VEGFA gene locus (6p12) amplification and colorectal cancer: implications for patients' response to therapy

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Abbreviations

5-FU 5-Fluorouracil

AJCC American Joint Committee on Cancer

APC Adenomatous polyposis coli

BAC Bacterial artificial chromosome

BER Base excision repair

CIMP CpG island methylator phenotype

CIN Chromosome instability

CGH Comparative genomic hybridization

CR complete response

CRC Colorectal Cancer

DLL4 Delta like ligand 4

ECM extracellular matrix

EPC endothelial progenitors cells

ERBB2 erythroblastosis oncogene B

ESR1 binding to the estrogen receptor-1

FAP Familial adenomatous polyposis

FDA Food and Drug Administration

FGF-2 Fibroblast growth factor-2

FFPE Formalin-Fixed, Paraffin-Embedded

FISH Fluorescent in situ Hybridization

FOLFIRI 5-FU plus leucovorin plus irinotecan

FOLFOX 5-FU plus leucovorin plus oxaliplatin

HBV Hepatitis B virus

HCV Hepatitis C virus

HCC Hepatocellular carcinoma

HE Hemotoxylin and Eosin Stain

HER2 human epidermal growth factor receptor 2

HGF hepatocyte growth factor

hMLH Human MutL Homolog

hMSH Human MutS Homolog

HNPCC Hereditary non-polyposis colorectal cancer

HP hyperplastic polyps

hPMS Human Post Meiotic Segregation

IDL Insertion/deletions loop

IHC Immunohistochemistry

KRAS Kirsten rat sarcoma viral oncogene homolog

LOH Loss of Heterozygosity

MAPK Mitogen-Activated Protein Kinase

mCRC Metastatic colorectal cancer

MGMT O-6-methylguanine-DNA methyltransferase

MMP Metalloproteinase

MMR Mismatch Repair

MSI Microsatellite Instability

MSS Microsatellite stable

MYC Myelocytomatosis viral oncogene homolog

NCI National Cancer Institute

NICD Notch intracellular domain

NSCLC Non small cell lung cancer

OCR one close relative

ORR objective response rate

OS overall survival

PCR Polymerase Chain Reaction

PD progressive disease

PDGF platelet-derived growth factor

PFS progression – free survival

PIK3CA Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha

PLC- γ phospholipase C gamma

PR partial response

Rb Retinoblastoma

RCC renal cell cancer

RECIST Response Evaluation Criteria in Solid Tumours

ROS reactive oxygen species

SD stable disease

SSA sessile serrated adenoma/polyp

TGF-β Tumour Growth Factor β

TCR two close relative

TSA traditional serrated adenomas

uPA uroplasminogen activator

UICC International Union Against Cancer

VEGF vascular endothelial growth factor

VPF vascular permeability factor

VEGFR vascular endothelial growth factor receptor

WHO World Health Organization

XELOX Capecitabine plus oxaliplatin

1. GENERAL INTRODUCTION

1.1 Cancer: an overview

Cancer is a leading cause of death worldwide. In normal tissue when the cells become old or damaged, they undergo apoptosis. In healthy tissue, the ratio between cell growth and cell death is in balance but it is completely lost in cancer. Cancer is essentially a genetic disease; the DNA can get damaged, acquiring mutations that affect normal cell growth, division and apoptosis. When this occurs, cells may start to grow uncontrollably and form a mass of tissue called tumor (1). Multiple types of cancer susceptibility genes have been identified. Although they differ in various tumors, the loss or abnormal functions of them will render most cancers to acquire the same set of capabilities during their development. These capabilities are: 1. self-sufficiency in growth signals; 2. evading apoptosis; 3. insensitivity to antigrowth signals; 4. tissue invasion and metastasis; 5. inducing angiogenesis; and 6. limitless replicative potential (2). Cancer susceptibility genes can be generally categorized into three classes-gatekeepers, caretakers and landscapers (3).

1.1.1 Gatekeepers

Gatekeepers include oncogenes and tumor suppressor genes (4). The mutations of both of them operate similarly at the physiological level: they drive the neoplastic process by increasing tumor cell number through the stimulation of cell growth or the inhibition of cell death or cell cycle arrest (5).

Oncogenes

Oncogenes are frequently activated by gain of function mutations or fusions with other genes, or they are aberrantly expressed due to amplification, increased promoter activity, or protein stabilization (6), hence they play important roles in diverse signaling pathways that are involved in various stages of human cancer initiation, progression, angiogenesis and metastasis (4). An activating somatic mutation in one allele is generally enough to confer a selective growth advantage on the cell. A large number of cellular oncogenes have been identified to play a role in colorectal cancer, such as *KRAS, MYC, SRC*, *β-catenin, BRAF*. Systematic mutational analyses showed that a minimum of 30% of CRCs contain at least one mutation in the tyrosine kinase family (7), and 32% of CRCs contain a mutation in the *PIK3CA* gene (8).

Tumor suppressor genes

As the name indicates, tumor suppressor genes' function is to suppress neoplastic cell growth. Mutation in these genes compromises this growth-suppressor mechanism by removing the inhibitions from the cell and result in uncontrolled growth. Examples are the genes *Rb1* (the first isolated human tumor suppressor gene) *p53* and *p16*. Tumor suppressor genes regulate diverse cell activities, including cell cycle checkpoint responses, detection and repair of DNA damage, protein ubiquitination and degradation, mitogenic signaling, cell specification, differentiation and migration, and tumor angiogenesis (9). Mutations (both point mutations and deletions) are not the only way through which a tumor suppressor gene can be inactivated. In recent years, it has become obvious that epigenetic changes, and haplo-insufficiency, can also help to switch off tumor suppressor genes.

1.1.2 Caretakers

Caretakers, or stability genes, act to maintain the genomic integrity of the cell by regulating DNA repair mechanisms, chromosome segregation, and cell cycle checkpoints (3). Caretaker defects lead to genetic instabilities that contribute to the accumulation of mutations in other genes, such as oncogenes and tumor suppressor genes that directly affect cell proliferation and survival, thus promoting tumorigenesis indirectly (10).

1.1.3 Landscaper

Landscaper defects do not directly affect cellular growth, but generate an abnormal stromal environment that contributes to the neoplastic transformation of cells (10). Their dysfunction or deregulation can disrupt normal tissue homeostasis, reduce host immune surveillance and defense, induce angiogenesis and inflammation, and promote tumor growth and migration (11). Some examples of landscaper genes are metalloproteinases (MMPs) uroplasminogen activator (uPA), friboblast growth factor-2 (FGF-2) and platelet-derived growth factor (PDGF).

1.2 General background on colorectal cancer

The colon is part of the digestive system, which is responsible for the final stages of the digestive process. The colon consists of ~10 crypts, each of which contains several thousand differentiated cells and a small number (between 1 and 10) of stem cells. Stem cells reside at the bottom of the crypts and divide slowly and asymmetrically, whereas differentiated cells divide rapidly and travel to the top of the crypt, where they undergo apoptosis. Each day, a total of approximately 10 cells are shed by the colon and have to be replaced, and each cell division represents a risk for cancer because of the mutational events that can occur during normal DNA replication and chromosome segregation (12). Colorectal cancer is the third most common cause of cancer-related death in the western world. Majority of CRC arises from malignant transformation of an adenomatous polyp. Based on familial clustering studies, it is estimated that 20-30% of CRCs have a potentially identifiable genetic cause(13).

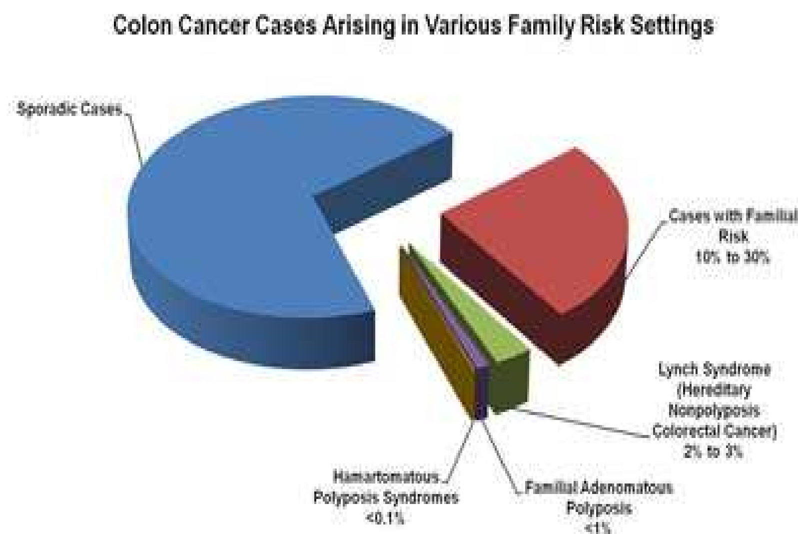


Figure 1: Frequencies of different types of CRC.

In the European Union the incidence and mortality of CRC are 58/100 000 and 30/100 000 per year, respectively. The American Cancer Society's most recent estimates for the number of colorectal cancer cases in the United States for 2011 are: 101,700 new cases of colon cancer and 39,510 of rectal cancers with 49,380 deaths. Risk factors according to the American Cancer Society for the development of CRC are:

a. **Age**: peak incidence in the 6Th/7Th decade.

b. **Nutrition**: a diet that is high in red meat, processed meats and not enough fruits and vegetables consumption is a colon cancer risk.

c. **A family history of colorectal cancer**: A family history of colorectal cancer or adenomatous polyps increase the risk of colorectal cancer.

d. **Member of certain racial or ethnic groups**: African Americans get colon cancer more often than other racial groups in the U.S. and are nearly twice as likely to die from it.

e. **Inherited conditions such as familial adenomatous polyposis**, which causes the development of 100-1000 of polyps in the colon, also raises the risk of colorectal cancer.

f. **Inflammatory bowel disease**: such as ulcerative colitis and Crohn's disease (1).

Prognostication of newly diagnosed CRC predominantly relies on stage or anatomic extent of disease based on the International Union Against Cancer (UICC-TNM) (14) and American Joint Committee on Cancer (AJCC) (15) staging classifications. However, CRC should be regarded as a heterogeneous, multi-pathway disease, an observation substantiated by the fact that histologically identical tumours may have neither similar prognoses nor response to therapy (16). CRC progresses through a series of clinical and histopathological stages ranging from single crypt lesions through small benign tumors (adenomatous polyps) to malignant cancers (carcinomas). Stages are usually defined by TNM classification, where **T** describes the size of the tumor and whether it has invaded nearby tissue, **N** describes regional lymph nodes that are involved; **M** describes distant metastasis (spread of cancer from one body part to another). The depth of tumor invasion defines the T stage and increases from T1 (invasion of the submucosa) to T4 (invasion into the serosa or adjacent structures) (17). Grading considers the architectural arrangement and the differentiation grade of the neoplastic cells. Grade 1 is the most differentiated, with well-formed tubules and the least nuclear polymorphisms and mitoses. Grade 3 is the least differentiated, with only occasional glandular structures, pleomorphic cells and a high incidence of mitoses. Grade 2 is intermediate between Grades 1 and 3 (18).

Colorectal cancer is thought to be initiated by inactivation of the adenomatous polyposis coli (APC) tumour-suppressor pathway in a cell somewhere within the colon. In ~85% of cases, the APC gene is mutated, whereas the β-catenin gene is

mutated in approximately half the remaining cases. The β-catenin protein is regulated by APC, and mutations in either gene have the same physiological effects. In few colorectal cancers without known mutations in APC or β-catenin, it is likely that other genes in the same pathway, or unusual mutations of APC or β-catenin, are to blame (12). The crypt in which the APC-mutant cell resides becomes dysplastic as abnormal cells accumulate to slowly produce a polyp. The development of a large polyp probably requires the acquisition of further mutations — for example, in the KRAS or BRAF oncogenes. Subsequently, 10–20% of these large polyps will progress to cancer by acquiring additional mutations in genes of the TGF-β pathway, the p53 pathway and other pathways that are still being actively researched. Individual cells within the bulk population that acquire such mutations are clonally selected on the basis of their improved fitness. This clonal selection creates a bottleneck in the development of the cancer, as mutations within this individual cell — advantageous and random — become fixed in future generations as its daughters overtake the rest of the cells in the tumour. Despite clonal selection, tumours are heterogeneous because of the continuing accrual of genetic changes. The whole process — from the occurrence of the first APC mutation to the development of a metastatic cancer — generally takes 20–40 years, and genetic instability develops at some point during this time (12).

1.3 Etiology

1.3.1 Genetic factors and family history

CRC can be diveded into familial and sporadic cases, with sporadic cases accounting for the majority. Epidemiological studies have long indicated that the predisposition for CRC in the population may account for a substantial fraction of CRCs. First-degree relatives of patients with sporadic CRC have been shown to have to three-fold increased risk for both colorectal adenomas and CRC compared to the general population (19).

1.3.2 High-risk familial CRC

Several high-risk inherited forms of CRC syndromes are known and the predisposing genes have been characterized for the majority of the cases. These syndromes include familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC).

1.3.3 Low-risk familial CRC

Low-risk CRC families can be classified into two categories, two close relative (TCR) and one close relative (OCR). TCR refers to families having two affected first-degree relatives, and OCR refers to families having only one affected first-degree relative with an early age of tumor onset. TCR are in general MSI-negative. Epidemiological studies demonstrated that risk individuals in TCR and OCR damilies have a lifetime risk of CRC of 10-20% and 20-40%, respectively. (20).

1.3.4 Non-genetic risk factors

Epidemiological studies have recognized the contribution of non-genetic factors in the etiology of CRC for many years. Compelling evidence is given by the observation that, when Japanese have moved from their home country of low incidence to the USA of high incidence, their CRC risk increases to the same as that of their adopted country in 20-30 years. It should be pointed out the association of some dietary components (fiber, meat and fat) with CRC risk is still controversial, which requires large meta-analyses and prospective cohort studies in the future.

1.4 Molecular aspects of CRC

1.4.1 Genomic instability

Most human malignacies are recognazed cytogenetically by marked aneuploidy and complex chromosomal rearrangements that include non-reciprocal translocations, DNA fragmentation and chromosome fusions (21). This chromosome instability (CIN) can be found in approximately 85% of CRCs. The remaining 15% of CRCs show a different genomic instability that is characterized by mismatch repair (MMR) deficiency. As this instability was first found in short repetitive DNA sequences known as microsatellites, it is named microsatellites instability (MSI, also known as MIN) (22). In addition, the third type of genomic instability, base excision repair (BER) instability, was identified recently to operate in a small proportion of CRCs (23).

1.4.2 Chromosome instability (CIN)

Polyploidy and aneuploidy are both frequent features of cancer cells (24). The chromosomal aberrations found in CRC are so complex that is too difficult to sum up all the chromosomal changes with a few representative karyotypes. The chromosomal instability — an accelerated rate of gains or losses of whole or large

portions of chromosomes (CIN) phenotype, which accounts for 85% of sporadic cases, exhibits gross chromosomal abnormalities such as aneuploidy and loss of heterozygosity (LOH) (12). Disruption of mitotic checkpoint assembly genes can lead to CIN because checkpoint defective cells can complete mitosis with inappropriately aligned chromosomes (12). Data strongly support the hypothesis that carcinogen exposure determines the type of instability in CIN cancers (7). Experimental evidence indicates that aneuploidy arises in cancers because of CIN. It refers to the rate with which whole chromosomes or large portions of chromosomes are gained or lost in cancers. Although it is believed that CIN is a process that drives most cancers to aneuploidy, the presence of aneuploidy per se does not imply the existence of CIN. There are several ways in which a cancer cell could become aneuploid in the absence of CIN. First, the cell could have gone through many more cell divisions than normal cells within a tissue, without a difference in the rate of chromosomal change per division. It is known that gross chromosomal changes occur in normal cells, so this possibility is a real one. Second, it is possible that the cancer cell was exposed to an endogenous or exogenous agent that induced aneuploidy, perhaps by interfering with proper spindle formation. The resultant daughter cells would be aneuploid, but not chromosomally unstable, in future generations. It is also possible that cancer cells develop chromosomal changes at the same rate as normal cells, but that gross chromosomal changes are lethal to the latter, but not the former. This possibility is consistent with the idea that oncogene and tumour-suppressor-gene mutations often seem to reduce apoptosis in the cancer cell. Although the ability to survive chromosomal changes might be scored as CIN in some assays, the mechanisms underlying this process would be very different. For example, mutations in genes that control the G2 checkpoint might result in CIN by stimulating chromosomal changes, whereas mutations in genes that control apoptosis would have no effect on the rate at which such changes occur if the rate in all cells was measured, rather than simply in surviving cells. CIN has only been formally shown for the gain or loss of whole or large portions of chromosomes in cancers. There is no assay at present that can reliably measure the rate of other chromosomal changes, such as rearrangements, deletions, insertions, inversions and amplifications .These latter changes are at least as common as losses or gains of whole chromosomes.

1.4.3 Microsatellite instability (MSI)

The primary function of post-replicative mismatch repair (MMR) is to eliminate base-base mismatches and insertion/deletions loops (IDLs) that arises as a consequent of DNA polymerase slippage during DNA synthesis (25, 26).

The hallmark of MSI is widespread mutation of insertions and deletions in repetitive DNA sequences know as microsatellites. Microsatellites are scattered throughout the human genome and comprise tandemly repeated DNA sequences of 1-6 bases, most commonly as (CA)n, so are very prone to undergo slippage and replication errors in MMR-deficient cells (27). MSI was initially described in association with HNPCC (28) and soon found in 12-15% of sporadic CRCs (29). In addition, MSI is also detected in extra colonic cancers, such as gastric, breast, endometrial and upper urinary tract carcinomas (30, 31). The “mutator phenotype” conferred by MSI is beleved to contribute to the initiation and promotion of multistage carcinogenesis (32), but the simple inactivation of an MMR gene is not thought to be by itself a transforming event. There are many targets of MSI, especially those conteining repetitive sequences in the coding region. In 1995 TGFβRII was reported as the first target gene for instability in MSI tumors (33). Since then, many other important genes have been identified, including apoptosis –releted genes like BAX and Caspase-5 (34), the cell cycle regulator genes E2F4, TCF4 and PTEN (35, 36), the DNA repair genes hMSH3 and hMSH6 (37). In 1997, a National Cancer Institute (NCI) Workshop meeting held in Bethesda proposed a panel of five markers for the uniform detection of MSI tumors. This panel of markers includes two mononucleotides, BAT 25 and BAT 26, and three dinucleotide repeats, D2S123, D5S346 and D17S250 (38). Tumors with instability at two or more of these markers were defined as being MSI-high (MSI-H), whereas those with instability at one repeat or showing no instability were defined as MSI-low (MSI-L) and microsatellite stable (MSS) tumors, respectively (38) (39). Dinucleotide markers are sensitive to MSI-L status, whereas mononucleotide are relatively specific for MSI-H cancers (40).

The commonly used diagnostic MSI test based on polymerase chain reaction (PCR) sometimes produces unexpected and overlooked results, due to several reasons such as contamination of the tumor with normal cells and intralesional heterogeneity. (41). Another recently introduced rapid and not expensive technique to test MSI is immunohistochemistry (IHC) staining of the tumors (42). Tumors that have lost the function of one of the MMR genes show negative staining for the protein product of

that gene by IHC. In almost all evaluation studies using cases with already known MSI status and known pathogenic mutations, IHC always showed a concordant detection rate of more than 90% (41, 43, 44). It should be kept in mind that IHC cannot completely replace MSI analysis based on PCR as long as the role of other putative MMR genes in CRC will not be elucidated (45). MSI-H in HNPCC is caused by germline mutations in DNA MMR genes, hMSH2, hMLH1, hMSH6, and hPMS2 (46, 47). MSI-H and MSI-L in sporadic CRCs are attributable to methylation and inactivation of the DNA repair genes hMLH1 and MGMT, respectively (39).

1.4.4 Base-excision Repair (BER)

The BER pathway plays a significant role in the repair of mutations caused by reactive oxygen species (ROS). These ROS can react with DNA to produce a variety of genotoxic lesions that have been implicated in many degenerative diseases such as aging, cancer, and neurodegenerative disorders. Three human BER components have been identified: OGG, MYH, and MTH; of these only the MYH gene has been demonstrated to have a pathogenic role in CRC development (23). CRCs with germline biallelic mutations in MYH contain an excess od G:C>T:A transversions in APC or KRAS in tumor cells tested, consistent with defective BER funtion (23, 48).

1.5 Epigenetics

Epigenenetic changes are modifications of the genome heritable during cell division that do not involve a change in DNA sequence (49). Since its discovery in 1983, the epigenetics of human cancer has been in the shadows of human cancer genetics. The genomic screening with a microarray-based strategy that combines gene expression status and epigenetic regulation has identified a large number of genes that are preferentially hypermethylated in CRC (50), substantiating the idea that epigenetic events play a key role in colorectal tumorogenesis. Global genomic hypomethylation at CpG dinucleotides is the first epigenetic abnormality identifies in cancer cells and involves every tumor type studied, both benign and malignant (49). It has been shown that hypomethylation occurs at an early stage in colorectal neoplasia (51). Hypomethylation can lead to gene activation by demethylating normally methylated CpG islands where genes reside. In addition, hypomethylation might favor mitotic recombination leading to LOH, as well as promoting karyotypically detectable rearrangement (52). CpG island are GC-and CpG areas of areas of 0.5 to

several Kb in size, usually located in the vicinity of genes and often found near the promoter of widely expressed genes (53). It has long ben recognized that CpG island hypermethylation plays a key role in silencing genes that are as critical for, and possibly as frequent in, tumorigenesis as mutations in coding regions. In sporadic CRC, it has been shown that the interruption of Ras signaling can be achieved by either genetic activation of the KRAS oncogene or epigenetic silencing of RAS effectors RASSF1 and RASSF2 (54, 55). Almost half of the tumor-suppressor genes that cause familial cancers through germline mutations can be inactivated through promoter hypermhetylation (56). In CRC, at least three predisposing genes for hereditary cases, MLH1, APC and LKB1, can undergo trascriptional inactivation by promoter hypermethylation in sporadic cases (57). Through the comparison of methylation profiles between MSI and MSS CRCs, a subset of colorectal tumors were shown to have a so-called CpG island methylator phenotype (CIMP), characterized by having higher incidence of hypermethylation at multiple promoter regions tested (58).

1.6 Metastasis

Most deaths in human cancer including CRC are due to metastatic disease that remains resistant to chemo-radiation-therapy (59). Metastasis consist of a series of sequential steps including shedding of cells from a primary tumor into the circulation, survival of the cells in the circulation, arrest in a new organ, extravasation into the surrounding tissue, initiation and maintenance of growth, and vascularization of the metastatic tumor (60). Despite the obvious importance of metastasis, the process remains incompletely characterized at the molecular and biochemical levels (61). It has long been recognized that certain tumor types tend to metastasize to specific organs. For example, breast cancer metastasizes to bone, liver, brain and lung while CRC preferentially spreads to liver (60).

Metastatic cells are believed to be rare in the primary tumor mass that happen to acquire metastatic capability during late stages of tumor progression (62). However, recent expression profiling studies on human tumors reported that exist a gene expression signature strongly predictive of a short interval to distant metastases (63). Through comparing expression profiles of human primary tumors versus metastases, a common expression signature of only 17 genes was found to be associated with metastasis in different adenocarcinomas of various origin (lung, breast, prostate,

colorectal, uterus, ovary) (64). An expression profiling study specific to CRC also identified a set of 194 known genes and 41 ESTs that discriminated well between samples with or without metastasis at dignosis or during follow-up (65). These studies suggested that a gene-expression program of metastasis may already be present in the bulk of some primary tumors at the time of diagnosis and could therefore be used to predict which tumors would become metastatic (64).

1.7 Genetic alterations and tumorigenesis pathways

Tumorigenesis is a multistep process, each step reflecting genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives (2). The most of CRCs are believed to occur trough a well-know adenoma to carcinoma sequence identified almost three decades ago. In 1990, Fearon and Vogelstein proposed a stepwise genetic model of colorectal tumorogenesis (66). In this model, CRC was supposed to develop in a series of genetic alterations, corresponding with histological progression from normal colonic epithelium to adenomatous dysplasia through microinvasion, adenocarcinoma and, finally, metastasis. This model has been generally validated and some essential components of this model seem to occur in a predictable manner, such as APC, KRAS and p53. The APC gene, localized on chromosome 5q21, is an important component of the canonical Wnt/wingless trasduction pathway. Mutations in APC have been demonstrated in the majority of FAP patients in the germline (67) and in more than 80% of sporadic CRC, somatically (68).These mutations are thought to occur in the earliest stage in colorectal tumorigenesis, and precede the other alterations observed during CRC formation (69). It has been reported that even dysplastic aberrant foci, a presumptive precursor lesion to CRC, harbor APC mutation (70). In addition, LOH of 5q21 could be observed in at least 30% of colonic adenomas and adenocarcinoma, and it was presumed that APC was the most likely target of these events (69). Thus, APC inactivation appears to be the initial step in colorectal tumorigenesis. In this context, APC can be classified as a gatekeeper gene.

KRAS, localized on chromosome 12p12, belongs, together with HRAS and NRAS, to a family of GTPases, which mediates cellular response to growth signals. In CRC, KRAS is the preferential target for genetic mutation compared with the other two

family members. Only a small percentage of CRCs have been reported to harbor mutations in N-RAS (71). KRAS is found mutated in approximately 50% of CRCs and a similar percentage of adenomas greater that 1cm in size, almost uniformly occurring as activating point mutations in codons 12, 13, and to a lesser extent, 61, as in other human tumors (69, 72). It has been noticed that small adenomas with APC mutations carry KRAS nutations in approximately 20% of tumors, whereas approximately 50% of more advanced adenomas have KRAS mutations (73), indicating KRAS mutations could promote clonal expansion during the early to advanced adenoma stage. However, KRAS mutations do not seem to initiate the tumorigenesis as APC mutations do. There have been several reports of KRAS mutations in histologically normal mucosa. In addition, it has been shown that KRAS mutations are not associeted with dysplasia (70).

Mutation in p53 are the most common genetic alterations reported in a variety of human cancer. p53, as one of the crucial tumor suppressor genes in maintaining cell homeostasis, integrates numerous signaling pathways; however, the most important censequence of p53 inactivation in tumorigenesis is probably a complete loss of the DNA damage checkpoints in controlling cell cycle and apopthosis, leading to widespread genomic instability. In CRC, p53 mutations and allelic losses on 17p manifested as LOH are found in more than 75% of the tested cases (69). Furthemore, mutation of p53 coupled with LOH of 17p was found to coincide with the appearance of carcinoma in an adenoma, pointing out to its role for malignant transformation as a late event (74).

1.8 Serrated pathway

Two distinct molecular pathways underlie most CRCs. Approximately 70% arise from the way of the well know and characterized chromosomal instability pathway. Over the last two decades, many of the molecular mechanisms of the “serrated neoplasia pathway”, so called for the pattern of crypts in the precursor polyps, accounting for approximately 30% of CRCs have been determined (75). In 1980 the only recognized serrated polyps were considered non-neoplastic lesion (76) but today the “serrated neoplastic pathway” describes the progression of serrated polyps to CRC (Figure 2). Early reports defined the molecular features of the serrated neoplasia pathway: (i) MAPK (Mitogen-Activated Protein Kinase) pathway activation, involving KRAS and BRAF; and (ii) CIMP (CpG island methylator phenotype). Currently, three major

categories of serrated polyp are recognized in the World Health Organization (WHO) classification: hyperplastic polyps (HP) sessile serrated adenoma/polyp (SSA) and traditional serrated adenomas (TSA). In 2007, Jass proposed three broad molecular profiles for serrated pathway carcinoma which have been modified as follow:

1. BRAF mutant, CIMP-H; with (a) MSI-H, or (b) MSS.
2. KRAS mutant, CIMP-L, MSS.

The group 1 is the most strongly linked with serrated neoplasia pathway compared to the group 2.

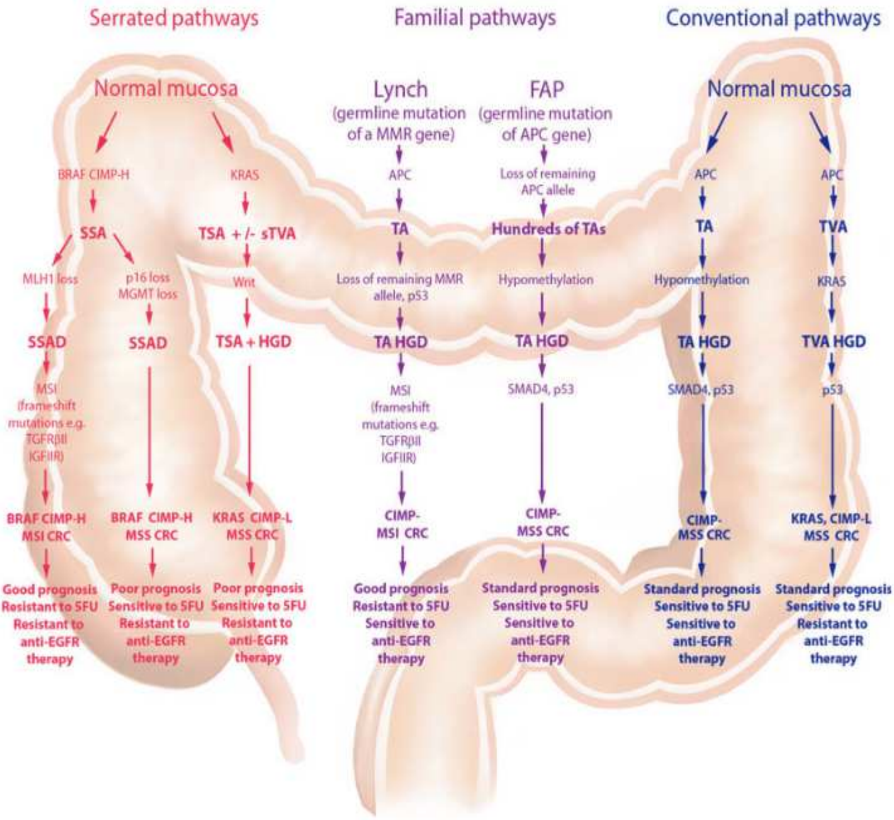


Figure 2 : Serrated, Familial and Conventional pathways to colorectal cancer.

1.9 The angiogenic vascular system

1.9.1 The vascular endothelial growth factor family and its receptors

In the developing embryo, initial blood vessels appear through a process known as vasculogenesis, in which blood vessels form de novo by differentiation and coalescence of individual progenitor cells (77). These progenitors are mesoderm-derived and can generate only endothelial cells (angioblasts) in response to vascular endothelial growth factor (VEGF), or can differentiate both in endothelial and hematopoietic cells (hemangioblasts) (78). After commitment to the endothelial lineage, angioblasts generate specialized structures, called blood islands, which can then fuse and remodel in response to haemodynamic forces to generate the first primitive plexus of vessels (78). Later in development, during differentiation, endothelial cells in the plexus become committed to either arterial or venous fate (78) (Figure 3).

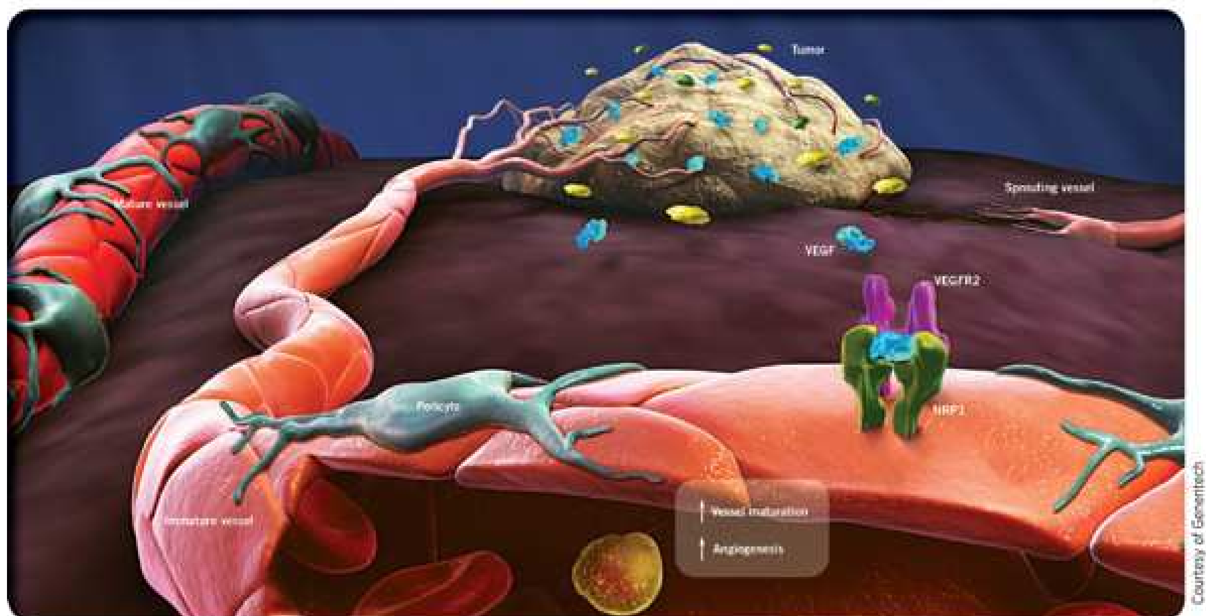


Figure 3: Representative pictures of the vessels development and differentiation.

Recent studies identified several signaling pathways controlling arterial and venous identities of endothelial cells, such as the complex Eph-Ephrin system. In addition, VEGF, Notch, angiopoietins, platelet derived growth factor (PDGF) and transforming growth factor (TGF)--☐beta are also key molecular determinants of vascular morphogenesis both in embryo and in adults (79).

After endothelial cell specification, the vascular plexus expands giving rise to a functional network of vessels by sprouting. After the formation of the immature plexus, mural cells that interact with the outer surface of the vessel are recruited (Figure 3). Most often, these cells are pericytes and invest the vast majority of capillary-size vessels in the body. Smooth muscle cells indeed cover large vessels, such as arteries and veins. During adult life, neovascularization occurs predominantly through *angiogenesis*, the growth of blood vessels from pre-existing capillaries. Blood vessel formation by angiogenesis is an extremely complex multistep process, which requires the tight control and coordination of endothelial cell behavior in all its phases (79).

The generation of new capillaries from pre-existing ones can be represented as a process in two phases: 1) tube formation, in which endothelial cells react to growth factor gradients and local concentration, migrating, proliferating and generating the new sprout and 2) vascular maturation, in which the nascent vessels are then stabilized by recruiting mural cells (pericytes or vascular smooth muscle cells) and by generating extracellular matrix (ECM) (80). Pericytes play a crucial role in regulating the physiology of the microvasculature. Their association with the newly-induced vessels render them independent of VEGF stimulus, whereas vessels lacking perictyes tend to regress following VEGF withdrawal (80).

In adult life, neovascularization is not only due to angiogenesis. Recent studies revealed that postnatal vasculogenesis can occur through the recruitment of endothelial progenitors cells (EPC) circulating in peripheral blood (81). These cells can be recruited at the site of revascularization and incorporated within the forming vasculature in distant organs in various disorders, such as hypoxic conditions or tumors (81). Most of these circulating EPCs reside in the bone marrow and can be mobilized in response to various stimuli including VEGF, GM--☐CSF, FGF--☐2 and angiopoietins (82). In addition, it has been reported that bone marrow derived -☐macrophages can also contribute to neovascularization by in situ trans-differentiation to endothelial cells (83). Recently, another population of recruited bone--☐marrow circulating cells (RBCCs) has been described. These cells do not function as EPC but contribute to neovessel formation. Their homing and retention in close proximity of angiogenic vessels is mediated by VEGF and SDF1, a chemokine induced by VEGF. These cells are able to enhance in situ proliferation of resident endothelial cells and therefore to promote adult neovascularization (84).

1.9.2 Molecular regulation of angiogenesis

The first suggestion of the existence of angiogenic factors comes from the pioneering work of Gordon Ide and collaborators in the early 20th century. Using a transparent chamber inserted into the rabbit ear as a model of tumor transplantation, they observed that tumor growth was accompanied by a strong angiogenic response (85). This observation led them to postulate for the first time that tumor cells released growth factors able to induce vessels growth. Subsequently, several other studies contributed to the discovery and characterization of numerous angiogenic factors such as VEGF, angiopoietins, TGF--☐β, fibroblast growth factor (FGF), hepatocyte growth factor (HGF) (86). Among them, VEGF is the most potent and specific angiogenic factor. In 1989, it was isolated for the first time from medium conditioned by bovine pituitary follicular cells as specific endothelial cell mitogen (87). At the same time, other research groups reported the identification of a protein that induced vascular leakage that was named tumor vascular permeability factor (VPF) (88) and isolated an endothelial mitogen from mouse pituitary cell line called “vasculotropin”. Later on, cloning and sequencing of those molecules revealed that VEGF and VPF was the same molecule whereas vasculotropin was the mouse orthologue of VEGF (89).

The mammalian Vascular Endothelial Growth Factor (VEGF) family is the best characterized and includes five members: VEGF-A, -B, -C, -D (also known as FIGF) and placenta growth factor (PIGF). In addition, ORF viruses (an epitheliotropic DNA parapoxvirus) can produce a VEGF homologue called VEGF-E.

Currently, three main subtypes of VEGF receptor tyrosine kinases have been described: VEGFR-1, VEGFR-2, and VEGFR-3. All of them are defined by seven immunoglobulin homology domains in the extracellular tyrosine kinase domain. VEGFs are secreted as dimers, the binding to their receptors leads to dimerization and trans-autophosphorylation of VEGFRs.

VEGF-A represent the best-studied VEGF family members (commonly referred to as VEGF). It has been described as a glycosylated mitogen protein that specifically acts on endothelial cells and has various effects, including increased vascular permeability, inducing angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis in both physiological and pathological conditions. It is expressed as various isoforms owing to alternative splicing that leads to mature 121-, 165-, 189- and 206-amino-acid proteins, although

proteolytic cleavage of these isoforms can lead to other, smaller isoforms. VEGF165 is the predominant isoform, commonly overexpressed in a variety of human solid tumours. As its name implies, VEGF-A activity has been studied mostly on cells of the vascular endothelium, although it acts on a number of other cell types (e.g., stimulation monocyte/macrophage migration, neurons, cancer cells, kidney epithelial cells).

Inherent or acquired resistance to anti-VEGF therapy is frequently observed in tumors, thus illustrating the need for targeting additional angiogenesis pathways to fully exploit the promise of anti-angiogenic cancer therapy. Notch signaling has recently emerged as a critical regulator of developmental and tumor angiogenesis. Notch signaling in both endothelial and smooth muscle cells appears to provide critical regulatory information to these cells downstream of the initiating signal induced by VEGF (90).

The Notch pathway is an evolutionary conserved signaling system that regulates cell fate specification, tissue patterning and morphogenesis by modulating cell differentiation, proliferation, apoptosis and survival (91). The Notch gene, first characterized in Drosophila melanogaster, encodes a 300-kD single-pass trans-membrane receptor (91). Functional studies in mice, zebrafish and cultured endothelial cells have demonstrated a critical role for Notch signaling during formation of the vascular system (90). Of the four Notch receptors, Notch1 and Notch4 are expressed by endothelial cells (92). Gene targeting studies in mice have demonstrated that Notch1 is the primary functional Notch receptor during developmental angiogenesis (90). Except for Dll3, expression of all Notch ligands has been detected in endothelial cells. Dll4 is the first Notch ligand to be expressed during mouse development, and Dll4 transcripts were detected in most capillary beds and arterial vessels (90). Lack of a single Dll4 allele in mice leads to early embryonic lethality characterized by severe defects in arterial differentiation and vascular remodeling (93).

The best characterized consist of vessels sprouting, relies on a balanced formation of tip cells, which migrate towards the VEGF gradient, and stalk cells, which instead proliferate behind the tip. These initial morphogenic events require the generation of an alternate pattern of Dll4 expression on tip cells and consequent Notch1 activation on neighboring endothelial cells, which become stalk.

VEGFA mediated tumor-induced angiogenesis has been reported to be a critical step in both tumor growth and metastasis formation (94). Concerning CRC, to date the majority of studies using either immunohistochemistry (IHC) for the detection of VEGFA protein in tumor tissue, mRNA or soluble plasma VEGFA levels all point towards a significant negative prognostic survival effect of increased VEGFA expression. On the other hand, VEGFC (Vascular endothelial growth factor C) mediated tumor-induced lymph/angiogenesis plays an important role in regional lymph nodes metastasis formation (95). VEGFC has been described as the main pro-lymphangiogenic factor (95). VEGFC can induce lymphatic endothelial cell proliferation and migration as well as vascular permeability (96). Angiogenesis has been demonstrated to be a landmark step for tumor disease progression, on one hand sustaining primary tumor growth and on the other promoting distant metastasis formation. Thus considering the importance of angiogenesis regulation in cancer progression, several anti-angiogenic agents have been developed in the last few years. Initially, results in preclinical models clearly demonstrated tumor response to anti-VEGFA therapy in terms of tumor shrinkage and metastatic incidence reduction. However, more recent data reported that a number of tumors although initially responding to VEGFA inhibition become resistant at later stages of treatment (97).

1.9.3 Anti-angiogenic therapy : Bevacizumab

In human patients, in last years the main anti-angiogeninc therapeutic agent brought into the clinic has been Bevacizumab® (BV) (Roche; Figure 4).

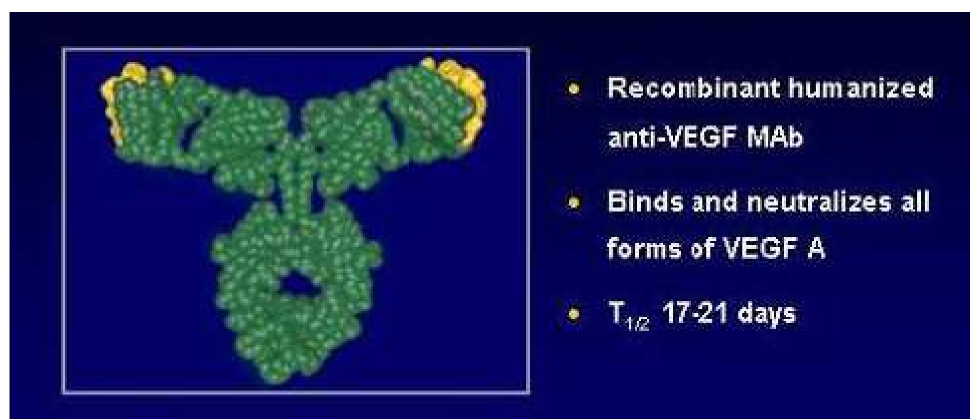


Figure 4: Avastin (Bevacizumab) San Francisco, Calif. Genetech, Inc; 2004.

In 1997, Ferrara et al. reported the humanization of the murine anti-VEGF monoclonal antibody A.4.6.1. Like its murine counter-part, BV binds to and neutralizes all human VEGF-A isoforms and bioactive proteolytic fragments, but not mouse or rat VEGF; it prevents VEGFA binding to the VEGF receptors (VEGF-R1 and VEGF-R2) mainly expressed on endothelial cells, thus inhibiting angiogenesis and inducing normalization of immature and abnormal blood vessels (98). In addition, the VEGF-R2 has been found to be expressed in CRC cells in about 50 % of published studied (99). Since 2004 in the United States, BV has been approved in addition to first line-therapy for mCRC patients' treatment. BV administration as single therapeutic agent has not shown significant anti-tumoral activity. However, if combined with IFL (irotencan/5FU/leucovorin) or FOLFOX (oxaliplatin/5FU/leucovorin), BV results in better tumor response rates and enhanced patients' progression free-survival (100, 101). Furthermore, BV has been also approved as second line therapeutic agent for irinotecan-refractory mCRC patients. In combination with FOLFOX it results in patients overall survival (OS) increase up to 12.5 months compared to 10.7 months in the control only FOLFOX treated arm (102). Nevertheless, despite BV use beneficial effects in CRC affected patients', it has to be considered that such a therapy produces only marginal OS improvement despite its considerable additional cost. Moreover, even if BV is generally well tolerated, without increasing the chemotherapy toxicity, its administration presents some dangerous and possibly fatal adverse effects. Notably, thrombo-embolic complications (2.4% incidence compared to 0.7% in the control arms), gastro-intestinal perforation (between 0.3 to 2.4% across clinical studies) and slower or incomplete wound healing (15% of treated patients) have been observed (103). Finally, the occurrence of anti-angiogenic therapy resistance, possibly explaining the poor OS benefits in BV treated mCRC patients, suggests that this therapeutic approach needs appropriate diagnostic tools in order to optimize its efficacy. Among the possible key players mediating anti-angiogenic therapy resistance, recently DLL4-Notch signalling has gained a great deal of interest. DLL4, one of the Notch ligands, has been found up regulated in a variety of human cancers and in CRC as well (90). When the ligand DLL4 binds to its receptor Notch, these results in the release of the Notch intracellular domain (named NICD). NICD goes to the nucleus where it functions as a transcriptional regulation of targets genes as Hes1, Hey1, and EprinB2. Notably it has been shown that Notch can directly inhibit angiogenesis via regulating the

number of endothelial tip cells therefore controlling the angiogenic sprouting processes (90). DLL4 over-expression in tumor models has been shown to reduce the overall vessels number but on the other hand to increase the vascular lumen size. In a randomised phase III trial, where breast cancer patients were treated with combination of capecitabine and BV, low DLL4 tumor expressing patients had a significant prolongation of PFS compared to high DLL4 tumor expressing patients (90). Furthermore, in a pre-clinical glioma xenograph model over expressing DLL4, tumors were found to be insensitive to BV treatment (104), thus corroborating clinical results. Most importantly, it has been demonstrated that large vessels rather than small capillaries play a major role in the BV resistance. It is known that BV play its major effect on small vessels regression, but has virtually no effect on bigger size stabilized vessels that are less depended to VEGF stimuli. A number of other pathways and molecules such as FGF2/FGFR, VEGF-C/VEGFR3 axes, EphrinB4 and EphB4, have been involved in the occurrence of anti-angiogenic resistance, however the global picture of this phenomenon is overall still unclear.

2. Aim of the study

The aims of this study was to assess the presence of VEGFA genomic alterations in colorectal cancer (CRC) and clarify how these genomic alterations can modulate CRC patients' response to BV treatment in addition to first line therapy (5fluorouracil, leucovorin, capecitabine, oxaliplatin, mephedrone). Among our goals we aim to find out predictive biomarkers to improve anti-angiogenic therapy efficacy and possibly to develop new therapeutic approaches by setting out new rational drug combinations. Of importance, this study allowed us to contribute to the improving patient's treatment efficiency as well as reducing the economic cost of ineffective therapy. To conclude, we believe that CRC patients will greatly benefit from our research activities. Altogether, our study helped the setting of personalized therapeutic strategies by: i) identifying those patients who really will profit of BV treatment, ii) sparing unnecessary side effects and costs for the non-responder CRC patients especially and iii) planning future alternative and/or combination treatments for CRC patients based on the functional results.

Chapter I

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

1.1 Patients

1.1.1 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 postoperative 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.

1.1.2 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.

1.2 Specimens

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

1.2.1 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 homemade 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.

1.2.2 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.

1.3 Assay Methods

1.3.1 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 (105). 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).

1.3.2 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.

1.4 Results

1.4.1 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 5). 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; see paper below 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 not-amplified cases (*P*<0.001; Figure 6a). 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).

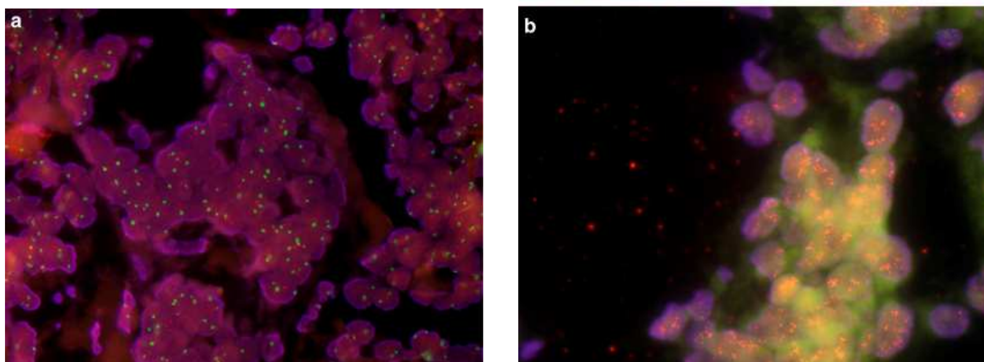


Figure 5: Fluorescence *in situ* hybridization (FITC+Rhodamine+DAPI) of the *VEGFA* gene locus 6p12 in colorectal cancer. (**a**) Negative case with no amplification. (**b**) Amplified colorectal cancer.

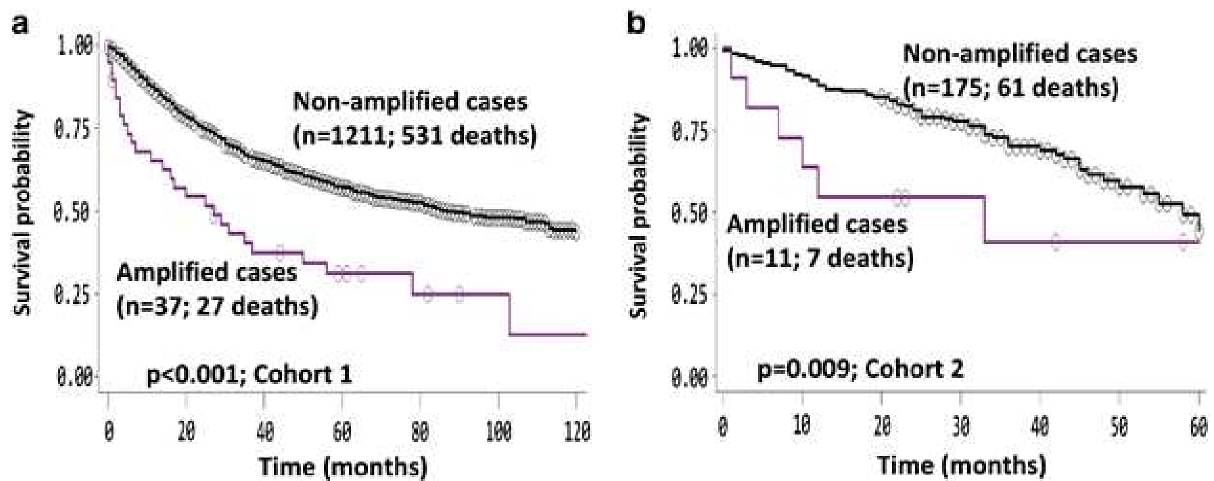


Figure 6: Kaplan–Meier survival curves showing the highly negative prognostic effect of *VEGFA* gene locus amplification in (**a**) cohort 1 and (**b**) cohort 2.

1.4.2 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 (see paper below Table 2). 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 not-amplified cases, particularly at earlier time points (*P*=0.009; Figure 6b). No multivariable analysis could be performed for this small-amplified subgroup.

Chapter II

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

2.1 Materials and methods

2.1.1 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 (106, 107). 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).

2.1.2 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).

2.1.3 FISH analysis

TMAs were subjected to pre-treatment as previously described (105). 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 (108). 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 (109-112). 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.

2.2 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.).

2.3 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.

2.4 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).

2.5 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 (AluI + RsaI) 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.

2.6 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 (113) and the Bioconductor software framework (114), using the snapCGH package (115) 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 (116) already implemented in the Bioconductor package DNAcopy (117).

2.7 Results

2.7.1 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 Table 2 for complete list). Representative pictures of amplified samples as detected using FISH are shown in Fig. 7. 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. (112). We observed polysomy of Chr6 in 18 of the 80 analysed tumour types (Table 3 and Table 4). 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 (Table 5 and Table 6). 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 3).

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=1)	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)

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

Organ	Tumor type	Amplification (%)	Polysomy (%)
Adrenal gland (n=32)	Adenoma(n=13)	0	0
	Pheochromocytoma(n=19)	0	0
Brain (n=141)	Meningeoma(n=42)	0	0
	Astrocytoma(n=39)	0	0
	Glioblastoma multiforme(n=36)	0	0
	Oligodendroglioma(n=17)	0	0
	Normal(n=7)	0	0
Breast (n=139)	Ductal cancer(n=30)	0	3.3 (n=1)
	Lobular cancer(n=30)	0	0
	Medullary cancer(n=50)	0	2 (n=1)
	Tubular cancer(n=12)	0	0
	Mucinous cancer(n=16)	0	6.2 (n=1)
	Normal(n=1)	0	0
Colon (n=150)	Adenoma, mild dysplasia(n=19)	5.3 (n=1)	0
	Adenoma, moderate dysplasia(n=64)	0	0
	Adenoma, severe dysplasia(n=25)	0	0
	Adenocarcinoma(n=39)	0	2.3 (n=1)
	Normal(n=3)	0	0
Endometrium (n=92)	Endometroid carcinoma(n=47)	0	0
	Serous carcinoma(n=31)	3.2 (n=1)	12.9 (n=4)
	Normal(n=14)	0	0
Esophagus (n=48)	Adenocarcinoma(n=9)	0	0
	Squamous cell carcinoma(n=32)	0	3.1 (n=1)
	Small cell carcinoma(n=1)	0	0
	Normal(n=6)	0	0
Gall bladder (n=41)	Adenocarcinoma(n=36)	2.8 (n=1)	5.5 (n=2)
	Normal(n=5)	0	0
Kidney (n=102)	Clear cell cancer(n=40)	0	0
	Papillary cancer(n=24)	4.2 (n=1)	0
	Chromophobic cancer(n=9)	0	0
	Oncocytoma(n=11)	0	0
	Normal(n=18)	0	0
Larynx (n=31)	Squamous cell carcinoma(n=31)	3.2 (n=1)	3.2 (n=1)
Liver (n=77)	Hepatocellular carcinoma(n=68)	1.5 (n=1)	2.9 (n=2)
	Normal(n=9)	0	0
Lung (n=172)	Squamous cell carcinoma(n=39)	0	2.5 (n=1)
	Adenocarcinoma(n=66)	0	1.5 (n=1)
	Large cell cancer(n=20)	5 (n=1)	5 (n=1)
	Small cell cancer(n=36)	0	0
	Bronchoalveolar carcinoma(n=6)	0	0
	Normal(n=5)	0	0
Lymphatic tissue (n=108)	NHL, diffuse large B(n=10)	0	0
	NHL, others(n=49)	0	0
	Hodgkin lymphoma, mixed cell(n=16)	0	0
	Hodgkin lymphoma, nodular sclerosis(n=23)	0	0
	Normal(n=10)	-	0
Myometrium (n=33)	Leiomyoma(n=27)	0	0
	Normal(n=6)	0	0
Neuroendocrine tissue (n=23)	Paraganglioma(n=7)	0	0
	Carcinoid tumor(n=16)	0	0
Oral cavity (n=52)	Squamous cell carcinoma(n=45)	0	0
	Normal(n=7)	0	0
Ovary(n=82)	Serous cancer(n=38)	2.6 (n=1)	5.2 (n=2)
	Mucinous cancer(n=11)	0	0
	Endometroid cancer(n=33)	3 (n=1)	3 (n=1)
Pancreas(n=54)	Adenocarcinoma(n=44)	4.5 (n=2)	0
	Normal(n=10)	0	0
Parathyroid (n=31)	Adenoma(n=29)	0	0
	Normal(n=2)	0	0
Peripheral nerves (n=49)	Neurofibroma(n=12)	0	0
	Schwannoma(n=37)	0	0
Pleura (n=24)	Malignant mesothelioma(n=24)	0	0
Prostate (n=98)	Adenocarcinoma, castration-resistant(n=34)	0	2.9 (n=1)
	Adenocarcinoma, untreated(n=46)	0	0
	Normal(n=18)	0	0
Salivary gland (n=88)	Adenolymphoma(n=20)	0	0
	Pleomorphic adenoma(n=31)	0	0
	Cylindroma(n=28)	0	0
	Normal(n=9)	-	0
Skin (n=228)	Basalioma(n=53)	0	0
	Squamous cell cancer(n=27)	0	0
	Benign appendix tumor(n=12)	0	0
	Malignant melanoma(n=65)	0	0
	Benign nevus(n=23)	0	0
	Benign histiocytoma(n=14)	0	0
	Kapillary hemangioma(n=22)	0	0
	Kaposi Sarcoma(n=7)	0	0
	Normal(n=5)	0	0
Small intestine (n=17)	Adenocarcinoma(n=16)	0	0
	Normal(n=1)	0	0
Soft tissue (n=124)	Lipoma(n=1)	0	0
	Liposarcoma(n=19)	0	0
	Malignant fibrous histiocytoma(n=27)	0	0
	Leiomyosarcoma(n=32)	0	0
	GIST(n=9)	0	0
	Tendon sheet, giant cell tumor(n=27)	0	0
	Normal (fat)(n=3)	0	0
	Normal (scelettal muscle) (n=4)	0	0
	Normal (smooth muscle)(n=2)	0	0
Stomach (n=65)	Diffuse adenocarcinoma(n=18)	0	0
	Intestinal adenocarcinoma(n=42)	4.8 (n=2)	2.4 (n=1)
	Normal(n=5)	0	0
Testis (n=95)	Seminoma(n=43)	0	0
	Non-seminomatous cancer(n=36)	0	0
	Normal(n=16)	0	0
Thymus (n=38)	Thymoma(n=36)	0	0
	Normal(n=2)	-	0
Thyroid (n=98)	Adenoma(n=26)	0	0
	Follicular cancer(n=42)	0	0
	Papillary cancer(n=25)	0	0
	Normal(n=5)	0	0
Urinary bladder (n=102)	TCC non-invasive (pTa)(n=35)	0	0
	TCC invasive (pT2-4)(n=66)	0	0
	Normal(n=1)	0	0
Uterus, cervix (n=24)	CIN III(n=24)	4.2 (n=1)	0
Vulva (n=19)	Squamous cell cancer(n=19)	0	0

Table 2: Rates of *VEGFA*: Rates of VEGFA gene locus amplification in the analysed tumour types as evaluated using FISH.

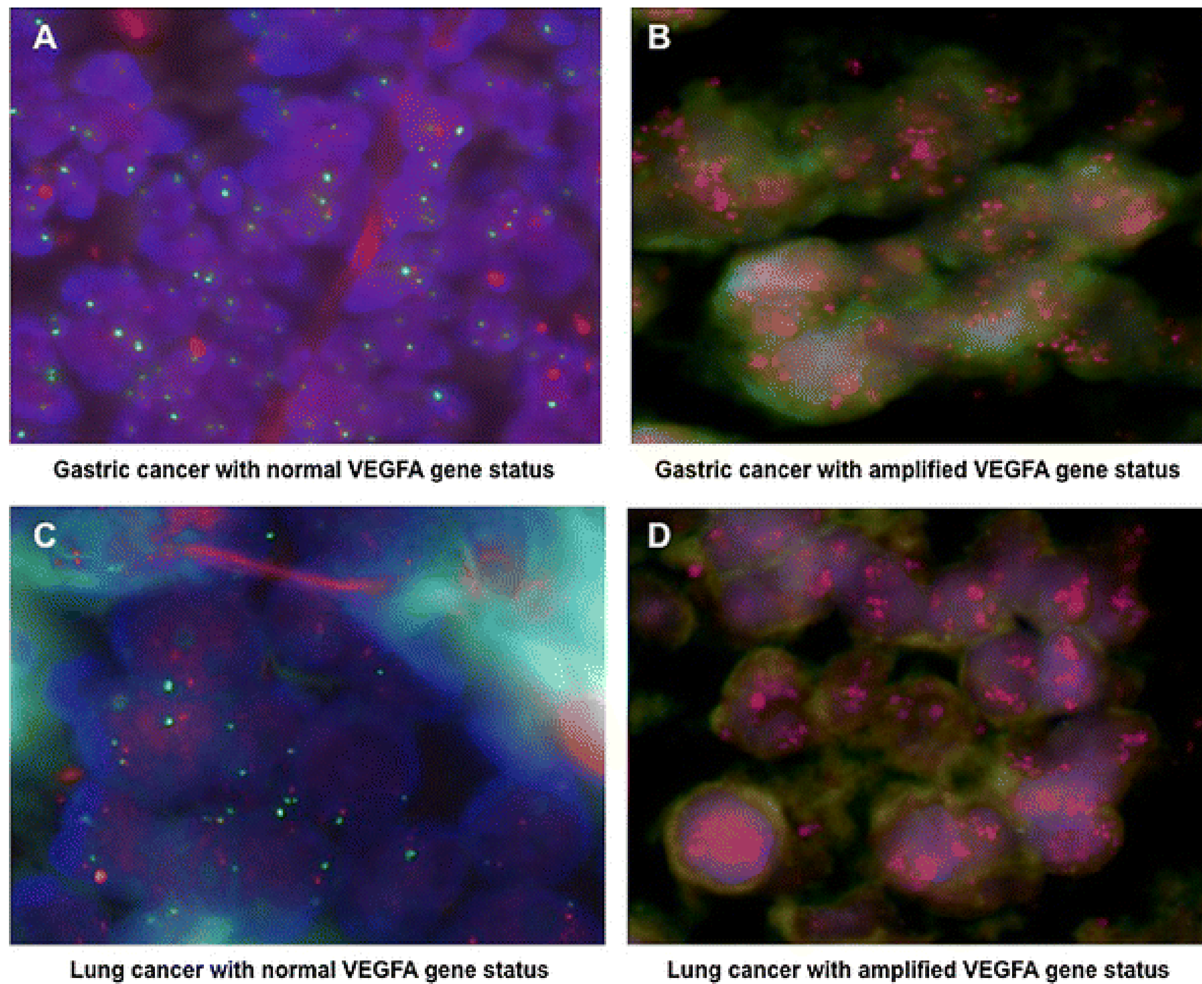


Figure 7: Visualization of VEGFA gene locus 6p12 amplification by FISH. The green and red signals correspond to centromere 6 and VEGFA gene region, respectively. a Representative pictures of gastric cancer with normal and b amplified VEGFA gene status. c Representative pictures of lung cancer with normal and d amplified VEGFA gene status.

Organ	Tumor type	*VEGFA* gene/chromosome 6 ratio (5 - 10) Amplification (%)	*VEGFA* gene/chromosome ratio > 10 Amplification (%)	Absolute CEP6 copy number (4 - 10) Polysomy (%)
Breast (n=139)	Ductal carcinoma (n=30)	0	0	3.3 (n=1)
	Medullary carcinoma (n=50)	0	0	2 (n=1)
	Mucinous carcinoma (n=16)	0	0	6.2 (n=1)
Endometrium (n=92)	Serous carcinoma (n=31)	3.2 (n=1)	0	12.9 (n=4)
Esophagus (n=48)	Squamous cell carcinoma (n=32)	0	0	3.1 (n=1)
Gall bladder (n=41)	Adenocarcinoma (n=36)	2.7 (n=1)	0	5.5 (n=2)
Kidney (n=102)	Papillary carcinoma (n=24)	4.1 (n=1)	0	0
Larynx	Squamous cell carcinoma (n=31)	0	3.2 (n=1)	3.2 (n=1)
Liver (n=77)	Hepatocellular carcinoma (n=68)	1.5 (n=1)	0	2.9 (n=2)
Lung (n=172)	Large cell carcinoma (n=20)	5 (n=1)	0	5 (n=1)
	Squamous cell carcinoma (n=39)	0	0	2.6 (n=1)
	Adenocarcinoma (n=66)	0	0	1.5 (n=1)
Ovary (n=82)	Endometroid carcinoma (n=33)	3 (n=1)	0	3 (n=1)
	Serous carcinoma (n=38)	0	2.6 (n=1)	5.2 (n=2)
Pancreas (n=54)	Adenocarcinoma (n=44)	4.5 (n=2)	0	0
Prostate (n=98)	Adenocarcinoma, castration-resistant (n=34)	0	0	2.9 (n=1)
Stomach (n=65)	Intestinal adenocarcinoma (n=42)	4.7 (n=2)	0	2.3 (n=1)
Uterus, cervix	CIN III (n=24)	4.1 (n=1)	0	0

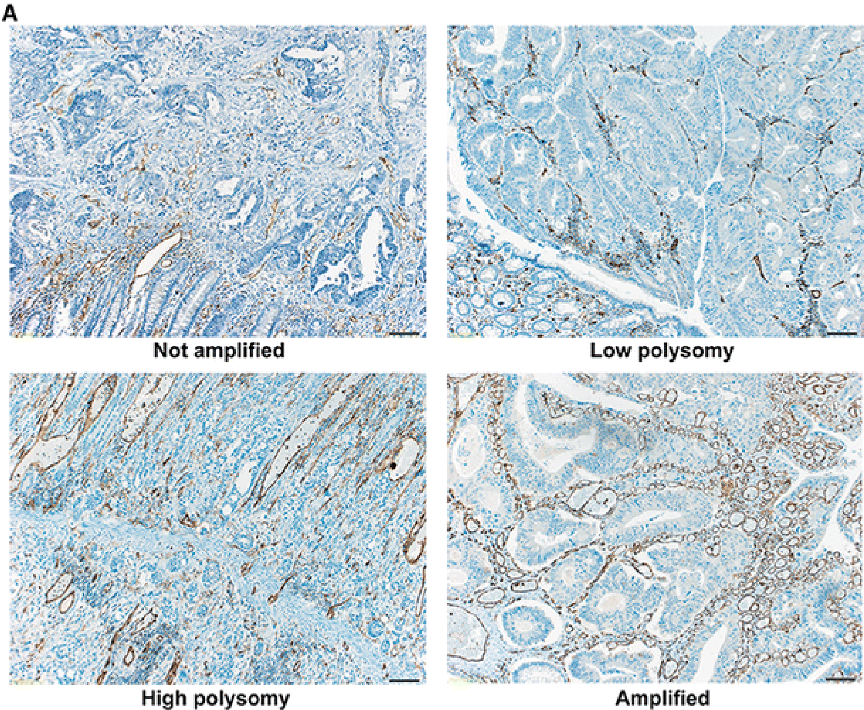
Table 3: Rates of *VEGFA* gene locus amplification using the alternative cut off criterion in selected tumour types as evaluated using FISH

		VEGFA gene/chromosome 6 ratio (5 - 10)	VEGFA gene/chromosome ratio > 10	Absolute CEP6 copy number (4 - 10)
Organ	**Tumor type**	**Amplification (%)**	**Amplification (%)**	**Polysomy (%)**
Adrenal gland (n=32)	Adenoma(n=13)	0	0	0
	Pheochromocytoma(n=19)	0	0	0
Brain (n=141)	Meningeoma(n=42)	0	0	0
	Astrocytoma(n=39)	0	0	0
	Glioblastoma multiforme(n=36)	0	0	0
	Oligodendroglioma(n=17)	0	0	0
	Normal(n=7)	0	0	0
Breast (n=139)	Ductal carcinoma (n=30)	0	0	3.3 (n=1)
	Lobular carcinoma(n=30)	0	0	0
	Medullary carcinoma(n=50)	0	0	2 (n=1)
	Tubular carcinoma(n=12)	0	0	0
	Mucinous carcinoma(n=16)	0	0	6.25 (n=1)
	Normal(n=1)	0	0	0
Colon (n=150)	Adenoma, mild dysplasia(n=19)	5.3 (n=1)	0	0
	Adenoma, moderate dysplasia(n=64)	0	0	0
	Adenoma, severe dysplasia(n=25)	0	0	0
	Adenocarcinoma(n=39)	0	0	2.56 (n=1)
	Normal(n=3)	0	0	0
Endometrium (n=92)	Endometroid carcinoma(n=47)	0	0	0
	Serous carcinoma(n=31)	3.2 (n=1)	0	12.9 (n=4)
	Normal(n=14)	0	0	0
Esophagus (n=48)	Adenocarcinoma(n=9)	0	0	0
	Squamous cell carcinoma(n=32)	0	0	3.1 (n=1)
	Small cell carcinoma(n=1)	0	0	0
	Normal(n=6)	0	0	0
Gall bladder (n=41)	Adenocarcinoma(n=36)	2.8 (n=1)	0	5.5 (n=2)
	Normal(n=5)	0.0	0	0
Kidney (n=102)	Clear cell carcinoma(n=40)	0.0	0	0
	Papillary carcinoma(n=24)	4.2 (n=1)	0	0
	Chromophobic carcinoma(n=9)	0	0	0
	Oncocytoma(n=11)	0	0	0
	Normal(n=18)	0	0	0
Larynx (n=31)	Squamous cell carcinoma(n=31)	0	3.2 (n=1)	3.2 (n=1)
Liver (n=77)	Hepatocellular carcinoma(n=68)	1.5 (n=1)	0	2.9 (n=2)
	Normal(n=9)	0	0	0
Lung (n=172)	Squamous cell carcinoma(n=39)	0	0	2.5 (n=1)
	Adenocarcinoma(n=66)	0	0	1.5 (n=1)
	Large cell carcinoma(n=20)	5 (n=1)	0	5 (n=1)
	Small cell carcinomar(n=36)	0	0	0
	Bronchoalveolar carcinoma(n=6)	0	0	0
	Normal(n=5)	0	0	0
Lymphatic tissue (n=108)	NHL, diffuse large B(n=10)	0	0	0
	NHL, others(n=49)	0	0	0
	Hodgkin lymphoma, mixed cell(n=16)	0	0	0
	Hodgkin lymphoma, nodular sclerosis(n=23)	0	0	0
	Normal(n=10)	-	-	0
Myometrium (n=33)	Leiomyoma(n=27)	0	0	0
	Normal(n=6)	0	0	0
Neuroendocrine tissue (n=23)	Paraganglioma(n=7)	0	0	0
	Carcinoid tumor(n=16)	0	0	0
Oral cavity(n=52)	Squamous cell carcinoma(n=45)	0	0	0
	Normal(n=7)	0	0	0
Ovary (n=82)	Serous cancer(n=38)	0	2.6 (n=1)	5.2 (n=2)
	Mucinous cancer(n=11)	0	0	0
	Endometroid cancer(n=33)	3 (n=1)	0	3 (n=1)
Pancreas (n=54)	Adenocarcinoma(n=44)	4.5 (n=2)	0	0
	Normal(n=10)	0	0	0
Parathyroid (n=31)	Adenoma(n=29)	0	0	0
	Normal(n=2)	0	0	0
Peripheral nerves(n=49)	Neurofibroma(n=12)	0	0	0
	Schwannoma(n=37)	0	0	0
Pleura (n=24)	Malignant mesothelioma(n=24)	0	0	0
Prostate (n=98)	Adenocarcinoma, castration-resistant(n=34)	0	0	2.9 (n=1)
	Adenocarcinoma, untreated(n=46)	0	0	0
	Normal(n=18)	0	0	0
Salivary gland (n=88)	Adenolymphoma(n=20)	0	0	0
	Pleomorphic adenoma(n=31)	0	0	0
	Cylindroma(n=28)	0	0	0
	Normal(n=9)	-	-	0
Skin (n=220)	Basalioma(n=53)	0	0	0
	Squamous cell cancer(n=27)	0	0	0
	Benign appendix tumor(n=12)	0	0	0
	Malignant melanoma(n=65)	0	0	0
	Benign nevus(n=23)	0	0	0
	Benign histiocytoma(n=14)	0	0	0
	Kapillary hemangioma(n=22)	0	0	0
	Kaposi Sarcoma(n=7)	0	0	0
	Normal(n=5)	0	0	0
Small intestine (n=17)	Adenocarcinoma(n=16)	0	0	0
	Normal(n=1)	0	0	0
Soft tissue (n=124)	Lipoma(n=1)	0	0	0
	Liposarcoma(n=19)	0	0	0
	Malignant fibrous histiocytoma(n=27)	0	0	0
	Leiomyosarcoma(n=32)	0	0	0
	GIST(n=9)	0	0	0
	Tendon sheet, giant cell tumor(n=27)	0	0	0
	Normal (fat) (n=3)	0	0	0
	Normal (sceletal muscle) (n=4)	0	0	0
	Normal (smooth muscle) (n=2)	0	0	0
Stomach (n=65)	Diffuse adenocarcinoma(n=18)	0	0	0
	Intestinal adenocarcinoma(n=42)	4.8 (n=2)	0	2.4 (n=1)
	Normal(n=5)	0	0	0
Testis (n=95)	Seminoma(n=43)	0	0	0
	Non-seminomatous carcinoma(n=36)	0	0	0
	Normal(n=16)	0	0	0
Thymus (n=38)	Thymoma(n=36)	0	0	0
	Normal(n=2)	-	-	0
Thyroid (n=98)	Adenoma(n=26)	0	0	0
	Follicular carcinoma(n=42)	0	0	0
	Papillary carcinoma(n=25)	0	0	0
	Normal(n=5)	0	0	0
Urinary bladder (n=102)	TCC non-invasive (pTa)(n=35)	0	0	0
	TCC invasive (pT2-4)(n=66)	0	0	0
	Normal(n=1)	0	0	0
Uterus, cervix (n=24)	CIN III(n=24)	4.2 (n=1)	0	0
Vulva (n=19)	Squamous cell carcinoma(n=19)	0	0	0

Table 4: Rates of *VEGFA* gene locus amplification using the alternative cut off criterion in the analysed tumour types as evaluated using FISH.

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 3). As expected neither genomic amplification of VEGFA nor polysomy was detected in any of the normal control samples (Table 4).

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. 7a. Our analysis revealed that CRC samples harbouring VEGFA amplification present with increased MVD compared to both not amplified and polysomic specimens (Fig. 8b).



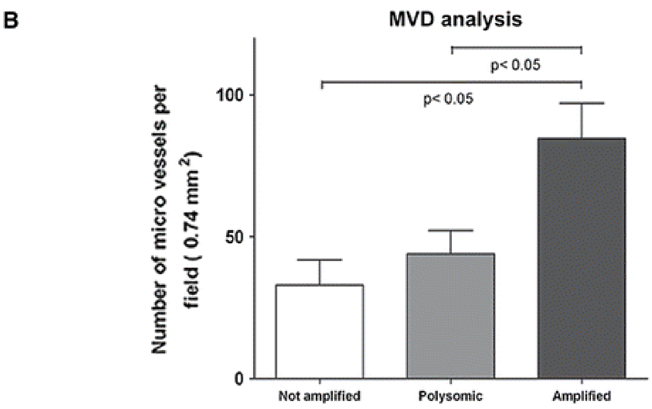


Figure 8: Micro-vessels density is increased in CRC VEGFA amplified samples. a Representative pictures of CD31 stained CRC specimens. Scale bar correspond to 100 µm. b MVD quantification

2.7.2 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 5 and Table 6 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 (Table 7 and Table 8). FISH results were confirmed for all 4 samples by CGH. Figure 9 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. 9b) showed a larger amplified region (approx. 20 Megabases), the NSCLC sample (Fig. 9c) was characterized by a focal amplicon covering a small region of 5.85 Mb.

Organ	Tumor Type	Amplification (%)	Polysomy (%)	Amplification + Polysomy (%)
Bladder	Bladder cancer (n=6)	0	16 (n=1)	16 (n=1)
Breast	Mammary cancer (n=12)	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 call 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)

Table 5: 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)
Brain	Glioblastoma (n=1)	0	0	0
Breast	Mammary cancer (n=12)	8 (n=1)	0	8 (n=1)
Head & neck	Head & neck cancer (n=7)	0	0	0
Intestine (n=30)	Anal carcinoma (n=1)	0	0	0
	Colon Cancer (n=29)	0	3 (n=1)	3 (n=1)
Kidney	Renal cancer (n=9)	0	0	0
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 sub-type (n=11)	0	27 (n=3)	27 (n=3)
	Non small cell lung cancer, large cell sub-type (n=7)	0	0	0
	Small cell lung cancer (n=5)	0	20 (n=1)	20 (n=1)
	Pleuramesothelioma (n=6)	0	16 (n=1)	16 (n=1)
Ovary	Ovarian cancer (n=8)	0	0	0
Pancreas	Pancreatic cancer (n=22)	0	0	0
Prostate	Prostate cancer (n=1)	0	0	0
Skin (n=12)	Melanoma (n=1)	0	0	0
	Skin cancer (n=1)	0	0	0
	Sarcoma (n=10)	10 (n=1)	0	10 (n=1)
Stomach	Gastric cancer (n=6)	16 (n=1)	0	16 (n=1)
Testis	Testicular Cancer (n=3)	0	0	0
Uterus, cervix (n=3)	Cancer of the uterine body (n=1)	0	0	0
	Uterine cervix Cancer (n=2)	0	0	0

Table 6: Rates of *VEGFA* gene locus amplification in human analysed tumour xenografts as evaluated by FISH

		VEGFA gene/chromosome 6 ratio (5 - 10)	*VEGFA* gene/chromosome ratio > 10	Absolute CEP6 copy number (4 - 10)
Organ	**Tumor Type**	**Amplification (%)**	**Amplification (%)**	**Polysomy (%)**
Bladder	Bladder carcinoma (n=6)	0	0	16.6 (n=1)
Breast	Mammary carcinoma (n=12)	8.3 (n=1)	0	0
Intestine	Colon carcinoma (n=29)	0	0	3.4 (n=1)
Lung(n=43)	Non small Cell lung carcinoma (n=20)	5 (n=1)	0	15 (n=3)
	Non small call lung carcinoma epidemoid (n=11)	0	0	27.3 (n=3)
	Small cell lung carcinoma (n=5)	0	0	20 (n=1)
	Pleuramesothelioma (n=6)	0	0	16.6 (n=1)
Skin	Sarcoma (n=10)	10 (n=1)	0	0
Stomach	Gastric carcinoma (n=6)	16.6 (n=1)	0	0

Table 7: Rates of *VEGFA* gene locus amplification in human selected tumour xenografts using the alternative cut off criterion as evaluated by FISH

		VEGFA gene/chromosome 6 ratio (5 - 10)	*VEGFA* gene/chromosome ratio > 10	Absolute CEP6 copy number (4 - 10)
Organ	Tumor Type	Amplification (%)	Amplification (%)	Polysomy (%)
Bladder	Bladder cancer (n=6)	0	0	16.6 (n=1)
Brain	Glioblastoma (n=1)	0	0	0
Breast	Mammary cancer (n=12)	8.3 (n=1)	0	0
Head & neck	Head & neck cancer (n=7)	0	0	0
Intestine (n=30)	Anal carcinoma (n=1)	0	0	0
	Colon Cancer (n=29)	0	0	3 (n=1)
Kidney	Renal cancer (n=9)	0	0	0
Lung (n=43)	Non small cell lung cancer (n=20)	5 (n=1)	0	15 (n=3)
	Non small cell lung cancer, epidemoid sub-type (n=11)	0	0	27.3 (n=3)
	Non small cell lung cancer, large cell sub-type (n=7)	0	0	0
	Small cell lung cancer (n=5)	0	0	20 (n=1)
	Pleuramesothelioma (n=6)	0	0	16.6 (n=1)
Ovary	Ovarian cancer (n=8)	0	0	0
Pancreas	Pancreatic cancer (n=22)	0	0	0
Prostate	Prostate cancer (n=1)	0	0	0
Skin (n=12)	Melanoma (n=1)	0	0	0
	Skin cancer (n=1)	0	0	0
	Sarcoma (n=10)	10 (n=1)	0	0
Stomach	Gastric cancer (n=6)	16.6 (n=1)	0	0
Testis	Testicular Cancer (n=3)	0	0	0
Uterus, cervix (n=3)	Cancer of the uterine body (n=1)	0	0	0
	Uterine cervix Cancer (n=2)	0	0	0

Table 8: Rates of *VEGFA* gene locus amplification in human tumour xenografts using the alternative cut off criterion as evaluated by FISH

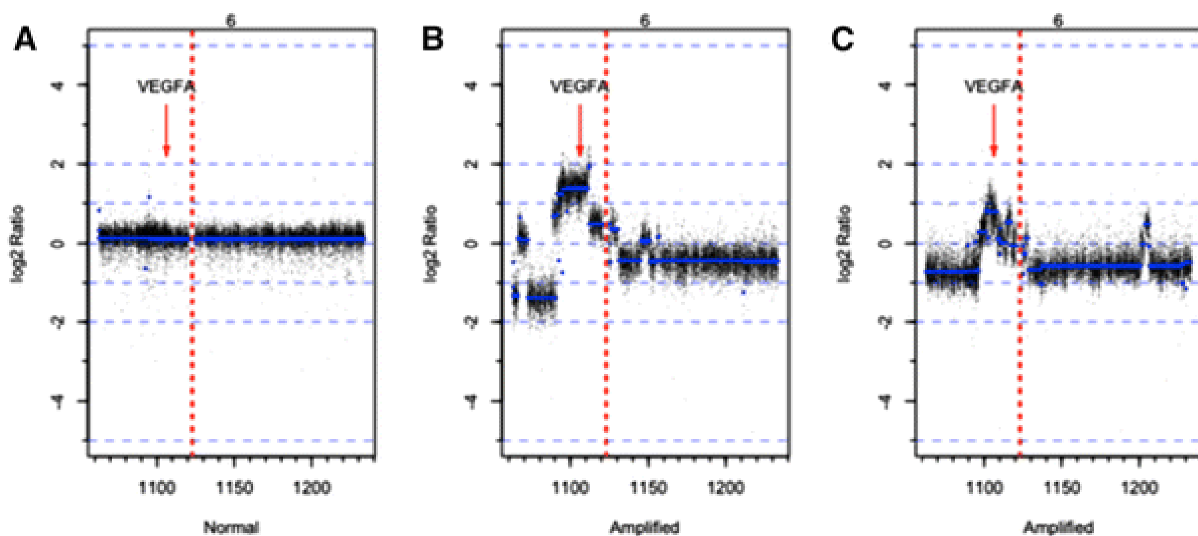


Figure 9: Representative CGH profiles of the VEGFA genomic region. Depicted area focus on Chromosome 6—VEGFA locus. a Melanoma sample with normal VEGFA gene copy number. b, c Gastric cancer and NSCLC samples showing VEGFA gene amplification, respectively. Red arrows point towards the VEGFA gene position 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.

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 7 and Table 8). Moreover our analysis revealed that among all tested tumour types only NSCLC, showed both amplified and polysomic cases.

Chapter III

VEGFA gene locus amplification in colorectal cancer and response to Bevacizumab treatment.

3.1 Materials and Methods

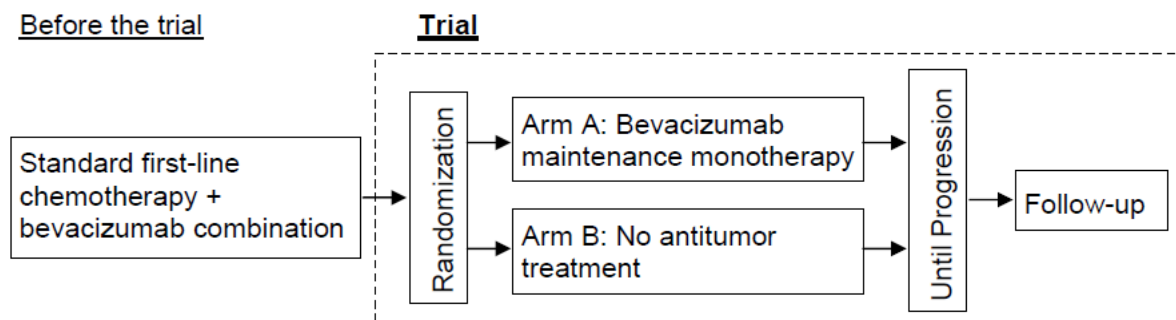
3.1.1 CRC Patients

A cohort of 86 patients with CRC diagnosed at the Cantonal Hospital of Aarau (Zentrum für Onkologie, Hämatologie u. Transfusionmedizin, Kantonsspital Aarau) and at Cantonal Hospital St.Gallen, were analysed into this study.

All pateints have been treated with surgical resection and selected according to:

- Histologically or cytologically confirmed metastatic colorectal cancer (CRC).
- First-line chemotherapy with oral or intravenous fluoropyrimidine alone, or in combination with irinotecan or oxaliplatin. Chemotherapy must have been given in combination with standard dose of bevacizumab for at least 16, but not longer than 24 weeks as part of the first-line treatment for metastatic CRC.
- Stable disease (SD), partial response (PR) or complete response (CR) after end of chemotherapy/bevacizumab first-line treatment.
- No anti-EGFR-antibody treatment (e.g. cetuximab) during first-line treatment.

After all patients have been stratified as following:



Clinico-pathological information included age, pT classification, pN classification, pM and tumor grade were available for the most of the patients (Table 9). All histomorphological data were reviewed from the corresponding hematoxylin and eosin (H&E)-stained slides.

Clinicopathological features	
Age (years range)	23-76
Gender	
female	35
male	51
Tstage	
pT1-2	5
pT3-4	35
Nstage	
pN0	5
pN1-2	30
Tumor grade	
G1-2	15
G3	8
Metastasis	
Absent	72
Present	14
Kras mutations	
G12A	8
G12C	1
G12D	2
G12R	1
G12V	5
G13D	2
G13R	1
Negative	25

Table 9: Clinico-pathological features of colorectal cancer patients.

3.1.2 RECIST criteria

The assessment of efficacy of bevacizumab-based therapy relies on Response Evaluation Criteria in Solid Tumours (RECIST) criteria (118). According to these criteria, response is measured on a patient based level, determined as the total change in sum of diameters of all pre-defined target lesions following treatment. Patients are then classified into one of four categories: complete response (CR), partial response (PR), progressive disease (PD) and stable disease (SD) (119). The RECIST criteria have been validated, are well-described, easy to use, implemented worldwide and useful for evaluation of response in clinical studies to identify patients showing disease progression. However, the RECIST criteria apply rather broad cut-

off values for the different response categories, thereby grouping large cohorts of patients who might show rather significant differences in response to therapy and possibly also in survival. This influences the accuracy of treatment efficacy evaluation. Furthermore, the RECIST criteria do not take into account individual lesion response, while several studies have demonstrated variations in intratumoural genetic alterations between different metastatic lesions within a single patient, suggesting differences in tumoural behavior between metastases.

3.1.3 Tissue sections

To assess the VEGFA gene amplification by FISH (Fluorescent in situ Hybridization) whole tissue sections from patients, treated with chemotherapy plus bevacizumab between 2003 and 2007 at the Hospital of Aarau and Hospital of St. Gallen were selected according to the response to the BV. Usage of the clinical samples for tissue microarray construction was approved by the ethical committee of the University of Basel and Aarau and St. Gallen hospital.

3.1.4 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). 1µg 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).

3.1.5 FISH analysis

86 FFPE whole tissue sections were subjected to pre-treatment as previously described (105). FISH probe was applied and after a denaturation step (10 minutes 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 (1000 ng/ml) (Vysis Inc. Abbott Molecular, Abbott Park, USA). As reference, a Spectrum Green-labelled chromosome 6 centromeric probe (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). A minimum of 100 tumour nuclei signals in four separate regions of the tissue section was counted. FISH results were interpreted according to: (1) absolute VEGFA gene copy number or (2) the ratio *VEGFA* gene/Chr6 copy number. We classified as not amplified samples with a *VEGFA*/Chr6 ratio of less than 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 (120). Polysomy of chromosome 6 was defined as an average of the chromosome 6 copy number. When the average was included between 2.26-3.75, the polysomy 6 was defined as low whereas, when the average was > 3.75 the polysomy 6 was defined high (109-112).

3.2 Results

3.2.1 *VEGFA* gene amplification in CRC patients selected according to response to adjuvant Bevacizumab in combination with chemotherapy

To demonstrate the potential role of *VEGFA* gene amplification and increased gene copy number as predictive biomarker of clinical response to BV in colorectal cancer, we enrolled a total of patients treated with Bevacizumab in addition to chemotherapy (5fluorouracil, leucovorin, capecitabine, oxaliplatin, mephedrone). 51 were male and 35 were female. The age was comprised between 23-76 years. (Table 9) The patients were stratified according to TNM classification as reported in Table 9 and they were selected on the basis of their response to bevacizumab, according to RECIST criteria. 14 were classified as responders and 72 as not-responders. The length of the therapy with bevacizumab was comprised between 14-511 days. 13 of 86 patients (15.1%) showed a significant response to Bevacizumab, whereas 73 of 86 patients (84.9 %) had disease progression. We performed the FISH analysis of 86 colorectal cancer patients in a blinded manner. VEGFA gene amplification was

observed in specimens of four patients (4.65 %) of 86 patients. The high polysomy 6 was observed in 10 (11.63 %) of 86 patients and the low polysomy in 13 (15.11 %) of 86 patients. The amplification, high polysomy and low polysomy were not found in 59 (68.6 %) of 86 patients. The high polysomy and low polysomy were observed in combination with *VEGFA* gene amplification in one and two cases, respectively. The four patients harbouring VEGFA gene locus (6p12) amplification in their primary CRC showed a significant differential response to BV treatment compared to patients with no gene amplification or either low/high polysomy. Indeed all four patients (100%) with VEGFA gene amplification were found to be responders; only 4 (40%) out of 10 patients and 5 (38%) out of 13 patients harbouring high polysomy and low polysomy of chromosome 6, respectively responded to BV. Only 1 (1.69%) out of the 59 patients with normal VEGFA gene presence seemed to profit from BV treatment (Figure 10).

3.2.2 Statistical analysis

The VEGFA gene amplification influence on the response to BV was calculated by means of logistic regression. The observed dependent variable being zero in case of absence and one in case of response to BV. The predicted value of the logit was converted into predicted odds via the inverse of the natural logarithm, namely the exponential function. Two-tailed P-values <0.05 were regarded as significant for all analyses. Statistical analyses were performed using R i386 Version 2.15.2 (http://www.R-project.org). Logistic regression analysis of these data is significant $p<0.000001$ and indicates an increase in response 1.29 (1.22-1.38) folds depending on the VEGFA gene status. These results suggest that VEGFA gene locus amplification could modulate CRC patients’ response to BV treatment depending on its genomic levels.

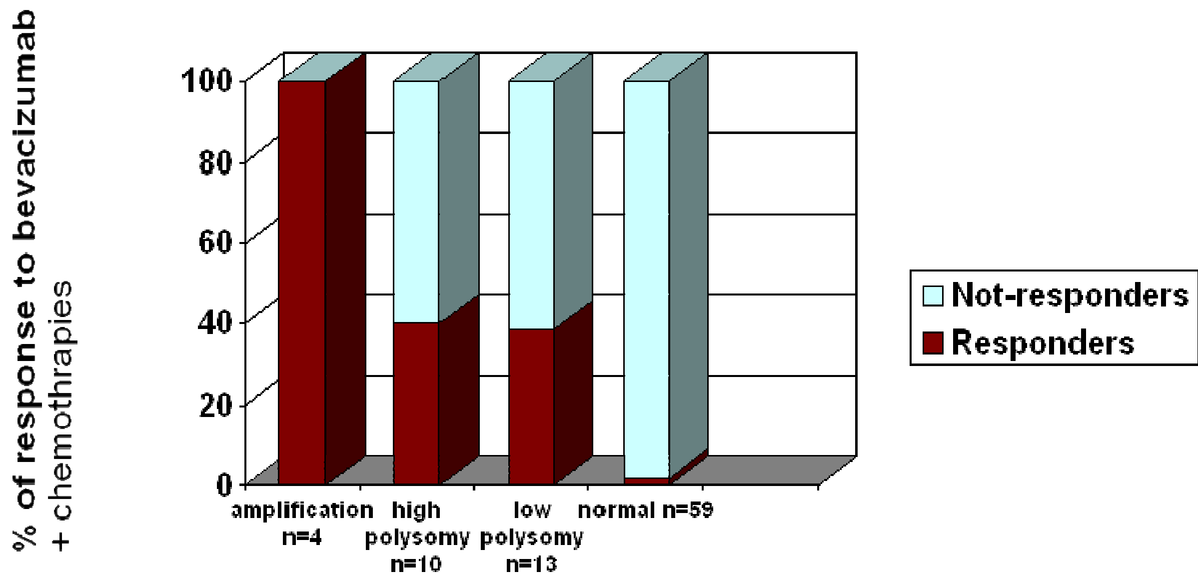


Figura 10: Histogram indicating the percentage of patients responding or not responding to BV treatment in combination with chemotherapeutics. Each plot represents a subset of patients according to their VEGFA gene presence (normal, low-, high- polysomy, VEGFA amplification).

3.2.3 Oncogenomic studies of a mouse model and human patients reveal a biomarker for sorafenib response in hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality worldwide, with the highest increase rate in northern America (121-124). Sorafenib is the mainstay of therapy for advanced HCC and the only drug that has shown any survival advantage in HCC so far (125, 126). However, patient's response is modest, and sorafenib treatment is associated with side effects (125-129). Thus, several studies looked for predictive markers for sorafenib response (130-133), yet none such biomarkers have yet entered the clinical setting. Predictive biomarkers, identifying patient subsets to guide treatment choices, are usually based on distinct pathogenetic mechanisms, and are a cornerstone of personalized medicine (134, 135). Prominent examples include ERBB2 amplification and K-RAS mutations that serve as key determinators of treatment with trustuzumab or tetuximab, respectively(136, 137). Sorafenib is a multikinase inhibitor, the targets of which include: B-Raf, C-Raf, PDGFR2, c-Kit and VEGF receptors (VEGFRs) (131, 138).

While VEGFRs were thought to be good candidates, testing for VEGF serum levels was not proven predictive (131).

Here, in collaboration with the Department of Pathology, Hadassah-Hebrew University Medical Center, Jerusalem, Israel, we characterize a subset of mouse and human HCC harboring genomic gains of VEGF-A. These tumors display distinct morphological and biological characteristics. Mouse amplicon-derived VEGF-A appears to work via heterotypic paracrine interactions: stromal VEGF receptors (VEGFRs) responding to tumor VEGF produce hepatoycte growth factor (HGF) reciprocally affecting tumor cells. This is therefore an example of genomic amplification driving tumorigenesis indirectly, through the microenvironment. Amplicon-positive mouse HCCs respond to VEGF inhibition, with HGF downregulation and a sharp decrease in proliferation. Sorafenib, a multikinase inhibitor, which is the first-line drug in human HCC targets the VEGFRs, but has only an overall mild beneficial effect in HCC. We found that genomic gains in VEGF-A specify mouse and human HCCs that are distinctly sensitive to sorafenib, suggesting they could serve as a biomarker for HCC response to VEGF-A blocking drugs.

Previous studies have delineated a hepatocyte-endothelial crosstalk taking place in non-neoplsatic liver, wherein VEGF-A stimulates endothelial cells to secrete several mitogens including Hepatocyte Growth Factor (HGF) (139, 140). We hypothesized that Amp^{pos} HCCs exploit this interaction for promoting tumor cell proliferation. Following this notion, we detected a 3-fold elevation of HGF mRNA levels in Amp^{pos} vs. Amp^{neg} HCCs.. We did not find significant changes in other angiocrine produced molecules – Wnt2, IL-6 and HB-EGF. Immunostaining detected HGF expression only in Amp^{pos} tumors, restricted to non-neoplastic tumor infiltrating cells. Coimmunofluorescent staining for HGF and F4/80 showed that macrophages are the major cell type expressing HGF.

To understand the VEGFA-HGF relationship we isolated hepatocytes and macrophages from $Mdr2^{-/-}$ livers at the age of 8 months, a time point signified by marked dysplasia, yet no overt HCC formation (141). mRNA profiling of these fractions portrayed that VEGF receptors (FLT1, KDR) and coreceptors (Neuropillin1 and 2) were higher in macrophages while the HGF receptor c-MET was more abundant in hepatocytes. This aligns with previous work showing that hepatocytes are inert to direct activation with VEGF-A (139). Immunostaining for KDR in Amp^{pos} tumors demonstrated that its expression in these tumors is restricted to non-

neoplastic cells. This correlated with a modest increase in mRNA levels of both KDR and FLT1 in total tumor lysates which is comparable to the increase in mRNA levels of recruited macrophage and endothelial markers Msr1 and CD105, respectively. This suggests that VEGF-A in Amp^{pos} tumors does not provide autocrine signals to hepatocytes, but rather acts through manipulation of the microenvironment to induce HGF secretion by TAMs.

3. GENERAL DISCUSSION

VEGFA binds to VEGFR1 and VEGFR2, which are both found predominantly on the surface of vascular endothelial cells, inducing vasculogenesis and angiogenesis. Studies in vitro suggest that VEGFR1 acts on the cell proliferation, survival and permeability and VEGFR2 contributes to cell migration (142, 143). Given its role in the growth and development of different tumor types, VEGFA is considered the most attractive target of anticancer therapy. The use of BV as antiangiogenic cancer treatment has been intensively reported and discussed in the literature. In 2004, Hurwitz et al report on a benefit for patients suffering from metastatic colorectal cancer (mCRC) when its administration is combined with chemotherapy (144). Recent data of several clinical trials, including more than 3000 patients, seem to confirm that the combination of BV and chemotherapy (Irinotecano, Fluorouracil, Leucovorin, Oxaliplatin) improves the progression – free survival (PFS) and the overall survival (OS) (145-147). However, only a subset of patients (22%) has a benefit, as compared with chemotherapeutic treatment alone (8.6%) and BV – based therapy alone (3.3%) (145-147). Besides CRC, BV has been used as antitumor agent in several other tumor types: Miller et al (148) showed that the early treatment with chemotherapy plus BV in the course of metastatic breast cancer prolongs the PFS and the objective response rate (ORR), but not the OS, when it is compared with paclitaxel alone. Sandler et al (149) reported a benefit of BV in addition of chemotherapeutic treatment (paclitaxel plus carboplatin) in selected patients with non-small-cell-lung-cancer (NSCLC). In fact, they showed a significant improvement in OS, PFS and ORR, but with the risk of increased deaths related to treatment. In patients with metastatic renal cell cancer (RCC), administration of high dose of BV led to significant increases of the time of disease progression, but not the OS. However, no significant difference was observed in patients who had received low-

dose of the drug and placebo (150). In patients with unresectable hepatocellular carcinoma (HCC), administration of BV led to an objective response rate only in 13% of the patients. However, a substantial part of patients showed evidence of minor tumor regression (151). At the moment BV is approved by the US Food and Drug Administration (FDA) for the treatment of mCRC, NSCLC and RCC. It remains approved for the treatment of metastatic breast cancer as well in multiple countries throughout the world, based largely on the results of the E2100 trial. In addition, anti-VEGF therapy with BV and other agents remains important for other tumor types (26–31). However, predictive biomarkers that identify which patients derive benefit and toxicity from anti-angiogenic agents are still needed (32).

The work presented here has been mainly focused on genomic aberrations of *VEGFA.* We analyzed a first cohort of 1420 patients with primary colorectal cancer diagnosed at the Institute of Pathology, University Hospital Basel, Institute of Clinical Pathology, Basel; 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. On a total number of 1501 primary unselected CRC patients we observed *VEGFA* gene locus amplification in 3% to 6% of two independent cohorts, respectively. The genomic aberration, in both cohorts, was significantly associated with unfavorable prognostic features and poorer survival time (152). Afterwards, we wanted to address whether *VEGFA* locus amplification is a phenomenon limited to CRC or it is a common alteration in several tumor types. We assessed the *VEGFA* gene copy number status in a total of 2837 tumors and 315 normal tissue specimens and we identified that *VEGFA* amplification is presented regularly in several tumor entities but with low incidence and it was always absent in normal tissues. The prevalence rates of *VEGFA* amplification in these tumors varied between 1.5% and 5%, based on CGH profiling studies, gain of chromosome 6p has been described in different tumor types, such as hepatocellular carcinoma, osteosarcoma, retinoblastoma, Merkel cell carcinoma and carcinosarcoma (153). Additionally, focal gain and amplification of the *VEGFA* gene was also reported in 4 out of 103 hepatocellular carcinomas (154) and in 4 out of 371 lung cancers (155, 156). Our 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.

To investigate how VEGFA gene locus amplification modulates CRC patients' response to BV treatment a cohort of 86 patients with CRC diagnosed at the Cantonal Hospital of Aarau and Cantobal Hospital I of St. Gallen with adjuvant BV treatments in combination with chemotherapeutic agents and known outcome were entered into our study. We found that CRC patients carrying the VEGFA gene locus amplification respond better to BV treatment compared to patients with no gene amplification. Despite if the biological mechanism is still to be elucidated it is tempting to speculate that the positive response to bevacizumab could act as already observed for HER2 amplification and response to trastuzumab in breast and gastric cancer. Slamon *et al*. (157) first described the importance of *HER2* in breast cancer in 1987 now it is well assessed its prognostic and predictive significance (158). Indeed, the *HER2/neu* overexpression is closely related to gene amplification in breast, ovarian, bladder, and stomach cancer (157) and it has been associated in 20% to 30% of breast cancers with a poor prognosis (157, 159). HER-2/neu overexpression/amplification has become clinically important in the management of metastatic breast cancer patients given to the availability of a humanized specific monoclonal antibody, trastuzumab (Herceptin, Roche, Basel, Switzerland), which has significant antitumor activity in these patients both as a single agent and in combination with chemotherapy (160, 161). For clinical HER-2/neu status determination immunohistochemistry according to the FDA-approved scoring system (0, 1+, 2+, and 3+) detects HER-2/neu protein overexpression whereas fluorescence in situ hybridization (FISH) assesses HER- 2/neu gene copy amplification. Both methods are recommended by national and international guidelines (162). The evaluation of genetic numerical aberration in tumor specimens is already a meaningful tool to predict response to therapy; therefore our results will pave the way to better understand the mechanism beside BV responses in order to reach personalized BV treatment modalities. This last point constitutes one of the most debated problems concerning cancer patients' treated with BV, also considering the monetary cost and toxicity of BV administration. Concerning mCRC treatment, BV has clearly demonstrated its efficacy. Nevertheless, since patients respond to this therapy in different manners, there is an urgent need to improve the understanding of its mechanism of action. Indeed, a constantly increasing number of preclinical studies are generating new insights in the mechanism of acquired resistance or intrinsic refractoriness to anti-angiogenic agents. Taking advantage of our experience with

human samples, with this project we aimed to validate preclinical data and set the basis for a future investigation concerning anti-angiogenic therapy resistance. With this work, we also validated the reliability of FISH test as a diagnostic tool to assess the *VEGFA* gene status. To conclude, if our preliminary data and future working hypotheses will be confirmed, CRC patients will greatly benefit from our research activities. Altogether, our research may lead to innovative personalized therapeutic strategies for those patients with aggressive forms of CRC disease which do not respond to anti-angiogenic therapies.

4. Appendix

General background on hepatocellular carcinoma

Unlike most malignancies, mortality from liver cancer has increased significantly over the past 20 years and epidemiologic evidence indicates that the medical and economic burden of liver cancer will increase significantly in Western populations during the next decades (163). Hepatocarcinogenesis is a multi-step process involving different genetic alterations that lead to malignant transformation of the hepatocyte (121). Most HCCs arise in a cirrhotic liver (164) and worldwide around 80% of them are related to a chronic infection with either hepatitis B (HBV) or hepatitis C virus (HCV) (165). Moreover, a rising proportion of HCCs is ascribed to alcohol abuse and metabolic disorders (166-168). In the last years, the extensive heterogeneity of genomic lesions reported in HCCs have suggested that liver cancer is the result of a combination of both, genetic and epigenetic, alterations which affect more than one regulatory pathway (124, 135, 169-175). Among these pathways the best described are Wnt/β-catenin, MAPK, p14ARF/p53, p16INK4A/Rb, transforming growth factor-β (*TGF-β*) and PTEN/Akt (163). In addition, previous studies have identified several oncogenes associated with HCC, such as *MDM4* (1q32), *MYC* (8q24), *Jab1* (8q), *cIAP1*, and *Yap* (11q22) and tumor suppressor genes such as *DLC1* (8p22), *RB1* (13q14), and *TP53* (17p13) (176-179). Furthermore, comparative genomic hybridization (CGH) has shown frequent DNA copy number gains at chromosomes 1q, 8q and losses at 1p, 4q, 8p, 13q, 16q and 17q in HCC specimens (121, 180-183). Interestingly, the chromosomal region surrounding 8p21-23 has been shown to be frequently lost in colorectal cancer (CRC) and other solid tumors (184) and thus has been generally linked to carcinogenesis (185). More recently, using an integrative approach of high-resolution array-based CGH and gene expression profiling, Roessler *et al.* characterized some of the genes located at 8p in HCC samples (180). Among these, *SH2D4A* (8p21.3) encodes for SH(2)A.

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December 2007 — **Master of Science (MSc) in Medical Biotechnology**
University "Federico II" of Naples - Italy

December 2005 — **Bachelor of Science (BSc) in Biotechnology for the Health**
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July 2002 — **Secondary school 91/100,**
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RESEARCH EXPERIENCE

2009-2013 **PhD student**
"The role of VEGFA gene amplification in the resistance to Bevacizumab therapy"

Research group: Prof. Dr. Luigi M Terracciano
Department of Molecular Pathology, Institute of Pathology.
University of Basel, Basel, Switzerland

2008-2009 **Postgraduate student**
Research field: metabolic diseases.

Research group: Prof. Dr. Margherita Ruoppolo
Department of Biochemistry and Medical Biotechnology (DBBM)
CEINGE Advanced Biotechnology
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2005-2007 **Master student (Master of Science)**
"Point Mutations in Catalytic Site ABL in Acute Lymphoid Leukemia Ph+ (Philadelphia chromosome –positive)"

Research group: Prof. Dr. Fabrizio Pane
Department of Biochemistry and Medical Biotechnology (DBBM)
CEINGE Advanced Biotechnology
Naples, Italy

2005-2006 **Traineeship**
U.O.C. Clinical Pathology, P.O. Moscati of Aversa (CE), Aversa, Italy.

2004-2005 **Bachelor student (Bachelor of Science)**
"Tetracycline-Controlled Transcriptional Activation: TET- on and TET - off gene expression system"

Research group: Prof. Dr. Mara Bevilacqua
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ACQUIRED PROJECT FUNDING

2013: Krebsforschung Schweiz (Oncosuisse) 220,000 CHF

Understanding the molecular mechanisms of anti-VEGFA treatment in colorectal cancer

HONORS

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2007: Master Degree: Cum Laude

LANGUAGE SKILLS

Italian: native

English: fluent (spoken and written)

TECHNICAL EXPERIENCE

- DNA manipulation: DNA extraction, qualitative and quantitative PCR, direct sequencing (Sanger's Method), standard and molecular (FISH) cytogenetics;
- RNA manipulation: RNA extraction, retrotranscription, in vitro transcription, quantitative PCR, RNA in situ Hybridization ;
- Protein manipulation: protein extraction, Western Blot, pull-down, ELISA immunodetection;
- Human cell cultures and isolation of mononuclear cells from bone marrow and peripheral blood: Technical buffy coat and stratification of the cell on Ficoll-hypaque and gradient centrifugation on density.
- Immunohistochemistry (IHC) analysis on human and mouse tissue, vessel structural analysis, in situ hybridization on human tissue.
- Chromatographic Tecniques: dHPLC.
- Mass-Spectrometry analysis: GS-MS.
- Bioinformatic skills: knowledge of nucleic acid databases, sequence alignment analysis.

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Publications

Mod Pathol
2011 Oct

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

Vlajnic T, **Andreozzi M**, Schneider S, Tornillo L, Karamitopoulou E, Lugli A, Ruiz C, Zlobec I, Terracciano L.

Diagn Mol Pathol
2012 Mar

KRAS mutation testing in colorectal cancer: comparison of the results obtained using 3 different methods for the analysis of codons G12 and G13.

Bihl MP, Hoeller S, **Andreozzi M**, Foerster A, Rufle A, Tornillo L, Terracciano L.

The Prostate
2012 Nov

High NRBP1 expression in prostate cancer is linked with poor clinical outcomes and increased cancer cell growth.

Ruiz C, Oeggerli M, Germann M, Gluderer S, Stocker H, **Andreozzi M**, Thalmann GN, Cecchini MG, Zellweger T, Stürm S, Koivisto PA, Helin HJ, Gelmann EP, Glass AG, Gasser TC, Terracciano LM, Bachmann A, Wyler S, Bubendorf L, Rentsch CA.

Angiogenesis
2013 Oct

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

Andreozzi M, Quagliata L, Gsponer JR, Ruiz C, Vuaroqueaux V, Eppenberger-Castori S, Tornillo L, Terracciano LM.

Eur J Cancer

2013 Dec

SH2D4A **is frequently downregulated in hepatocellular carcinoma and cirrhotic nodules.**

Quagliata* L, **Andreozzi* M**, Kovac M, Tornillo L, Makowska Z, Moretti F, Heim MH, Heinimann K, Piscuoglio S and Terracciano LM. *(*equally contributed first author)*

Clin Cancer Res

Oncogenomic studies of a mouse model and human patients reveal a biomarker for sorafenib response in hepatocellular carcinoma.

Horwitz E, Stein I, **Andreozzi M**, Nemeth J, Shoham A, Pappo O, Schweitzer N, Tornillo L, Kanarek N, Quagliata L, Zreik F, Porat RM, Reuter H, Mogler C, Shibolet O, Hess J, Breuhahn K, Schirmacher P, Vogl A, Terracciano LM, Angel P, Ben-Neriah Y & Pikarsky E.

Manuscript under review

Annals of Oncology PD-1 and PD-L1 expression in molecularly selected non-small-cell lung cancer patients

Armida D'Incecco, Mariacarla Andreozzi, Vienna Ludovini, Elisa Rossi, Alessandra Capodanno, Lorenza Landi, Carmelo Tibaldi, Gabriele Minuti, Jessica Salvini, Gabriella Fontanini, Maria Elena Filice, Roberto Incensati, Spartaco Sani, Lucio Crinò, Luigi Terracciano, and Federico Cappuzzo

PhD thesis

2013
University of Basel

VEGFA gene locus (6p12) amplification and colorectal cancer: implications for patients' response to therapy.

Manuscripts In Preparation (final results already presented at international meetings)

submitted

Enhanced expression of Thy1/CD90 and HMGA1 in human hepatocellular carcinoma correlates with poor prognosis.

Quagliata L, Benz D, Trapani F, **Andreozzi M**, Eppenberger- Castori S, Ruiz C, Pallante P, Heim MH, Tornillo L, Fusco A, Piscuoglio S, Terracciano LM.