) for amplified and nonamplified cases, respectively ($P<0.001$). Results were confirmed in cohort 2. In multivariable analysis, the relative risk for amplification was 2.09 (95% CI 1.4–3.1; $P<0.001$) and linked to more frequent *BRAF* mutation ($P=0.015$), overexpression of p-MAPK3/MAPK1 ($P=0.012$) and PLAU ($P=0.048$) and loss of metastasis suppressor protein PEBP1 ($P=0.047$). *VEGFA* gene locus amplification highlights a small but remarkably aggressive subgroup of colorectal cancers. Further studies are needed to elucidate the potential role of amplification as a prognostic or predictive biomarker in both metastatic and nonmetastatic patients.
Modern Pathology (2011) **24,** 1404–1412; doi:10.1038/modpathol.2011.96; published online 8 July 2011

Keywords: amplification; colorectal cancer; FISH; prognosis; *VEGFA*; 6p12

In the early 1970s, Folkman, based on previous observations by Ide and Algire,[1,2] hypothesized that tumor growth could be arrested by blocking a 'tissue angiogenic factor', an approach that would be useful in the treatment of cancer. In 1983, Senger *et al*[3] identified a protein capable of inducing vascular leakage, suggesting that this vascular permeability factor (VPF) might mediate the high permeability of tumor blood vessels. These experiments were followed up by several groups that independently cloned, purified and sequenced this molecule now known as vascular endothelial growth factor (VEGF).[4,5]

The VEGF protein (VEGFA) is a glycosylated mitogen that, in addition to its roles in vascular permeability and angiogenesis, acts on vasculogenesis, endothelial cell growth, cell migration and inhibition of apoptosis.[6] KDR (VEGFR-2), the receptor of VEGFA, is a receptor tyrosine kinase III, expressed on various types of endothelial cells. Ligand binding to the receptor activates the RAS/MEK/MAPK pathway, probably via phosphorylation of PLCG, thus leading to mitotic activation in endothelial cells.[7] The majority of studies to date, using either immunohistochemistry for the detection of VEGFA protein in tumor tissue, mRNA or soluble plasma VEGFA levels, all point toward a significant negative effect of increased VEGFA

Correspondence: Professor L Terracciano, Institute of Pathology, University Hospital Basel, Schoenbeinstrasse 40, Basel 4031, Switzerland.
E-mail: lterracciano@uhbs.ch
*These two authors contributed equally to this work.
Received 31 December 2010; revised 25 March 2011; accepted 25 March 2011; published online 8 July 2011

expression levels in patients with colorectal cancer.[6–12] Even in rectal cancer patients treated with preoperative radiotherapy based on long-course or short-course/hyperfractionated regimens, overexpression of VEGFA also indicates a poorer pathological response rate when evaluated in pre-operative biopsies.[13,14]

In 2004, Hurwitz *et al*[15] were the first to report a benefit of anti-VEGF therapy in combination with chemotherapy in the treatment of metastatic colorectal cancer patients. A recent meta-analysis of several trials including >3000 patients appears to confirm that the addition of bevacizumab to chemotherapy prolongs both progression-free and overall survival in most, but not all, cases.[16,17] These results support the notion set forth by Jain[10] that antiangiogenic agents may in fact function by 'normalizing' the tumor vasculature, rendering vessels less 'leaky' and thus allowing for more effective targeting of the tumor by chemotherapeutic agents and by irradiation.

However, several points regarding the role of VEGFA in colorectal cancer remain to be elucidated. It is unclear whether tumor cell expression of VEGFA may be clinically more important in early rather than later stages of colorectal cancer progression because of the so-called angiogenic switch.[9,18] Second, the potential role of VEGFA as a predictive biomarker of clinical response to anti-VEGF therapies and other treatments has been scarcely investigated.[19] Third, although immunohistochemical analysis of VEGFA is frequently performed for research, it may not be an 'ideal' approach to assessing VEGFA levels in the treatment setting for several reasons: interlaboratory variability, inter-observer reproducibility, possible tumor heterogeneity and selection of appropriate threshold values for assigning a 'positive' case are typical problems faced by potential prognostic or predictive immunohistochemical biomarkers.

Amplification or increased gene copy number detected by fluorescence *in situ* hybridization (FISH) may represent an alternative, and perhaps less subjective, method for investigating deregulation of VEGFA expression levels. Such an approach has previously highlighted gene copy number gain and amplification of the epidermal growth factor receptor (EGFR) as a predictor of clinical response in patients with non-small cell lung cancers, and metastatic colorectal cancer.[20,21] To date, however, the frequency and prognostic relevance of *VEGFA* gene locus (6p12) amplification in colorectal cancers as well as its correlation to other RAS/MAPK signalling molecules has not been investigated.[22]

Therefore, the aim of this study was: (1) to determine the frequency of *VEGFA* gene locus amplification in a large cohort of colorectal cancers, (2) determine the effect of gene amplification on clinical outcome using two independent colorectal cancer patient cohorts and (3) determine the relationship between *VEGFA* gene amplification and *KRAS* or *BRAF* gene mutation as well as with other protein markers of the RAS/MAPK signalling pathway.

Patients and methods

Patients

Cohort 1
A first cohort of 1420 patients with primary colorectal cancer diagnosed at the Institute of Pathology, University Hospital Basel, Institute of Clinical Pathology, Basel and the Triemli Stadtspital were entered into this study. Histomorphological and clinical information included age at diagnosis, tumor diameter, gender, tumor location, histological subtype, pT classification, pN classification, tumor grade, vascular invasion, mismatch repair status and survival time information. Information on post-operative therapy, distant metastasis and local recurrence were retrieved from patient records and available in 1/3 of the cases. Censored observations included patients who were alive at the last follow-up, died for reasons other than colorectal cancer or were lost to follow-up.

Cohort 2
A second cohort of 221 nonconsecutive patients treated at the 4th Department of Surgery, University of Athens Medical School, randomly selected from the archives of the 2nd Department of Pathology, University of Athens Medical School (Attikon University Hospital), Greece, were entered into this study. Patients were treated between 2004 and 2006. All histomorphological data were reviewed from the corresponding hematoxylin and eosin (H&E)-stained slides, whereas clinical data were obtained from corresponding reports. Clinicopathological information for all patients included age, tumor diameter, gender histological subtype, tumor location, pT stage, pN stage, pM stage, tumor grade, vascular invasion, lymphatic invasion and mismatch repair status. Information on postoperative therapy and follow-up time was available for all patients.

Specimens

The use of material was approved by the local ethics committees of the University Hospital of Basel and University of Athens, respectively.

Tissue microarrays
Paraffin-embedded tissue blocks from all patients in both cohorts 1 and 2 were retrieved and two tissue microarrays were constructed. For cohort 1, a single-punch tissue microarray consisting of 1420 colorectal cancer specimens and 57 normal mucosa samples was established. In cohort 2, in order to exclude bias due to possible tumor heterogeneity, each patient had multiple tissue and tumor punches

taken from formalin-fixed, paraffin-embedded blocks using a tissue cylinder with a diameter of 0.6 mm that were subsequently transferred into one recipient paraffin block (3 × 2.5 cm) using a home-made semiautomated tissue arrayer. Tissues were obtained from the tumor center, the invasive tumor front, the normal adjacent mucosa (if available), and the transitional zone where tumor and normal adjacent mucosa first interact (if available). Each patient on average had 5.1 tissue punches included on this array with an average of four tumor punches. The final tissue microarray contained 1079 tissues: namely, 437 tissues from the tumor center, 430 from the invasive front, 90 from normal adjacent mucosa and 122 from the transitional zone.

Whole tissue sections
Additionally, in order to assess the intratumoral heterogeneity of *VEGFA* amplification, 25 whole tissue sections from patients with metastatic colorectal cancer treated between 2003 and 2010 at the University Hospital Basel were selected from the archives of the Institute for Pathology.

Assay Methods

FISH (cohorts 1 and 2 and whole tissue sections)
Tissue microarray and whole tissue sections were used for dual-labelling FISH. The genomic BAC clone RPCIB753M0921Q (imaGENES GmbH, Berlin, Germany), which covers the *VEGFA* gene region, was used for preparation of the FISH probe. A starter culture of 2–5 ml LB medium was inoculated with the BAC clone and 0.5 ml of the starter culture was diluted in 500 ml selective LB medium. BAC-DNA was isolated using the Large-Construct Kit (Qiagen, Hombrechtikon, Switzerland) according to the instructions of the manufacturer. BAC identity was verified by sequencing using 1 μg of isolated DNA and 20 pmol of SP6, respectively, T7 primers (EuroFins MGW Operon, Ebersberg, Germany). Isolated BAC-DNA (1 μg) was digested with *Alu*I restriction enzyme (Invitrogen, Lucerne, Switzerland) and labelled with Cy3-dUTP (GE Healthcare, Buckinghamshire, UK) using the BioPrime Array CGH Kit (Invitrogen). Labelling reaction was assessed by usage of a Nanodrop assay (Nanodrop, Wilmington, DE, USA). The labelled DNA was purified by using the FISH Tag DNA Kit (Invitrogen). Tissue microarrays and whole tissue sections were subjected to pretreatment as previously described.[23] FISH probe was applied and after a denaturation step (10 min at 75 °C), the slides were incubated overnight at 37 °C. Washing of the slides was performed with the Wash Buffer (2 × SSC, 0.3% NP40, pH 7–7.5) and slides were counterstained with DAPI I solution (1000 ng/ml; Vysis Abbott Molecular, Abbott Park, IL, USA). As reference, a Spectrum Green-labelled chromosome 6 centromeric probe (Vysis Abbott Molecular) was used. Images were obtained by usage of a Zeiss fluorescence microscope using a 63 × objective (ZEISS, Feldbach, Switzerland) and the Axiovision software (ZEISS).

Immunohistochemistry (cohort 1)
Immunohistochemistry was performed on the tissue microarray for protein markers p-MAPK3/MAPK1 (clone 20G11, dilution 1:100; Cell Signaling Technology, Danvers, MA, USA), RAF-1 kinase inhibitor protein (PEBP1; dilution 1:1000; Upstate, New York, NY, USA), receptor for hyaluronic acid-mediated motility (HMMR (RHAMM); clone 2D6; dilution 1:25, Novocastra, Newcastle, UK), T-cell-originated protein kinase (PBK; PBK/TOPK, rabbit polyclonal, dilution 1:50, Cell Signaling Technology), p-AKT (clone 244F9, dilution 1:00; Cell Signaling Technology), urokinase plasminogen activator (PLAU; no. 3689; dilution 1:25; American Diagnostica, Stamford, CT, USA) and its receptor (PLAUR, no. 3936; dilution 1:25; American Diagnostica) as well as for TP53 (DO-7; Dako Cytomation, Glostrup, Denmark) and VEGFA (polyclonal; 1:300; Santa Cruz, CA USA). Cutoff scores for 'overexpression/positivity' compared with 'loss/negativity' were previously established and determined to be: 70% for PEBP1, 90% for HMMR (RHAMM), 0% for pMAPK3/MAPK1, 0% for p-AKT, 90% for PBK, 60% for PLAU, 75% for PLAUR and 90% for VEGFA.

KRAS and BRAF gene analysis (cohort 1)
Genomic DNA was obtained from 404 colorectal tissue blocks using NucleoMag 96 Tissue Kit (Macherey Nagel) protocol and processed in the Xiril X-100 robot (Xiril, Hombrechtikon, Switzerland). Briefly, punched tissue was lysed in proteinase K. B-beads and MB2 buffer were added to the cleared lysate and shaken for 5 min at RT. The supernatant was removed and MB3 was added followed by shaking and supernatant removal. The genomic DNA was eluted with MB6 buffer and amplified by PCR using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA). *KRAS* (exon 2; codons 12 and 13) and *BRAF* (exon 15; codon 600) were amplified by a first and a nested PCR. Residual primers were removed using the EXOSAPit (Amersham). Samples were then subjected to direct sequencing of single-stranded PCR products using the BigDye® Terminator v1.1 cycle sequencing kit (Applied Biosystems) and the ABI Prism® 3130 genetic analyzer (Applied Biosystems). All products were sequenced bidirectionally.

Study Design

The study design is summarized in Figure 1. A total of 1420 colorectal cancers mounted onto a tissue microarray (cohort 1). This tissue microarray was then analyzed for *VEGFA* gene locus amplification, and protein expression of p-MAPK3/MAPK1, PEBP1, HMMR (RHAMM), PBK, PLAU, PLAUR

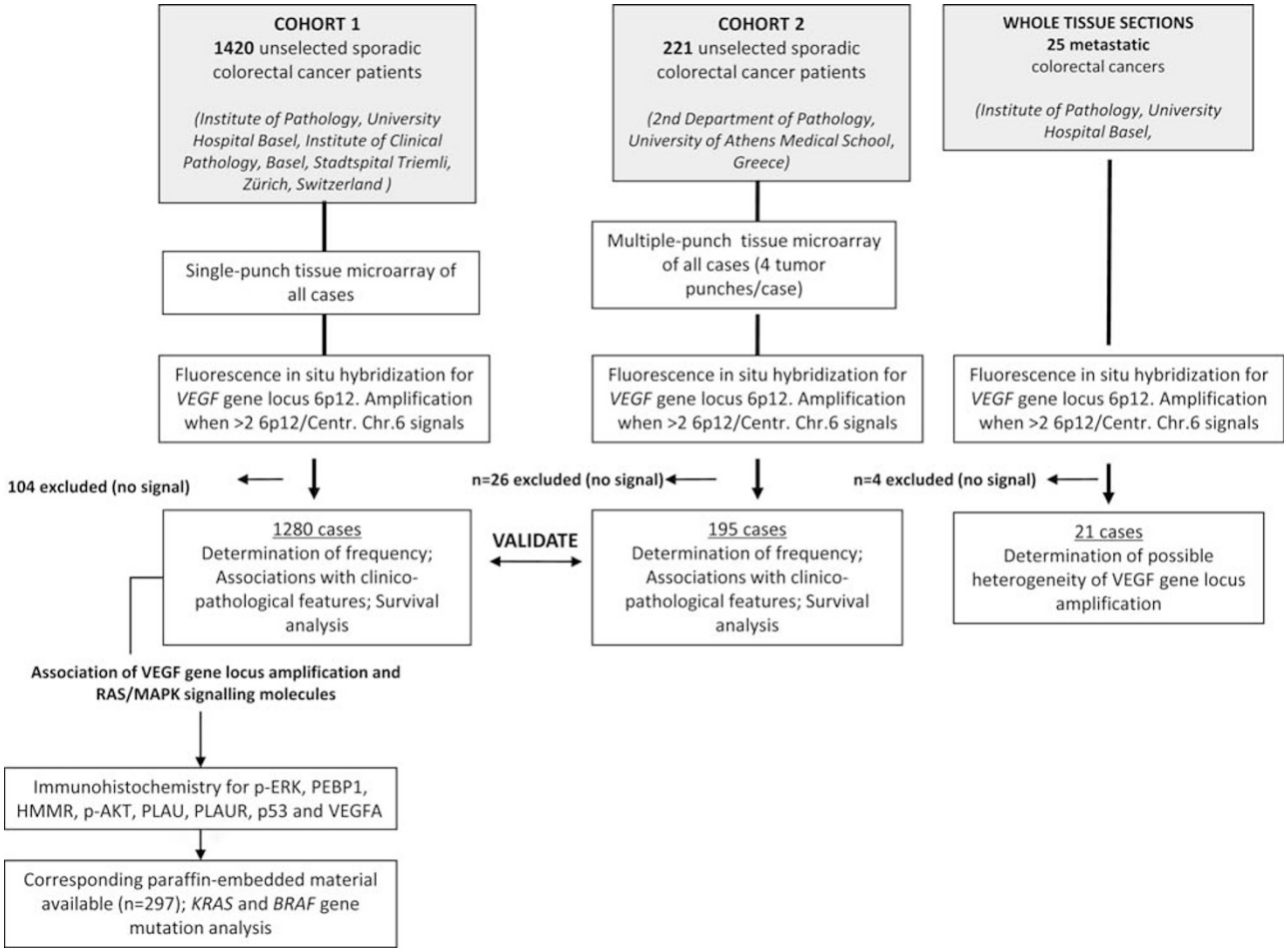

Figure 1 Study design. A total of 1420 colorectal cancers mounted onto a tissue microarray (cohort 1) were analyzed for *VEGFA* gene locus amplification, and protein expression of p-MAPK3/MAPK1, PEBP1, HMMR (RHAMM), PBK, PLAU, PLAUR, TP53 and VEGFA. For a subgroup of patients, molecular analysis of *KRAS* and *BRAF* gene status was performed. A second independent set of 221 colorectal cancers was mounted onto a multiple-punch tissue microarray. *VEGFA* amplification using FISH analysis was performed and correlated with clinicopathological features and clinical outcome. Additionally, FISH analysis was performed on whole tissue sections from 25 selected patients with metastatic colorectal cancer.

and TP53. For a subgroup of patients, paraffin-embedded tissue blocks could be retrieved and molecular analysis of *KRAS* and *BRAF* gene status performed. Associations of *VEGFA* gene locus amplification with clinicopathological features and prognosis were determined. A second, independent cohort of 221 patients was additionally entered into this study. A multiple-punch tissue microarray containing on average four tumor punches per patient was constructed. *VEGFA* amplification using FISH analysis was performed and correlated with clinicopathological features and clinical outcome. Additionally, FISH analysis was performed on whole tissue sections from 25 patients with metastatic colorectal cancer.

Statistical Analysis

Differences in categorical clinicopathological parameters and *VEGFA* gene locus amplification were analyzed using the χ^2 and Fischer's exact test, where appropriate. For age and tumor diameter, the Wilcoxon rank-sum test was used. Survival curve were plotted using the Kaplan–Meier method and differences assessed using the Wilcoxon and log-rank tests in univariate survival analysis. Multiple Cox regression analysis was undertaken for multivariable survival time analysis after verification of the proportional hazards assumption. No imputation was performed for missing variables, rather case-wise deletion was performed. This led to 37 cases of *VEGFA* amplification (and 27 events) in the amplified group. In order to prevent overfitting, adjustments were carried out for pN stage and vascular invasion only. Hazard ratios (HRs) and 95% confidence intervals (CIs) were used to determine the effect of each variable in multivariable analysis, with HR >1.0 indicating an increased relative risk of death in the case of amplification, higher pN stage and for the presence of vascular invasion compared with negativity (nonamplified

cases), lymph node negativity and absence of vascular invasion. Because of multiple hypothesis testing from the analysis of *VEGFA* amplification with 27 different clinicopathological, molecular or protein features, the level of significance was adjusted to $\alpha = 0.05/27$. Hence, only *P*-values <0.002 were considered statistically significant whereas others <0.05 were considered as trends. Analyses were carried out using SAS (V9.1, SAS Institute, Cary, NC, USA).

Results

Cohort 1

Frequency of VEGFA amplification and association with clinicopathological parameters

FISH analysis of the *VEGFA* gene locus was evaluable in 1280 of 1420 (90%) colorectal cancer punches. Amplification was found in 39/1280 (3%; Figure 2). Amplified tumors were more frequently of larger diameter ($P = 0.045$), right sided ($P = 0.016$) and were of higher pT stage ($P = 0.022$), higher tumor grade ($P = 0.024$) and had vascular invasion ($P = 0.003$; Table 1). No particular pattern of tumor recurrence was observed. *VEGFA* amplification was significantly linked to unfavorable prognosis with 5-year disease-specific survival rates of 31% (95% CI 17–46) compared with 57.1% (95% CI 54–60) for nonamplified cases ($P < 0.001$; Figure 3a). Taking into account the number of patient deaths in the amplified group ($n = 27/37$; 1 patient had no survival time information), multivariable survival time analysis was performed to determine the effect of *VEGFA* amplification when adjusting for pN stage and vascular invasion. Despite the small number of amplified cases, the highly negative impact of *VEGFA* gene locus amplification on survival time was maintained (HR 2.09; 95% CI 1.4–3.1; $P < 0.001$) when controlling for the effects of pN stage (HR 2.58; 95% CI 2.1–3.1; $P < 0.001$) and vascular invasion (HR 2.17; 95% CI 1.8–2.6; $P < 0.001$).

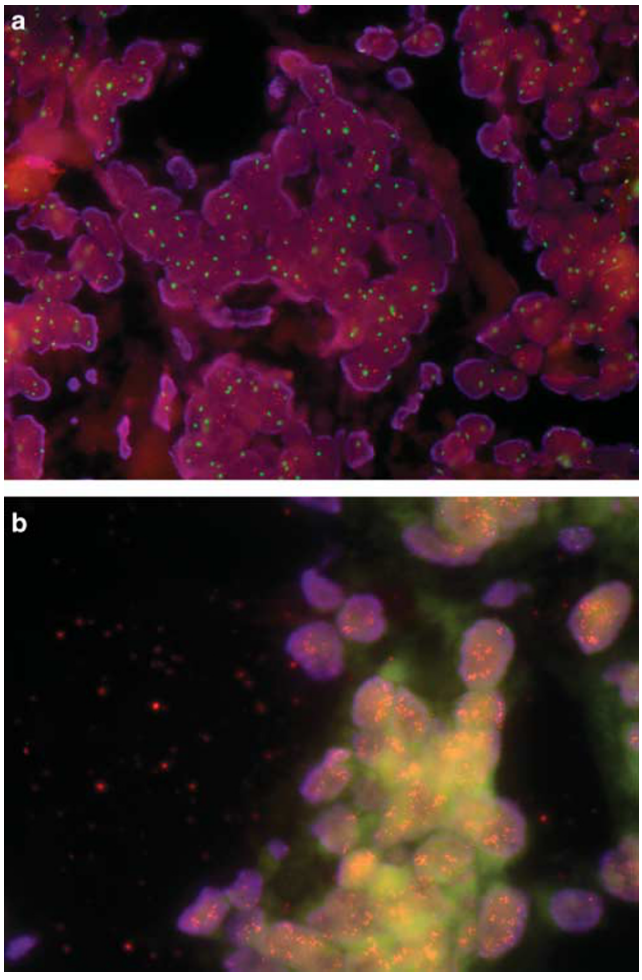

Figure 2 Fluorescence *in situ* hybridization (FITC + Rhodamine + DAPI) of the *VEGFA* gene locus 6p12 in colorectal cancer. (**a**) Negative case with no amplification. (**b**) Amplified colorectal cancer.

VEGFA amplification and association with molecular and protein markers in RAS/MAPK signalling

VEGFA gene locus amplification showed a trend toward more frequent *BRAF* mutation ($P = 0.015$), overexpression of p-MAPK3/MAPK1 ($P = 0.012$), PLAU ($P = 0.048$) and loss of the RAF1 inhibitor protein PEBP1 ($P = 0.047$; Table 2). No associations were found between *VEGFA* amplification and *KRAS* gene status or with the HMMR (RHAMM), PBK, p-AKT, PLAUR, TP53 or VEGFA protein expression.

Cohort 2

Validation of VEGFA and association with clinicopathological parameters and prognosis

Using a second external validation cohort of 221 patients, FISH analysis of the *VEGFA* gene locus was evaluable in 195 colorectal cancers of which 11/195 (5%) showed gene amplification. Trends toward a more frequent higher tumor grade ($P = 0.016$), vascular ($P = 0.032$) and lymphatic invasion ($P = 0.008$) were observed in patients with amplified tumors (Table 3). Of the 11 patients with *VEGFA* amplification, 7 (63%) died of disease compared with 61/175 (35%) of nonamplified cases. Moreover, a trend for negative prognostic impact was observed for patients with *VEGFA* amplification compared with nonamplified cases, particularly at earlier time points ($P = 0.009$; Figure 3b). No multivariable analysis could be performed for this small amplified subgroup.

Whole tissue sections

The *VEGFA* gene locus amplification on whole tissue sections was possible for 21/25 cases. High polysomy was observed in 2 (9%) and low polysomy

Table 1 *VEGFA* gene locus (6p12) amplification and clinicopathological features of colorectal cancer patients (cohort 1)

Clinicopathological feature	*VEGFA status,* N *(%)*		P-*value*
	Negative (n = *1241)*	*Amplification* (n = *39)*	
Age (years)			
Mean (range)	70, 30–96	70, 50–88	0.717
Diameter (mm)			
Mean (range)	49, 4–170	54, 25–110	0.045
Gender			
Female	660 (53)	18 (46)	0.387
Male	581 (47)	21 (53)	
Tumor location			
Left sided	797 (65)	18 (46)	0.016
Right sided	430 (25)	21 (53)	
Histological subtype			
Mucinous	95 (8)	1 (3)	0.357
Nonmucinous	1146 (92)	38 (97)	
T stage			
pT1-2	241 (20)	2 (5)	0.022
pT3-4	973 (80)	37 (95)	
N stage			
pN0	634 (53)	15 (40)	0.097
pN1-2	559 (47)	23 (60)	
Tumor grade			
G1-2	1061 (87)	28 (74)	0.024
G3	153 (13)	10 (26)	
Vascular invasion			
Absent	887 (73)	20 (51)	0.003
Present	326 (27)	19 (49)	
Local recurrence			
Absent	260 (59)	2 (40)	0.653
Present	183 (41)	3 (60)	
Metastasis			
Absent	368 (82)	3 (60)	0.231
Present	82 (18)	2 (40)	
MMR status			
Proficient	1047 (84)	34 (87)	0.633
Deficient	194 (16)	5 (13)	
Tumor border configuration			
Pushing	461 (38)	10 (26)	0.116
Infiltrating	751 (62)	29 (74)	
Peritumoral lymphocytic inflammation			
Absent	962 (79)	30 (77)	0.726
Present	252 (21)	9 (23)	
Postoperative therapy			
None	355 (80)	5 (100)	0.588
Treated	90 (20)	0 (0)	
Survival (months)			
5-year survival rate	57 (54–60)	31 (17–46)	<0.001

in 5 cases (24%). *VEGFA* gene locus amplification was found in 4/21 cases (19%) and heterogeneous expression was detected in 0/21 cases.

Discussion

The novel findings of this study suggest that (1) *VEGFA* gene locus (6p12) amplifications occur in 3–6% of colorectal cancers, (2) amplification identifies a subgroup of highly aggressive colorectal cancers and (3) may be more frequently associated with deregulation of RAS/MAPK signalling.

In this study we observe an amplification of the *VEGFA* gene locus 6p12 in 3–6% of primary colorectal cancer cases. A review of comparative genomic hybridization (CGH) studies reports that amplification of this locus is found in several types of malignancy, such as lymphomas, sarcomas, non-small cell lung carcinoma, bladder, breast and ovarian carcinomas and uveal melanoma.[24] Horlings *et al*[25] recently documented 6p12 copy number gains in basal-like tumors of the breast and furthermore related this copy number gain to unfavorable prognosis. Yaginuma *et al*[26] applied CGH to nine colon samples from patients with Dukes' C cancers and their corresponding lymph node metastases. They report a significantly greater number of 6p12 copy number gains in primary tumor compared with lymph node metastasis. However, to date, the frequency and prognostic relevance of 6p12 amplification have not been reported for patients with colorectal cancer.

In both independent cohorts of patients, *VEGFA* gene locus amplification was significantly linked to more unfavorable prognostic features including advanced stage, vascular and lymphatic invasion and significantly poorer survival time. Our results are in line with previous publications using different techniques to assess deregulation of VEGFA protein or mRNA expression in tumors as well as using plasma concentrations of soluble VEGFA or by analysis of single-nuclear polymorphism (SNP) in peripheral blood.[6,8,9,11,12,27] However, our group[8] in a previous study was not able to detect any relationship between VEGFA protein expression and the main clinicopathological parameters. Despite these associations, *VEGFA* gene locus amplification was not more frequent in patients with confirmed metastatic disease. These findings may indicate that 6p12 amplification is an early event in colorectal tumor progression, a finding similar to those reported by Hanrahan *et al*[9] studying mRNA expression levels, as well as those by Barresi *et al*[6] investigating protein expression in early colorectal cancers. Additionally, our findings suggest that *VEGFA* gene locus amplification could interfere with RAS/MAPK signalling particularly by implicating *BRAF*. Not only was amplification more frequent in *BRAF*-mutated tumors known for their poor prognostic effect but was also found more often in tumors showing a loss of the metastasis suppressor protein PEBP1 that functions as a RAF kinase inhibitor protein.[28,29] RAS/MAP kinase signalling has previously been found to be the predominant pathway through which VEGF exerts its effects.[5]

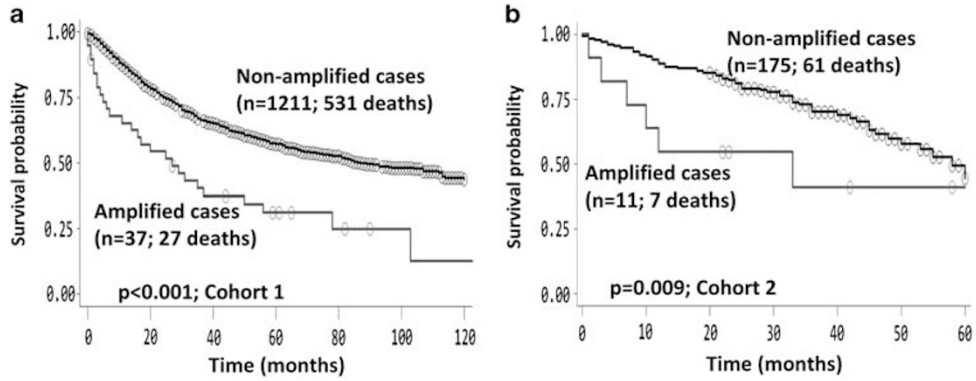

Figure 3 Kaplan–Meier survival curves showing the highly negative prognostic effect of *VEGFA* gene locus amplification in (**a**) cohort 1 and (**b**) cohort 2.

Table 2 *VEGFA* gene locus (6p12) amplification and distribution of molecular and protein markers in colorectal cancer patients (cohort 1)

	VEGFA status, N *(%)*		P-*value*
	Negative	*Amplification*	
KRAS			
Wild type	196 (73)	7 (70)	0.732
Mutation	72 (27)	3 (30)	
BRAF			
Wild type	243 (90)	6 (60)	0.015
Mutation	26 (10)	4 (40)	
pMAPK3/MAPK1			
Negative	993 (84)	27 (69)	0.012
Positive	185 (16)	12 (31)	
PEBP1			
Negative	246 (26)	14 (41)	0.047
Positive	704 (74)	20 (59)	
HMMR (RHAMM)			
Negative	587 (56)	15 (43)	0.122
Positive	460 (44)	20 (57)	
PBK			
Negative	637 (57)	24 (65)	0.311
Positive	491 (43)	13 (35)	
p-AKT			
Negative	428 (37)	15 (41)	0.663
Positive	728 (63)	22 (59)	
PLAU			
Negative	556 (56)	14 (39)	0.048
Positive	445 (44)	22 (61)	
PLAUR			
Negative	327 (34)	7 (21)	0.109
Positive	641 (66)	27 (79)	
TP53			
Negative	536 (48)	20 (56)	
Positive	572 (52)	16 (44)	0.396
VEGFA			
Negative	446 (42)	13 (35)	0.406
Positive	616 (58)	24 (65)	

In fact, binding of VEGFA to its receptors KDR (VEGFR-2), FLT-1 (VEGFR-1) and FLT-4 (VEGFR-3) activates multiple signalling pathways, in particular the RAS/MAPK pathway. Moreover, RAS mutations have been shown to be correlated with the expression of VEGFA *in vitro* and the disruption of *RAS*-mutant allele has been shown associated with reduced expression of VEGFA.[5] The relationship between amplification of *VEGFA* gene locus and molecules of RAS/MAPK signalling may further elucidate the mechanisms leading to tumor progression in patients with colorectal cancer.

It is unlikely that *VEGFA* amplification is associated with CIN. MSI and CIN seem to define pathogenetically different subsets of tumors and are described as mutually exclusive events;[30] most colorectal cancers with CIN are indeed microsatellite stable (MSS) with wild-type *BRAF*. In fact, according to the current model of colorectal tumorigenesis,[31] the majority of colorectal cancers are hypothesized to have both MSS and CIN. As *VEGFA* amplification is not associated with MMR proficiency, it is doubtful that it would also be linked with CIN. Together, these preliminary results suggest that *VEGFA* gene locus amplification may highlight only a small but remarkably aggressive subgroup of colorectal cancers.

Our study is limited by several factors. Nonetheless, given the large sample size of evaluable tissue punches ($n=1280$), it is unlikely that this heterogeneity has significantly influenced our findings. Second, because of the relatively infrequent occurrence of amplification in our tissue microarray series (3–6%), rigid statistical analysis of the effect of *VEGFA* amplification on survival time adjusting for other known confounders could not be adequately performed. However, using two of the most important essential prognostic factors proposed for colorectal cancer, namely lymph node metastasis and vascular invasion, the amplification at 6p12 remained a significant prognostic parameter.[32]

Despite the well-documented association of increased VEGFA expression and more unfavorable clinical outcome in colorectal cancer, our study is

Table 3 *VEGFA* amplification and clinicopathological features of patients with colorectal cancer (cohort 2)

Clinicopathological feature	*VEGFA status,* N *(%)*		P-*value*
	*Negative (*n = *184),* N *(%)*	*Amplification (*n = *11),* N *(%)*	
Age (years)			
Mean (range)	68.2, 35–91	66.3, 42–81	0.663
Diameter (cm)			
Mean (range)	4, 1–12	4, 2–8	0.415
Gender			
Male	82 (47)	6 (55)	0.62
Female	93 (53)	5 (45)	
Histological subtype			
Adenocarcinoma	157 (90)	9 (82)	0.336
Other	18 (10)	2 (18)	
Tumor location			
Left	108 (62)	6 (55)	0.815
Rectum	21 (12)	2 (18)	
Right	46 (26)	3 (27)	
pT stage			
pT1-2	45 (26)	1 (9)	0.298
pT3-4	130 (74)	10 (91)	
pN stage			
pN0	88 (51)	3 (27)	0.139
pN1-2	87 (49)	8 (73)	
pM stage			
pM0	158 (90)	9 (82)	0.312
pM1	17 (10)	2 (18)	
Tumor grade			
G1-2	110 (78)	4 (40)	0.016
G3	32 (22)	6 (60)	
Vascular invasion			
None	149 (83)	6 (55)	0.032
Present	30 (17)	5 (45)	
Lymphatic invasion			
None	109 (62)	2 (18)	0.008
Present	67 (38)	9 (82)	
Postoperative therapy			
None	63 (34)	3 (27)	0.753
Treated	121 (66)	8 (73)	
Survival (months)			
5-year survival rate	49 (36–60)	41 (11–68)	0.009

novel for several reasons. First, it appears to be the first analysis of the *VEGFA* gene locus using FISH on standard paraffin-embedded tissues from colorectal cancer patients. Thus, the quantification of gene copy number may be considered a more objective assessment when compared with immunohistochemical methods. Second, our study benefits from complete survival time information in >1000 cases and characterization of follow-up and treatment on >500 cases. Third, the low rate of amplification and its highly negative effect on prognosis were confirmed using two independent, large cohorts of colorectal cancer patients. We used both single-punch and multiple-punch tissue microarrays to determine the frequency of *VEGFA* amplification in colorectal cancers, thus conforming to the guidelines set by Goethals *et al*[33] for tissue microarray studies. Moreover, our evaluation of whole tissue sections suggests that *VEGFA* gene locus amplification is a homogeneous occurrence within the tumor, further supporting the use of tissue microarrays here.

It remains to be seen whether *VEGFA* gene locus amplifications could be useful as predictive biomarkers of clinical response in patients considered for bevacizumab or tyrosine kinase inhibitors in addition to chemotherapy. The effect of anti-VEGF agents appears to vary substantially with the type of chemotherapy regimen (first or second line, 5-FU or capecitabine/oxaliplatin).[17] As noted by Wagner *et al*,[17] the mechanisms of primary and secondary resistance to angiogenesis inhibitors require clarification, and *VEGFA* gene amplification may play a role in these processes.

In summary, *VEGFA* gene locus amplification identified a small, yet highly aggressive, subgroup of colorectal cancers. It is tempting to speculate that, as for other biomarker routinely assessed in pathology laboratories (*KRAS* mutation or EGFR by immunohistochemistry and/or ISH), *VEGFA* assessment will deserve a predictive role in colorectal cancer.[34] Further studies are needed to elucidate the potential role of amplification as a prognostic or predictive biomarker in patients with both metastatic and nonmetastatic disease.

Disclosure/conflict of interest

The authors declare no conflict of interest.

References

1 Algire GH, Legallais FY. Recent developments in the transparent-chamber technique as adapted to the mouse. J Natl Cancer Inst 1949;10:225–253, incl 8 pl.
2 Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182–1186.
3 Senger DR, Galli SJ, Dvorak AM, *et al.* Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983;219: 983–985.
4 Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 1989;161:851–858.
5 Keck PJ, Hauser SD, Krivi G, *et al.* Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 1989;246:1309–1312.
6 Barresi V, Di Gregorio C, Regiani-Bonetti L, *et al.* Stage I colorectal carcinoma: VEGF immunohistochemical

expression, microvessel density, and their correlation with clinical outcome. Virchows Arch 2010;457:11–19.
7 Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 2005;23:1011–1027.
8 Eppenberger M, Zlobec I, Baumhoer D, *et al.* Role of the VEGF ligand to receptor ratio in the progression of mismatch repair-proficient colorectal cancer. BMC Cancer 2010;10:93.
9 Hanrahan V, Currie MJ, Gunningham SP, *et al.* The angiogenic switch for vascular endothelial growth factor (VEGF)-A, VEGF-B, VEGF-C, and VEGF-D in the adenoma-carcinoma sequence during colorectal cancer progression. J Pathol 2003;200:183–194.
10 Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 2005;307:58–62.
11 Nakayama Y, Sako T, Shibao K, *et al.* Prognostic value of plasma vascular endothelial growth factor in patients with colorectal cancer. Anticancer Res 2002;22:2437–2442.
12 Yamamori M, Sakaeda T, Nakamura T, *et al.* Association of VEGF genotype with mRNA level in colorectal adenocarcinomas. Biochem Biophys Res Commun 2004;325:144–150.
13 Giralt J, Navalpotro B, Hermosilla E, *et al.* Prognostic significance of vascular endothelial growth factor and cyclooxygenase-2 in patients with rectal cancer treated with preoperative radiotherapy. Oncology 2006;71: 312–319.
14 Zlobec I, Steele R, Nigam N, *et al.* A predictive model of rectal tumor response to preoperative radiotherapy using classification and regression tree methods. Clin Cancer Res 2005;11:5440–5443.
15 Hurwitz H, Fehrenbacher L, Novotny W, *et al.* Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004;350:2335–2342.
16 Ellis LM, Hicklin DJ. Resistance to targeted therapies: refining anticancer therapy in the era of molecular oncology. Clin Cancer Res 2009;15:7471–7478.
17 Wagner AD, Arnold D, Grothey AA, *et al.* Anti-angiogenic therapies for metastatic colorectal cancer. Cochrane Database Syst Rev 2009; (3): CD005392.
18 Dallas NA, Fan F, Gray MJ, *et al.* Functional significance of vascular endothelial growth factor receptors on gastrointestinal cancer cells. Cancer Metastasis Rev 2007;26:433–441.
19 Jubb AM, Hurwitz HI, Bai W, *et al.* Impact of vascular endothelial growth factor-A expression, thrombospondin-2 expression, and microvessel density on the treatment effect of bevacizumab in metastatic colorectal cancer. J Clin Oncol 2006;24:217–227.
20 Hirsch FR, Varella-Garcia M, Cappuzzo F. Predictive value of EGFR and HER2 overexpression in advanced non-small-cell lung cancer. Oncogene 2009;28 (Suppl 1):S32–S37.
21 Siena S, Sartore-Bianchi A, Di Nicolantonio F, *et al.* Biomarkers predicting clinical outcome of epidermal growth factor receptor-targeted therapy in metastatic colorectal cancer. J Natl Cancer Inst 2009;101: 1308–1324.
22 Fang JY, Richardson BC. The MAPK signalling pathways and colorectal cancer. Lancet Oncol 2005;6: 322–327.
23 Sauter G, Moch H, Carroll P, *et al.* Chromosome-9 loss detected by fluorescence *in situ* hybridization in bladder cancer. Int J Cancer 1995;64:99–103.
24 Knuutila S, Bjorkqvist AM, Autio K, *et al.* DNA copy number amplifications in human neoplasms: review of comparative genomic hybridization studies. Am J Pathol 1998;152:1107–1123.
25 Horlings HM, Lai C, Nuyten DS, *et al.* Integration of DNA copy number alterations and prognostic gene expression signatures in breast cancer patients. Clin Cancer Res 2010;16:651–663.
26 Yaginuma Y, Unotoro J, Kamiyama H, *et al.* Genomic copy-number aberrations related to lymph-node metastasis of colon cancer. J Int Med Res 2006;34: 390–396.
27 Jain L, Vargo CA, Danesi R, *et al.* The role of vascular endothelial growth factor SNPs as predictive and prognostic markers for major solid tumors. Mol Cancer Ther 2009;8:2496–2508.
28 Odabaei G, Chatterjee D, Jazirehi AR, *et al.* Raf-1 kinase inhibitor protein: structure, function, regulation of cell signaling, and pivotal role in apoptosis. Adv Cancer Res 2004;91:169–200.
29 Walther A, Johnstone E, Swanton C, *et al.* Genetic prognostic and predictive markers in colorectal cancer. Nat Rev Cancer 2009;9:489–499.
30 Ogino S, Goel A. Molecular classification and correlates in colorectal cancer. J Mol Diagn 2008;10:13–27.
31 Leggett B, Whitehall V. Role of the serrated pathway in colorectal cancer pathogenesis. Gastroenterology 2010;138:2088–2100.
32 Compton CC, Fielding LP, Burgart LJ, *et al.* Prognostic factors in colorectal cancer. College of American Pathologists Consensus Statement 1999. Arch Pathol Lab Med 2000;124:979–994.
33 Goethals L, Perneel C, Debucquoy A, *et al.* A new approach to the validation of tissue microarrays. J Pathol 2006;208:607–614.
34 Hamilton SR. Targeted therapy of cancer: new roles for pathologists in colorectal cancer. Mod Pathol 2008;21 (Suppl 2):S23–S30.