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VEGFA gene locus analysis across 80 human tumour types reveals gene amplification in several neoplastic entities

**Mariacarla Andreozzi · Luca Quagliata · Joel R. Gsponer ·
Christian Ruiz · Vincent Vuaroqueaux · Serenella Eppenberger-Castori ·
Luigi Tornillo · Luigi M. Terracciano**

Abstract

Background Angiogenesis plays a pivotal role in neoplastic growth and metastasis formation. Vascular endothelial growth factor A (VEGFA) is a major player in physiological and tumour-induced angiogenesis and numerous human tumours have been show to overexpress VEGFA. Moreover increased *VEGFA* gene expression has been found frequently to correlate with tumour progression, recurrences and survival. Interestingly, several studies have demonstrated that gene amplification may result in protein overexpression and that amplification of the therapeutics' target gene can serve as an excellent predictive marker (i.e. HER2 and trastuzumab). However the impact of *VEGFA* gene amplification has been only recently assessed for some cancer types such as osteosarcoma, colorectal, breast and liver cancer.

Aims This study aimed to assess *VEGFA* gene amplification status using fluorescent in situ hybridization (FISH) in a large cohort of different tumour entities. Thus, we investigated the incidence of *VEGFA* amplification using a multi-tumour tissue microarray (TMA) containing 2,837 evaluable specimens from 80 different tumour entities and 31 normal tissue types. Moreover, we validated FISH analysis as reference method to evaluate *VEGFA* gene status by comparing it to comparative genomic hybridization (CGH).

Results We observed that *VEGFA* locus amplification and/or polysomy represented a small but regularly detected population in several tumour entities while was not present in normal tissues. *VEGFA* gene alterations were predominantly observed in hepatocarcinomas, adenocarcinomas of the pancreas and intestine, large cell carcinoma of the lung and in endometrium serous carcinoma. Furthermore our data demonstrated that *VEGFA* detection by FISH provided highly comparable results to those generated by CGH.

Conclusion Albeit with low percentage, *VEGFA* amplification is commonly observed across several tumour entities. Furthermore, our results demonstrated that FISH test could be used as a reliable diagnostic tool to evaluate *VEGFA* gene status in human specimens.

Keywords VEGFA · Gene amplification · 6p12 · FISH · TMA

M. Andreozzi · L. Quagliata · J. R. Gsponer · C. Ruiz ·
S. Eppenberger-Castori · L. Tornillo · L. M. Terracciano (✉)
Molecular Pathology Unit, Institute of Pathology, University Hospital of Basel, Schonbeinstrasse 40, 4003 Basel, Switzerland
e-mail: lterracciano@uhbs.ch

V. Vuaroqueaux
Oncotest GmbH, Freiburg, Germany

Introduction

Vascular endothelial growth factor A (VEGFA) is a growth factor acting as a key regulator of proliferation, survival, migration and permeability of blood endothelial cells (ECs) in both physiological and pathological angiogenesis [1]. In the human genome, the *VEGFA* gene is located on the short arm of chromosome 6 (Chr6), at position p12–21. Among the different VEGFA isoforms (up to 12 have been described), VEGFA *165* is the most prevalent one and it is overexpressed in several human solid tumours [1].

Vascular endothelial growth factor A tumour-mediated angiogenesis is a critical step in both tumour growth and metastasis formation [1]. The majority of studies using either

immunohistochemistry (IHC) or other approaches for the detection of VEGFA protein and mRNA in tumour tissues as well as the detection of VEGFA plasma levels, all point towards a significant negative prognostic survival effect of increased VEGFA expression in cancer patients [2]. Subsequently, in the last decade several anti-angiogenic agents have been developed and some, such as Bevacizumab® (BV) (Roche, Basel, Switzerland), a recombinant humanized monoclonal antibody that prevents VEGFA binding to both its receptors (VEGFR1 and VEGFR2), are in clinical use [3, 4]. Nevertheless, the clinical benefits of anti-angiogenic therapy are limited and recent data report that a number of tumours, although initially responding to VEGFA inhibition, become resistant at later stages of treatment [1]. Furthermore, it has been demonstrated that in some experimental models, anti-angiogenic therapy may even increase tumour aggressiveness and metastatic burden [5]. Therefore, there is an urgent need for the identification and validation of biomarkers that predict response towards anti-angiogenic treatments and allow the monitoring of therapeutic benefit and toxicity.

Recently, we analysed two independent colorectal cancer (CRC) cohorts using a non-commercial, house made fluorescent in situ hybridization (FISH) probe covering the VEGFA chromosomal region and reported that VEGFA gene locus (6p12) amplification identified a small but highly aggressive subset of CRCs [6]. In both cohorts, VEGFA gene amplification was significantly linked to more unfavourable prognostic features including advanced stage, vascular and lymphatic invasion and significantly poorer survival time [6]. Furthermore, it has been recently demonstrated that VEGFA amplification in univariate analysis was associated with poor outcomes in breast cancer, mainly in Her2+ as well as triple negative breast cancers [7]. However despite the fact that the evaluation of VEGFA gene status has been shown to be an important parameter to predict disease progression, a comprehensive study investigating its status in different tumours is still missing.

In this work, we investigated the incidence of VEGFA gene locus amplification across a large tissue collective made of 80 different cancer entities taking advantage of a multi-tumour tissue microarray (TMA) with 2,837 evaluable specimens. In addition, we compared our FISH data with comparative genomic hybridization (CGH) analysis data, in order to investigate the effectiveness and reliability of our FISH test as a diagnostic tool to assess the VEGFA gene status.

Materials and methods

Tissue microarrays

A pre-existing set of six TMAs with 3,417 tissue samples from 80 tumour entities and 31 normal tissue types was used in this study [8, 9]. All tissue samples were retrieved from the archives of the Institute of Pathology (University of Basel, Switzerland) and were reviewed by experienced pathologists (L.T. and L.M.T.). Briefly, to construct the TMAs, tissue samples were fixed in buffered 4 % formalin and embedded in paraffin. H&E-stained sections were made from each selected primary block (named donor blocks) to define representative tissue regions. Tissue cylinders (0.6 mm in diameter) were then punched from the region of the donor block with the use of a custom-made precision instrument (Beecher Instruments, Silver Spring, USA). Afterwards, tissue cylinders were transferred to a 25 × 35 mm paraffin block to produce the TMAs. The resulting TMA block was cut into 3-μm sections that were transferred to glass slides by use of the paraffin sectioning aid system (Instrumedics, Hackensack, USA). Sections from the TMA blocks were used for FISH analysis. The use of the clinical samples from the biobank of the Institute of Pathology for the TMA construction was approved by the Ethics Committee of the University Hospital of Basel (EKBB). In addition, we used a second set of three TMAs comprising a total of 194 tissue samples obtained from 24 different human tumour-derived xenograft types in nude mice models (Oncotest GmbH, Freiburg, Germany).

Preparation of FISH probe

The genomic BAC clone RPCIB753M0921Q (imaGENES GmbH, Berlin, Germany), which covers the VEGFA genomic region (6p12) 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 manufacturer's instructions. BAC identity was verified by sequencing using 1 μg of isolated DNA and 20 pmol of SP6, using T7 primers (EuroFins MGW Operon, Ebersberg, Germany). 1 μg of isolated BAC-DNA 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, Lucerne, Switzerland). Labeling reaction was assessed by usage of a Nanodrop assay (Nanodrop, Wilmington, USA). The labelled DNA was purified by using the FISH Tag DNA Kit (Invitrogen, Lucerne, Switzerland).

FISH analysis

TMAs were subjected to pre-treatment as previously described [10]. FISH probe was applied and after a denaturation step (10 min at 75 °C), the slides were incubated over night at 37 °C. Slides were afterward washed with

washing buffer (2X SSC, 0.3 % NP40, pH 7–7.5) and slides were counterstained with DAPI I solution (1,000 ng/ml) (Vysis Inc. Abbott Molecular, Abbott Park, USA). As reference, a Spectrum Green-labelled Chr6 centromeric probe (CEP6) (Vysis Inc. Abbott Molecolar, Abbott Park, USA) was used. Images were obtained by usage of a Zeiss fluorescence microscope using a 63X objective (ZEISS, Feldbach, Switzerland) and the Axiovision software (ZEISS, Feldbach, Switzerland). Two expert pathologists counted a minimum of 100 tumour nuclei signals in four separate regions of the tissue section independently; consensus on non-matching results was achieved.

FISH results were interpreted according to: (1) absolute Chr6 copy number or (2) the ratio *VEGFA* gene/Chr6 copy number. We classified as not amplified samples with a *VEGFA*/Chr6 ratio of <1.8; equivocal/borderline with a *VEGFA*/Chr6 ratio between 1.8 and 2.2, amplified with a *VEGFA*/Chr6 ratio higher than 2.2, as proposed by the ASCO/CAP guidelines for *HER2* amplification in breast cancer [11]. Polysomy of Chr6 was defined as an average of the Chr6 copy number. When the average was included between 2.26 and 3.75, the polysomy 6 was defined as low whereas, when the average was >3.75 the polysomy 6 was defined high [12–15]. In addition, we have further categorized the amplified samples using a second selection criterion (referred in the tables as: alternative cut off criterion) discriminating between high (i.e. >10 *VEGFA*/Chr6), average (5–10 *VEGFA*/Chr6) or low number of gene copies.

Micro-vessel density (MVD) quantification

To evaluate micro-vessel density MVD, CD31 Ab (Ventana, Cat. Num: 760-4378; pre-diluted) was used as marker of blood vessels. Immunostaining was performed using Benchmark® XT system (Ventana) according to the manufacturer's guidelines. MVD was assessed on a small cohort of CRCs (total n = 10, not amplified n = 3, polysomic n = 4, amplified n = 3). Staining evaluation and vessel counting (number of vessel per field—0.74 mm^2) were performed by two expert pathologists in a blinded manner (L.T. and L.M.T.).

Xenograft mouse

Human derived tumour tissue was cut into pieces of 4–5 mm edge length. For implantation 4–6 week old homozygous nude mice were used. Briefly, once mice were fully anesthetized, the skin and sub cutis were carefully separated by using scissors in order to form a "subcutaneous pocket" on the animal's flank, where one tumour fragment per side was placed. After equipping the animal with a unique identification tag (ear tag or transponder), the animal was disinfected and put to fresh cage for recovery from the anaesthesia. Afterwards, tumours growing subcutaneously in nude mice were explanted and prepared by removal of visible necrotic areas, large blood vessels and surrounding mouse tissue (pseudo-capsule). Immediately after, samples were snap frozen in liquid nitrogen and stored at −80 °C. Additional tissues were collected for TMAs preparation.

DNA preparation

DNA was extracted from snap frozen tumour xenografts. Tumours were digested with proteinase K at 55 °C overnight and the lysate treated with DNAse-free RNAse (Qiagen, Hombrechtikon, Switzerland). DNA was extracted by phenol:chloroform:isoamylalcohol and precipitated by ethanol. DNA pellets were then washed and resuspended in TElow. The integrity of each DNA preparation was checked on a 1.3 % agarose gel and the purity analysed using NanoDrop 2000 (Thermo Scientific, Canada).

CGH array profiling

DNA was hybridized to 244 K whole-genome Agilent arrays at Shangai Biochip Ltd, China, according standard internal procedure. In brief, 1.5 μg of DNA were fragmented by a double enzymatic digestion (*Alu*I + *Rsa*I) for 2 h at 37 °C followed by enzymes inactivation at 95 °C. Digested DNA checked on a 0.8 % agarose gel prior to labelling and hybridization. Digested DNA were labelled by random priming with CY5-dCTPs and CY3-dCTP, respectively, and hybridized at 65 °C for 40 h. The chips were scanned on an Agilent Scanner and image analysis was done using the Feature-Extraction V10.7.3.1 software (Agilent Technologies). Feature-Extraction was used for the fluorescence signal acquisition from the scans. Normalization was done using the ranking-mode method available in the Feature-Extraction V10.7.3.1 software.

Data analysis

FISH data were summarized into tables and relative percentage of gene amplification as well as chromosomal polysomy were calculated for each tumour. Concerning CGH results, array data were analysed and processed with R [16] and the Bioconductor software framework [17], using the snapCGH package [18] for normalization, as well as custom routines for data processing and visualizations. Segmentation for subsequent calling of chromosomal aberrations was done using the circular binary segmentation algorithm [19] already implemented in the Bioconductor package DNAcopy [20].

Results

VEGFA locus amplification is observed in a large subset of tumour types

In order to investigate the incidence of *VEGFA* gene locus amplification across several tumour types, we took advantage of a FISH *VEGFA* specific probe to screen a set of multi-tumour TMAs. Out of the 3,417 tissue samples composing our set of tissues (including 315 normal specimens as controls) and representing 80 different tumour entities, 2,837 were evaluable by FISH. Causes of exclusion were either the absence of tissue punch or poor hybridization quality.

Our analysis revealed *VEGFA* amplification in 10 different tumour types with prevalence rates between 1.5 % and 5 % (Table 1 and Supplementary Table 1 for complete list). Representative pictures of amplified samples as detected using FISH are shown in Fig. 1. Since an increased gene copy number can also be caused by polysomy instead of focal amplification, we further investigated the polysomic status of Chr6, as defined by Ma et al. [15]. We observed polysomy of Chr6 in 18 of the 80 analysed tumour types (Table 1 and Supplementary Table 1). In addition, we have further categorized the amplified samples using another selection criterion discriminating between high (i.e. >10 *VEGFA*/Chr6), average (5–10 *VEGFA*/Chr6) or low number of gene copies (Supplementary Table 2 and Supplementary Table 3). Some tumours such as gallbladder adenocarcinoma, squamous cell carcinoma of the larynx, ovary (both serous and endometrioid carcinoma subtypes) and stomach adenocarcinoma of intestinal type showed both amplified and polysomic cases with serous endometrium carcinoma and large cell lung carcinoma having the highest incidence of 16 and 10 % of cases, respectively. Of note, in our investigation none of the breast cancer samples from either ductal (n = 30), medullary (n = 50) or mucinous (n = 16) subtypes were found to feature *VEGFA* focal amplification, conversely polysomy was observed in all of them, with an incidence of 3, 2 and 6 %, respectively (Table 1).

Furthermore, across the all tumour species analysed, the sum of cases with either focal *VEGFA* gene amplification and/or polysomy of Chr6 revealed prevalence rates up to 16 % (endometrium–serous carcinoma, Table 1). As expected neither genomic amplification of *VEGFA* nor polysomy was detected in any of the normal control samples (Supplementary Table 1).

Finally, in order to investigate whether *VEGFA* amplification induces increased vessel density as a result of VEGFA overproduction, we performed MVD IHC-based evaluation on a small cohort of CRCs (total n = 10, not amplified n = 3, polysomic n = 4, amplified n = 3) specimens using CD31 as marker of blood vessels. Representative pictures of CD31 staining are presented in Fig. 1a. Our analysis revealed that CRC samples harbouring *VEGFA* amplification present with increased MVD compared to both not amplified and polysomic specimens (Fig. 2b).

Table 1 Rates of *VEGFA* gene locus amplification in selected tumour types as evaluated by FISH

Organ	Tumor type	Amplification (%)	Polysomy (%)	Amplification + polysomy (%)
Breast (n = 96)	Ductal cancer (n = 30)	0 (n = 0)	3.3 (n = 1)	3.3 (n = 1)
	Medullary cancer (n = 50)	0 (n = 0)	2 (n = 1)	2 (n = 1)
	Mucinous cancer (n = 16)	0 (N = 0)	6.2 (n = 1)	6.2 (n = 0)
Gall bladder	Adenocarcinoma (n = 36)	2.7 (n = 1)	5.5 (n = 2)	8.2 (n = 3)
Endometrium	Serous carcinoma (n = 31)	3.2 (n = 1)	12.9 (n = 4)	16.1 (n = 5)
Esophagus	Squamous cell carcinoma (n = 32)	0 (n = 0)	3.1 (n = 1)	3.1 (n = 1)
Kidney	Papillary cancer (n = 24)	4.1 (n = 1)	0 (n = 0)	4.1 (n = 1)
Larynx	Squamous cell carcinoma (n = 31)	3.2 (n = 1)	3.2 (n = 1)	6.4 (n = 2)
Liver	Hepatocellular carcinoma (n = 68)	1.5 (n = 1)	2.9 (n = 2)	4.4 (n = 3)
Lung (n = 125)	Large cell cancer (n = 20)	5 (n = 1)	5 (n = 1)	10 (n = 2)
	Squamous cell carcinoma (n = 39)	0 (n = 0)	2.6 (n = 1)	2.6 (n = 1)
	Adenocarcinoma (n = 66)	0 (n = 0)	1.5 (n = 1)	1.5 (n = 1)
Ovary (n = 71)	Endometroid cancer (n = 33)	3 (n = 1)	3 (n = 1)	6 (n = 2)
	Serous cancer (n = 38)	2.6 (n = l)	5.2 (n = 2)	7.8 (n = 3)
Pancreas	Adenocarcinoma (n = 44)	4.5 (n = 2)	0 (n = 0)	4.5 (n = 2)
Prostate	Adenocarcinoma, castration-resistant (n = 34)	0 (n = 0)	2.9 (n = 1)	2.9 (n = 1)
Stomach	Intestinal adenocarcinoma (n = 42)	4.7 (n = 2)	2.3 (n = 1)	7 (n = 3)
Uterus, cervix	CIN III (n = 24)	4.1 (n = 1)	0 (n = 0)	4.1 (n = 1)

Fig. 1 Visualization of *VEGFA* gene locus 6p12 amplification by FISH. The *green* and *red* signals correspond to centromere 6 and *VEGFA* gene region, respectively. **a** Representative pictures of gastric cancer with normal and **b** amplified *VEGFA* gene status. **c** Representative pictures of lung cancer with normal and **d** amplified *VEGFA* gene status. (Color figure online)

Gastric cancer with normal VEGFA gene status

Gastric cancer with amplified VEGFA gene status

Lung cancer with normal VEGFA gene status

Lung cancer with amplified VEGFA gene status
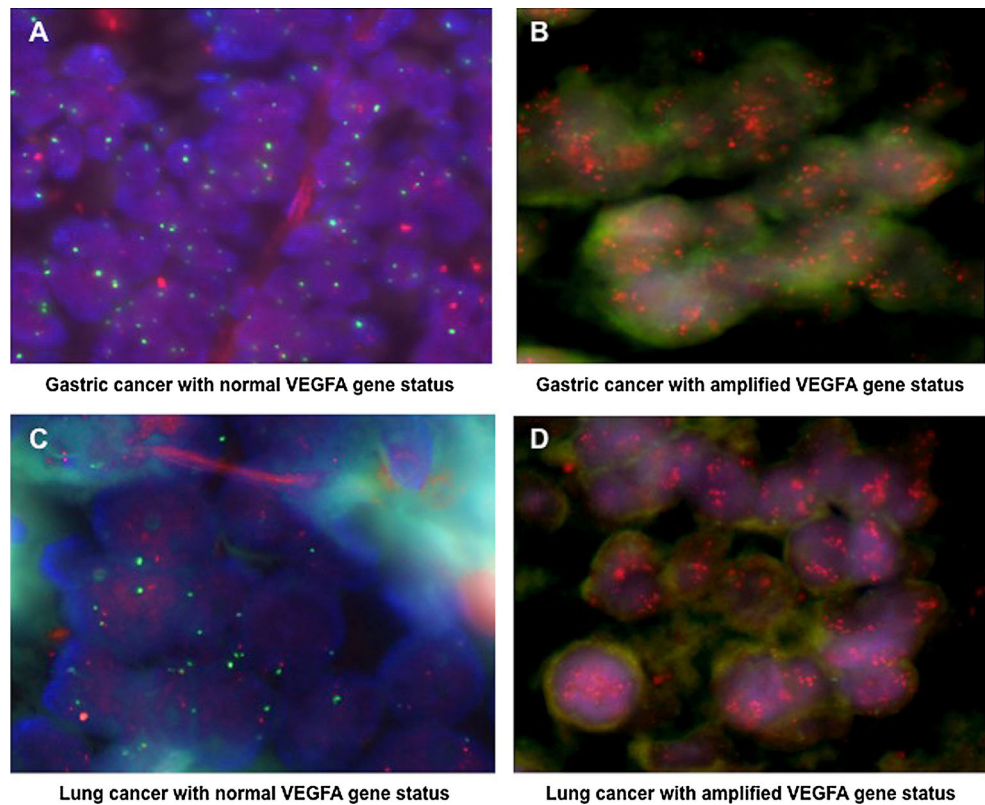
Analysis of the *VEGFA* gene amplification by FISH on TMA and high resolution CGH microarrays in human tumour xenografts

To confirm the presence of the *VEGFA* gene amplification by an independent method, we analysed 194 fresh-frozen tissue specimens obtained from human tumour-derived xenografts using CGH. The *VEGFA* gene locus status of these 194 models was previously analyses using corresponding FFPE samples by means of our FISH probe. FISH analysis revealed 4 samples harbouring *VEGFA* amplification namely specimens obtained from gastric, breast, non-small-cell lung cancer (NSCLC) and sarcoma, with prevalence rates between 5 and 16 % (Table 2 and Supplementary Table 4 for complete listing). In addition, as for the human multi tumour-TMA above described, we have further categorized the amplified samples using a second selection criterion discriminating between high (i.e. >10 *VEGFA*/Chr6), average (5–10 *VEGFA*/Chr6) or low number of gene copies (Supplementary Table 5 and Supplementary Table 6). FISH results were confirmed for all 4 samples by CGH. Figure 3 displays representative high-resolution CGH microarray profiles of one melanoma without amplification and two *VEGFA* amplified tumours (a gastric cancer and NSCLC). By means of CGH analysis, we also investigated the structure of the *VEGFA* amplicon. Whereas the gastric cancer sample (Fig. 3b) showed a larger amplified region (approx. 20 Megabases), the NSCLC sample (Fig. 3c) was characterized by a focal amplicon covering a small region of 5.85 Mb.

In addition, we investigated the copy number status of Chr6 and observed polisomy in 6 of the 24 analysed tumour types such as bladder, colon and small cell lung cancer, pleura mesothelioma, NSCLC and NSCLC of the squamous cell subtype (Table 2 and Supplementary Table 4). Moreover our analysis revealed that among all tested tumour types only NSCLC, showed both amplified and polysomic cases.

Discussion

In this study for the first time, we comprehensively surveyed the *VEGFA* gene status in a broad range of human cancers. Our analysis reviled that, albeit with low incidence, *VEGFA* amplification was detected regularly in several tumour entities, whereas it was always absent in normal tissues. Furthermore we also validated the reliability of FISH test as a diagnostic tool to assess the *VEGFA* gene status.

Given its role in the growth and development of different tumour types, VEGFA is considered to be one of the most attractive targets in cancer therapy. We previously reported that the presence of *VEGFA* gene locus (6p12) amplification
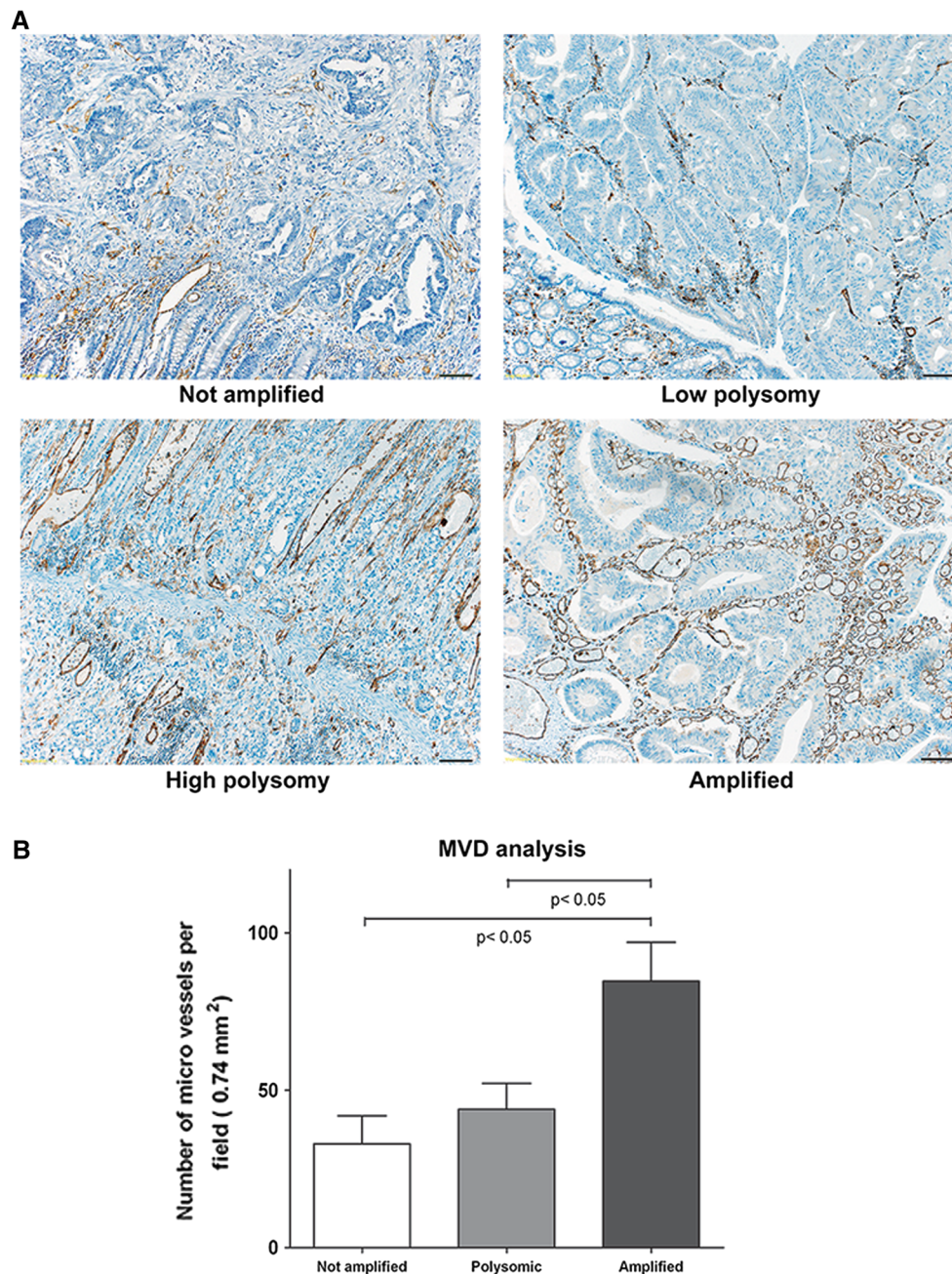
Fig. 2 Micro-vessels density is increased in CRC *VEGFA* amplified samples. **a** Representative pictures of CD31 stained CRC specimens. *Scale bar* correspond to 100 μm. **b** MVD quantification

characterizes a small but considerable aggressive subgroup of CRC [6]. On a total number of 1,501 primary unselected CRC patients we observed *VEGFA* gene locus amplification in 3–6 % of two independent cohorts, respectively. The genomic aberration, in both cohorts, was significantly associated with unfavourable prognostic features and poorer survival time [6]. Afterwards, we wanted to address whether *VEGFA* locus amplification is a phenomenon limited to CRC or is a common alteration in several tumour types. The study was as well motivated by the hypothesis that the *VEGFA*

gene amplification could be useful as predictive biomarker of clinical response in patients considered for BV or tyrosine kinase inhibitors treatment in addition to chemotherapy. Here, we assessed the *VEGFA* gene copy number status in a total of 2,837 tumours and 315 normal tissue specimens. *VEGFA* amplification was observed in different types of malignancies, such as those of the lung (large cell cancer), stomach (adenocarcinoma intestinal type), ovary (endometroid as well as serous carcinoma), gall bladder (adenocarcinoma), uterus cervix, endometrium (serous carcinoma),

Table 2 Rates of *VEGFA* gene locus amplification in selected human tumour xenografts as evaluated by FISH

| Organ	Tumor type	Amplification (%)	Polysomy (%)	Amplification + polysomy (%)
Bladder	Bladder cancer (n = 6)	0	16 (n = 1)	16 (n = 1)
Breast	Mammary cancer (n = l2)	8 (n = 1)	0	8 (n = 1)
Intestine	Colon cancer (n = 29)	0	3 (n = 1)	3 (n = 1)
Lung (n = 43)	Non small cell lung cancer (n = 20)	5 (n = 1)	15 (n = 3)	20 (n = 4)
	Non small cell lung cancer, epidemoid (n = 11)	0	27 (n = 3)	27 (n = 3)
	Small cell lung cancer (n = 5)	0	20 (n = 1)	20 (n = 1)
	Pleuramesothelioma (n = 6)	0	16 (n = 1)	16 (n = 1)
Skin	Sarcoma (n = 10)	10 (n = 1)	0	10 (n = 1)
Stomach	Gastric cancer (n = 6)	16 (n = 1)	0	16 (n = 1)
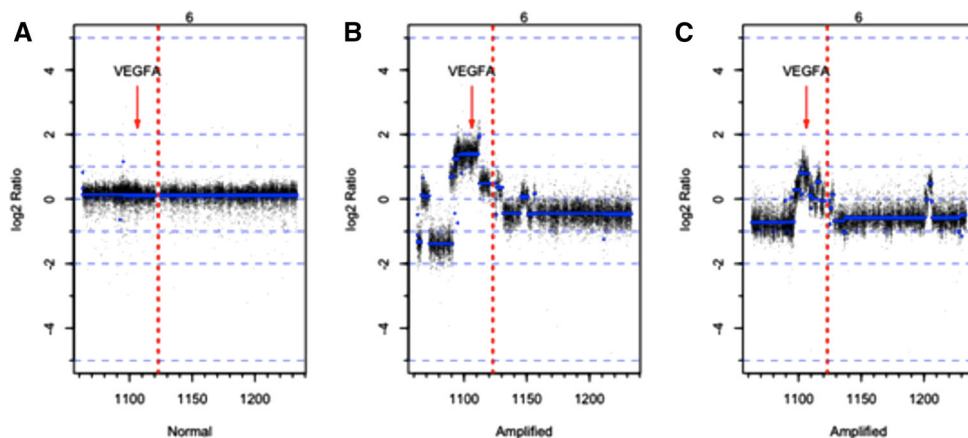
Fig. 3 Representative CGH profiles of the VEGFA genomic region. Depicted area focus on Chromosome 6—*VEGFA* locus. **a** Melanoma sample with normal *VEGFA* gene copy number. **b**, **c** Gastric cancer and NSCLC samples showing *VEGFA* gene amplification, respectively. *Red arrows* point towards the *VEGFA* gene position at 6p12. The vertical dotted *red line* denotes the position of the centromere of the Chromosome. Horizontal *blue lines* represent the segments inferred by the CGH calling algorithm. (Color figure online)

kidney (papillary renal cancer), larynx (squamous cell carcinoma) and liver (hepatocellular carcinoma).

The prevalence rates of *VEGFA* amplification in these tumours varied between 1.5 and 5 %. Based on CGH profiling studies, gain of Chr6p has been described in different tumour types, such as hepatocellular carcinoma, osteosarcoma, retinoblastoma, Merkel cell carcinoma and carcinosarcoma [21]. Additionally, focal gain and amplification of the *VEGFA* gene was also reported in 4 out of 103 hepatocellular carcinomas [22] and in 4 out of 371 lung cancers [23, 24]. This is in line with our findings from the multi-tumour TMA, in which we also report the genomic amplification of the *VEGFA* gene in these two tumour types at comparable frequency. Thus, our results are in line previously reported genomic profiling studies, which indicated the presence of specific genomic aberrations at rather low prevalence, but consistently observed throughout different tumour types.

Interestingly, some tumour types that did not show the *VEGFA* gene amplification were characterized by the polysomy of Chr6. Among these we observed breast cancer samples (mucinous and ductal cancer) and esophageal carcinoma specimens (squamous cell carcinoma). However, the impact of polysomy in cancer and its usage as a diagnostic marker is controversially discussed [25, 26]. Thus, additional studies specifically addressing this question are needed. Moreover, for the future we seek to correlate the *VEGFA* gene locus status (e.g. amplification or polisomy) with VEGFA protein levels.

Furthermore, we observed that *VEGFA* amplification was exclusively present in malignant epithelial tumours, whereas haematological neoplasias as well as soft tissue sarcomas were completely negative for such genomic aberrations. Concerning haematological neoplasias, our data are in line with recently reported results [27]. About soft tissue sarcomas, taking advantage of new generation

sequencing (NGS), previous work has globally investigated the incidence of genomic aberrations in soft tissue sarcomas, observing *VEGFA* amplification in about 1.9 % of tested specimens (n = 207) [28]. Furthermore, VEGFA overexpression has been reported to be a frequent event in Ewing's sarcoma, being associated with increased angiogenesis, MVD and modulating patient's survival [29, 30]. The discrepancy observed concerning the *VEGFA* status with our results could be explained by relative small number size of our investigated cohort of such cases. Interestingly, in osteosarcoma, *VEGFA* amplification has been ranked among the most frequent genetic amplifications being observed in more than 50 % of analysed samples [31]. However, unfortunately our TMA lacks of any osteosarcoma samples, thus we cannot make any direct comparison to previously reported data for this tumour entity. All together, these examples suggest that the role of *VEGFA* amplification in specific sub-class of malignancies deserves further and accurate investigations.

In addition, as *VEGFA* amplification in osteosarcoma has been associated to an increase in MVD due to VEGFA protein overproduction [31], we seek to evaluate MVD on a small cohort of CRCs comprising amplified samples. In accordance to previously reported data [31], we observed that CRC samples harbouring *VEGFA* amplification present with increased MVD compared to both not amplified and polysomic specimens. MVD density status could modulate the response to antiangiogenic therapy [32], thus our results further underline the importance of using *VEGFA* status as a potential tool to stratify patients. The use of anti-VEGFA treatments and the partial success of these agents in CRC, NSCLC and renal carcinoma (RCC), emphasizes the importance for further stratification of patients in potential responder and non-responders before therapy assignment. The evaluation of gene amplification and deletion in tumour specimens has been proven to serve as a meaningful tool to predict response to therapy. For example, it has been clearly demonstrated that breast cancer patients harbouring *HER2* amplifications are highly sensitive to therapeutic agents targeting HER2 protein (e.g. trastuzumab), while patients without *HER2* amplification receive little benefit from such a treatment [7]. Thus, it is tempting to speculate that *VEGFA* gene amplification might also be a useful biomarker for sensitivity towards VEGFA-targeted therapies, as for *HER2* amplification. Further studies specifically addressing this point are needed.

Most of our results were obtained using a FISH-based analysis. Importantly, in this work we also tested the consistency of our FISH probe by directly comparing FISH-obtained data to CGH results acquired analysing tumour xenograft samples. The two methodologies displayed high concordant results thus fostering the reliability of FISH data. To conclude, our results demonstrate that *VEGFA* gene locus amplification is not limited to CRC but is broadly observed across different tumour species. Moreover, we demonstrated that our FISH test is robust and reliable for clinical diagnostic tool, independently of tissue type, and clearly facilitates the identification of the *VEGFA* gene locus amplification as well as the polysomy of Chr6.

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