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"Dysregulation of EGFR pathway in EphA2 cell subpopulation significantly associates with poor prognosis in colorectal cancer"

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INDEX

TABLES' ABBREVIATIONS

CI	Confidence interval
CR	Complete remission
CSS	Cancer specific survival
HR	Hazard ratio
N	Number
NA	Not available
OS	Overall survival
PD	Progressive disease
PFS	Progression free survival
PR	Partial remission
SD	Stable disease
WT	Wild type

SUMMARY

The general experimental project object of the research activity of my PhD course was directed to investigate the complex hierarchical scenario of the altered pathways in colorectal carcinogenesis. In this perspective, the pathway activated by EphA2 and EphB2 have fundamental but opposite roles. Ephs (Ephrin receptors) are the largest group of Receptor Tyrosine Kinases (RTK) and detailed biochemical studies have revealed them as very attractive drug targets and diagnostic biomarkers. $EphA2_{high}$ cells in normal mucosa are positioned at the crypt top, where differentiated cells lie, while $EphB2_{high}$ cells are restricted to the crypt base and behave as intestinal stem cells. In colorectal cancer (CRC) progression, EphA2 expression is significantly increased exerting a crucial role in migration and invasion. On the contrary, EphB2 expression is significantly reduced in the tumor bulk. Nevertheless, as already demonstrated, $EphB2_{high}$ cancer cells do persist and retain stem-like signature, in vitro organoid formation ability and in-vivo high tumorigenic activity in orthotopic xenograft. This issue constitutes the "Eph paradox" that we tried to unveil and study.

Our hypothesis was that gene expression signatures of EphB2, EphA2 and other tumor cell subpopulations might help characterize their functional roles in the contest of the progressive hierarchical organization of the tumor, throughout the different phases of CRCarcinogenesis. Our experimental strategy predicted that moving from animal models to clinical specimens might help assess whether and to what extent $EphA2_{high}$ and $EphB2_{high}$ cells contribute to CRC progression.

With this aim we first developed and characterized the murine AOM/DSS model, a platform that reliably reproduces the causal progression of every single phase of CRCarcinogenesis, to study the pathways involved in the initiation and evolution of the malignancy (Oncotarget 2015: *Novel insights into Notum and glypicans regulation in colorectal cancer*).

In this landscape, my thesis work focused on the analysis of the genetic and epigenetic features of the EphA2high cell population in the context of colorectal cancer (CRC). Particularly, we investigated a possible correlation between EphA2 and EGFR (Epidermal Growth Factor Receptor) pathways in tumor development, finding an association to mechanisms of resistance to therapy in colorectal cancer patients (Clinical Cancer Reaserch 2017: *Dysregulation of EGFR pathway in EphA2 cell subpopulation significantly associates with poor prognosis in colorectal cancer*).

1. INTRODUCTION

1.1 Colorectal Cancer

1.1.1 Epidemiology

Colorectal cancer (CRC) is the third most common cancer worldwide, with between 1,4 million new cases being diagnosed every year and 700,000 deaths per year. CRC is the second most common cancer in women (9.2%) and the third in men (10%)[1]. Its incidence has risen by more than 200,000 new cases per year from 1990 to 2012 and predictions for 2016 are not encouraging, with 134,490 new cases and 49,190 death related to this cancer expected.

Worldwide, the probability of suffering from CRC is about 4%-5%, but this percentage can be raised by a number of risk factors. Between the most common non-modifiable risk factors of colorectal cancer we can enumerate age[2], a familiar or a personal history of colorectal cancer[3] and a personal history of inflammatory bowel disease (IBD) like ulcerative colitis or Crohn's disease[4]. Lifestyle-related risk factors include inactivity, obesity, smoking and alcohol consumption[5-7].

1.1.2 Molecular etiology

The molecular etiology of colorectal cancer can be found in point mutations involving oncogenes, tumor suppressors or DNA repair mechanisms. The nature of these mutations has determined the classification of CRCs in sporadic, hereditary and familial. 70% of colon cancers are sporadic, generated by point mutations that casually occur during life and in most cases follow a specific succession that leads to a specific morphologic sequence, evolving from adenoma to carcinoma state. Typically, the first mutation occurs in a tumor suppressor gene, Adenomatous Polyposis Coli (APC), and causes the formation of non-malignant adenomas, or polyps. 15% of these lesions undergoes mutations at the level of KRAS, TP53 and DCC, and in ten years is expected to evolve to carcinoma state[8]. Only the 5% of colorectal carcinomas is caused by inherited mutations and is classified in polyposis and non-polyposis forms. The most common polyposis form is the Familial

Adenomatous Polyposis (FAP), characterized by the presence of numerous potentially malignant polyps in the colon[9]. The non-polyposis form, or Hereditary Non-Polyposis Colorectal Cancer (HNPCC), is caused by mutations in the DNA repair mechanism's genes (MSH2, MLH1, MLH6, PMS1, PMS2), mainly related to the Lync Syndrome[10]. The familial class of colorectal cancers, finally, includes all the inherited variants that cannot be assigned to any of the inherited cancer categories[11].

Genomic instability underlies all the variants of colorectal cancer and includes pathogenic mechanisms like chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype. The most common (80%-85%) instability pathway is CIN[12] characterized by aneuploidy and loss of heterozygosity caused by alterations in chromosome segregation, telomere dysfunction and DNA damage response. This aberrant phenotype affects critical genes involved in the physiological function of the cell including APC, KRAS, PI3K and TP53, leading to tumor proliferation, invasion and metastasis[13].

Loss of DNA repair mechanisms, caused by spontaneous events or germinal mutations, is at the basis of MSI pathway and underlies a hypermutable phenotype affecting non-coding regions and codifying microsatellites. Generally MSI tumors have a better prognosis than sporadic tumors[10]. CIMP tumors are characterized by epigenetic instability: CpG island hypermethylation of oncogene promoters leads to genetic silencing and loss of protein expression. Genetic and epigenetic alterations are not mutually exclusive and together contribute to the development of colorectal cancer [14].

1.1.3 Models of carcinogenesis

CRC evolves from adenoma to dysplastic adenoma and adenocarcinoma through distinct phases of genetic and morphologic alterations. Taking as paradigmatic model the colorectal carcinogenesis Fearon and Vogelstein introduced in 1988 a multiphasic-multigenic clonal model[15] (Fig.1 A). According to them, cancer arises from a single cell and develops following progressive genetic mutations. Each mutation provides a selective advantage to the cell that proliferates and gives rise to a monoclonal population of cancer cells. The typical features of cancer biology, including invasion, metastasis and

pharmacoresistance, may be attributed to each of these mutations, whose accumulation, more than the frequency, has an essential importance in carcinogenesis process. Fearon and Vogelstein identified three phases in the carcinogenetic process: initiation, promotion and progression[8]. In the elaboration of this model, epigenetic alterations are only considered as an alternative to the "classical" genetic mutations that involve two classes of genes with different roles in tumor: oncogenes, that promote an autonomous cellular growth independently from external mytogenic stimuli, and oncosuppressor genes that, on the contrary, block cellular proliferation. Oncogenes and tumor-suppressor genes are named "gatekeepers" as they regulate the entrance of the cell in the oncogenic process. Among the oncogenes, which act in a dominant pattern and are switched on by point mutations, translocation, fusions and amplifications, we can enumerate transcription factors (MYC), chromatin modifiers (EZH2, Enhancer of Zeste Homologue 2), growth factors (TGFα, Tissue Growth Factorα) and their receptors (EGFR Epidermal Growth Factor Receptor), signal transductors (RAS, Rat Sarcoma) and apoptosis mediators (BCL-2, B-Cell Lymphoma 2).

Tumor-suppressor genes are inactivated through point mutations, deletions and translocations, as they negatively influence cellular growth regulating cell cycle (RB, Retino Blastoma gene), inhibiting cell duplication in presence of a genetic alteration (p53), or blocking cell migration and invasion (CDH1, Cadherin 1).

Caretakers genes are involved in DNA repair and genome stabilization and include: mismatch repair genes (MSH2, MutS homolog; MLH1, MutL-homolog), nucleotide excision repair genes (XP, Xeroderma Pigmentosum), recombination repair genes (ATM, Ataxia Telangiectasia Mutated)[16].

This model has been very useful for the comprehension of the different mechanisms involved in carcinogenesis, stating that the genetic alterations necessarily arise in the first phases of the disease. Thanks to this theory the most important therapeutic agents in anticancer therapy are now available acting versus the so-called gatekeeper genes to block tumor growth, as for example the monoclonal antibodies Bevacizumab®, against VEGF, Cetuximab® and Panitumumab® against EGFR, largely used in colorectal cancer therapy.

However, the model presents some important limitations: the absence of a mutation that is necessary and sufficient to trigger specific stages of tumor

progression; the impossibility to explain the extended latency period only with the succession of multiple mutations; the lack of a correlation between genes and environment.

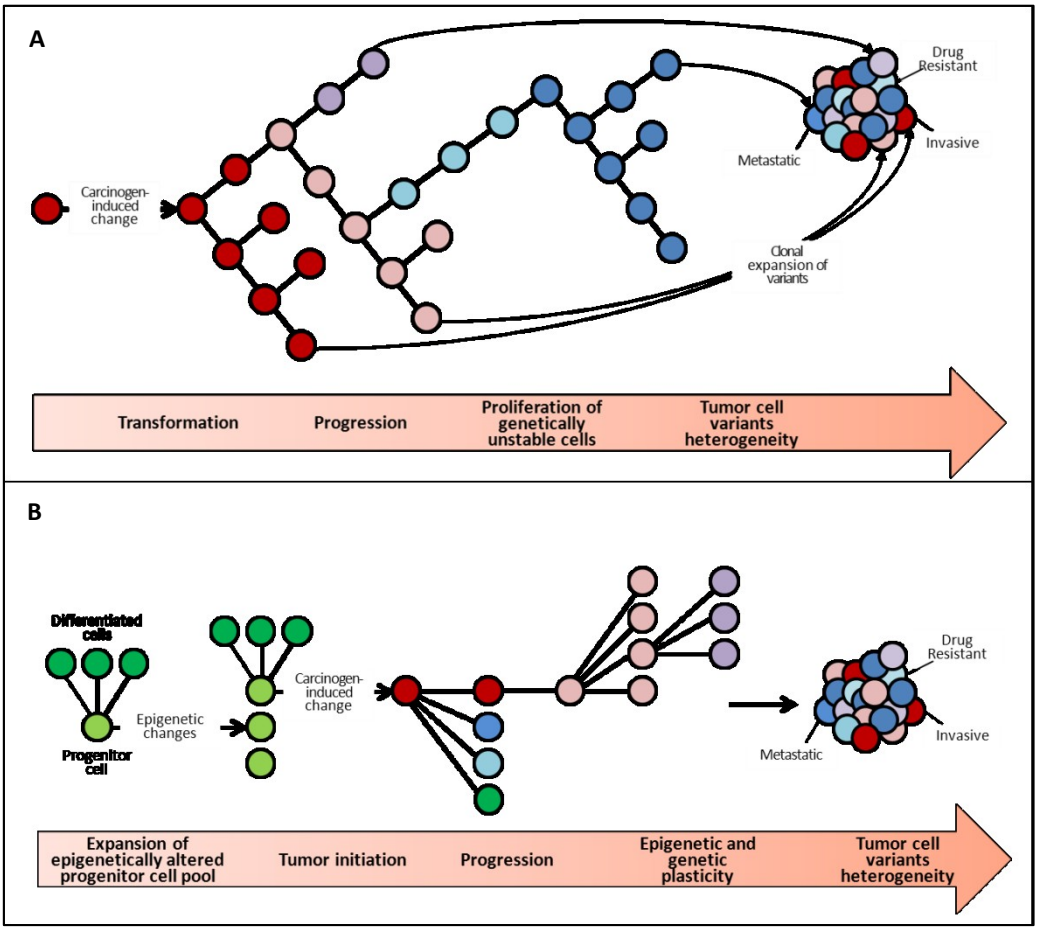

Figure 1: Models of carcinogenesis: (A) the clonal genetic model of cancer; (B) the epigenetic progenitor model of cancer.

Following a deeper comprehension of the epigenetic mechanisms involved in tumor progression, such as genome-wide demethylation (Vogelstein 1983), hypoacetylation of histonic proteins and gene-specific hypomethylation, in 2006 this globally accepted model was surpassed by Feinberg's epigenetic progenitor model[7] (Fig.1 B). Observing that stem cells are at the origin of cancer and that the principal difference with the somatic cell resides in the epigenetic status, Feinberg speculated that early epigenetic alterations of stem

cells could be the basis of cancer pathogenesis. Its model comprises three steps: for first, the stem cells of a given tissue undergo an epigenetic alteration mediated by tumor progression genes (TPGs) deregulation, triggered by an environmental damage or particular events involving the stem cell itself or the stromal compartment. TPGs are usually involved in stemness regulation: IGF2 (Insulin-like Growth Factor 2), when hit by loss of imprinting, promotes the expansion of the progenitor cells' compartment; APOBEC (Apolipoprotein B mRNA-editing Enzyme, Catalytic polypeptide) could be the responsible of the genome-wide demethylation of tumors; the transcription factors OCT4 (Octamer-binding Transcription factor 4), FOXD3 (Forkhead box D3) and Nanog normally maintain the balance between self-renewal and differentiation in the stem cell compartment; EZH2 influences chromatin structure. The perturbed equilibrium between non differentiated progenitors and differentiated cells causes the advent of a polyclonal precursors able to give rise to neoplasia. Tumor initiation is the second step of Feinberg's model and is triggered by a monoclonal mutation of gatekeeper genes in the context of the epigenetically deregulated progenitors. This mutation is cancer type-specific, and in colorectal cancer involves APC (Adenomatous Polyposis Coli) or β-catenin. Finally, the third step consists in genetic and epigenetic instability that sustains cancer evolution and explains tumor heterogeneity.

This model has important implications in the study and treatment of cancer. The presence of epigenetically deregulated progenitors implies that the early phases of carcinogenesis take place when a preneoplastic lesion is still not identifiable. Moreover the reversibility of epigenetic mutations makes them an interesting therapeutic target, as demonstrated by FDA approved drugs with demethylating activity like Azacitidine (Vidaza; Celgene, Summit, NJ, USA) and Decitabine (Dacogen; SuperGen, Dublin CA, USA), and the histone deacetylase inhibitors like Vorinostat and Romidepsin. So it could be possible to identify and treat tumors in the very early steps of their development, leading to disease remission.

Under this theory, the typical features of advanced tumors like invasion, metastasis and pharmacoresistance are not determined by the progressive mutations occurring during carcinogenesis, but are inherent in the epigenetically perturbed progenitors at the origin of cancer.

Moreover according to Feinberg cancer heterogeneity is attributable to the presence of tumor progenitor cells epigenetically modified at gatekeepers' level, which are phenotypically different from the tumor bulk and more similar to the early progenitors.

In this model is finally elucidated the role of the environment in the decade-lasting process of determining epigenetic alterations, that explains the insurgence of cancer mostly in adult age.

1.1.4 Colon Cancer Stem Cells

The failure of therapies directed against the proliferating fraction of cancer cells and the physiological loss of mutated cells during tissue renewing questioned the clonal model of carcinogenesis. More likely, cancer derives from less represented cells with a long half-life and the ability to undergo self-renewal, clonal expansion and accumulating mutation, namely the stem cells (SCs). When a tumorigenic mutation alters SCs' self-renewal program, they transform in Cancer Stem Cells (CSCs) to trigger and sustain cancer development. Tumor assumes the features of a neo-organ, sustained by a small fraction (<1%) of stem cells and mostly constituted by a bulk of cell populations at different levels of differentiation[18].

In physiological conditions, the colonic tissue consists of four distinct layers: mucosal, submucosal, muscular and serous. The mucosal epithelial layer faces the lumen and is made of a single sheet of columnar epithelial cells that form digitiform invaginations supported by the lamina propria to build the functional unit of the intestine, named Lieberkühn crypt. Intestinal stem cells (ISCs) are restricted to the crypt basis and give rise, through asymmetric cell division, to the transient amplifying cells (TA), characterized by a high proliferation rate but a reduced half-life. TA cells migrate to the top of the crypt, proliferate and differentiate in one of the three epithelial cells types that populate the intestinal wall: colonocytes, muciparous cells and enteroendocrine cells. Intestinal stem cells are sustained by a stem cell niche that in colon is composed by myofibroblasts at the crypt basis (Fig.2).

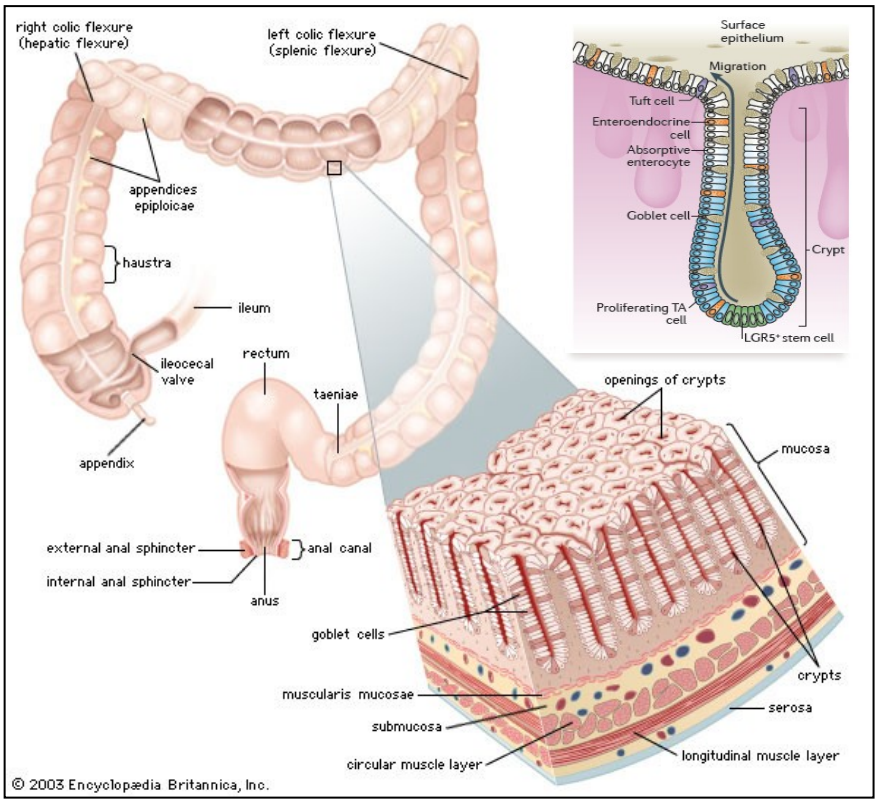

Figure 2: the structural organization of colon. Schematic representation of colon histology and crypt organization. Edited from Encyclopedia Britannica, 2008.

The main properties of stem cells are: differentiation, or the ability to give rise to an heterogeneous population of mature cells with short half-life that progressively specialize following a hierarchical process; self-renewal, or the capability to give rise to new stem cells with identical proliferative potential; homeostatic control, or the ability to balance the differentiation and self-renewal processes indulging environmental stimuli or tissue damages[19]. Stem cells perform symmetrical and asymmetrical cell divisions to maintain the exact number of stem cells in a population, generating respectively two identical stem cells or one stem cell and one more differentiated cell[20].

To identify these cells in the heterogeneous context of the colonic tissue, a number of markers have been proposed and validated in the last years.

The first putative marker of ISCs is Msh-1 (Musashi-1), a protein that controls at the post-transcriptional level the genes involved in maintaining the

undifferentiated status of the stem cells[21]. Nishimura and colleagues localized Msh-1+ cells at the crypt bottom, exactly where ISCs lie[22].

Lgr5 (Leucine-rich repeat containing G protein-coupled Receptor 5), is a target of the fundamental Wnt intestinal pathway and has been proposed as marker of the colon stem cells. Lgr5 is a transmembrane G-coupled protein whose expression is limited to proliferating intestinal cells at the bottom of the crypt. Lgr5+ cells are able to give rise to all the colonic epithelial cell lineages[23].

EphB2, another Wnt target, has been recently proposed as a stem cell marker. Its activity is essential for cell positioning during intestine development and high levels of EphB2 expression correlates with a stem-like phenotype in normal colon, as will be discussed later[24].

Also the integrin subunit β1 (CD29) is a candidate surface marker for the proliferative zone of the human colon crypt, that overexpresses this protein respect to the other colonic cells[25].

Cancer Stem Cells are defined as cells able to self-renew and maintain the ability to give rise, through asymmetric cell division, to tumorigenic and non-tumorigenic cancer cell offsprings. The complex tumor cellular system includes cell subpopulations with distinct tumorigenic ability: the high percentage of cells that form the tumor bulk, unable to initiate cancer, and the rare tumor initiating cells (TICs) that, when implanted in a xenograft, are able to generate a tumor that histologically and phenotypically resembles the original one.

Dieter and colleagues demonstrated in an animal xenograft model the presence of distinct stem cell subpopulations in CRC[26]: colorectal cancer biopsies-derived cells cultured in suspensions formed tumor spheres that, when xenografted, were able to generate the original tumor. These spheres consisted of a conspicuous number of cells unable to proliferate, that corresponded to the tumor bulk; a smaller cell populations with an intermediate proliferative potential (tumor transient amplifying cells, T-TACs) with a predominant role in tumor formation; a minimal cell population with high proliferative potential (long term tumor initiating cells, LT-TICs) capable of self-renewing and metastasis. Serial xenografts gave rise to a third stem population, named delayed contributing tumor initiating cells (DC-TICs), with late recruitment in the carcinogenesis process. Cancer stem cells are therefore included in the two cell subpopulations of LT-TICs and DC-TICs able to self-

renew, differentiate through asymmetric cell division and give rise to all the different cancer cell lines (Fig. 3).

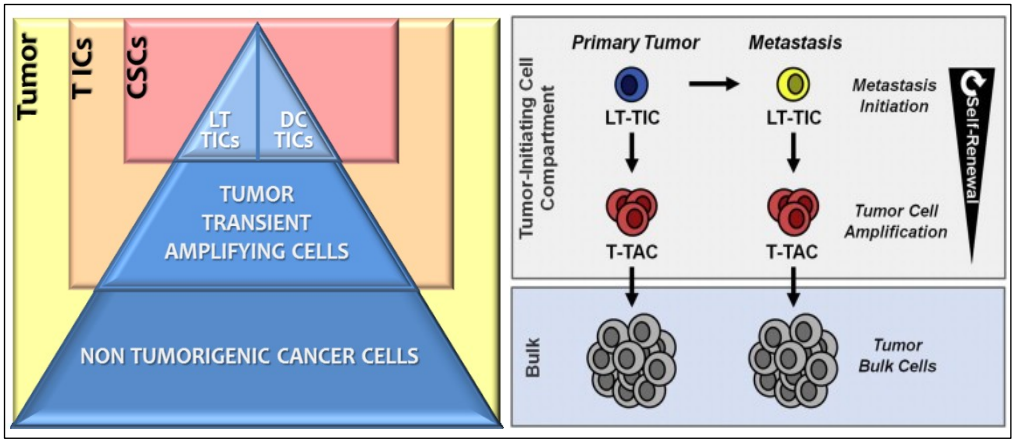

Figure 3: Hierarchy of colon cancer stem cells. Edited from Zeuner A and De Maria R *Not So Lonely at the Top for Cancer Stem* Cells Cell Stem Cell 9 (2011)

Among the different markers currently in use for colorectal CSCs, the transmembrane glycoprotein CD133 is one of the first proposed. Its role is still not totally clarified, but it is likely involved in asymmetrical cell division and self-renewal. Serial xenografts in immunodeficient mice demonstrated a marked increase of the tumorigenic potential in the small CD133+ cellular fraction (2.5%) respect to the non-dissociated tumor. However, a number of studies questioned CD133's specificity, showing an unexpected expression of the protein in intestinal cells distributed all along the crypt axis and a metastatic potential also in CD133- cell population[27].

The transmembrane glycoprotein CD44, restricted to the basolateral membrane of the colonocytes at the bottom of the crypt, is involved in cell survival, growth, differentiation and migration. CD44 is widely used as CSCs biomarker in a number of solid tumors, including CRC: CD44+ colon cancer cells are highly tumorigenic, even more if also CD133+, conversely CD44- colon cancer cells are unable to form tumors in immunodeficient mice[28].

CD166 is a mesenchymal stem cell marker with a role in cell-cell contact formation and has been related to negative prognosis in CRC. Cells that are positive for CD44 and CD166 show elevated tumorigenity in immunodeficient mice compared to CD44-CD166+, CD44+CD166- or CD44-CD166-[29].

Three molecules involved in the Wnt pathway are now in use as marker of colon CSCs.

Lgr5 has been found on the surface of colon cancer stem cells and is considered as a CRC-SC marker. Spheroid cultures derived from primary tumors were enriched for Lgr5 expression and Lgr5+ cells form CRC cell lines displayed colony forming, tumorigenic, and therapy resistance abilities[30].

Ascl2, homologous to the Drosophila Achaete-scute complex gene, is a transcription factor expressed in a Wnt-dependent and highly restricted fashion in intestinal stem cells. Ascl2 acts as a master regulator of crypt stemness by interpreting Wnt levels and specifying stem cells. When overexpressed, it induces stem cell genes and crypt neogenesis in vivo[31].

EphB2 has been firstly proposed as an intestinal stem cell marker by Battle's group as they demonstrated that EphB2+ colon cancer cells not only display a gene expression profiles that overlaps with the one of the intestinal stem cells, but also show organoid formation ability in vitro and high tumorigenic activity in vivo[25].

Finally also aldehyde dehydrogenase 1 (ALDH1) is in use as CSCs marker. It is a detoxifying enzyme that oxidizes intracellular aldehydes and identifies cells resistant to alkylating agents, which are protected from oxidative stress[32].

1.1.5 Molecular pathways of Colorectal Cancer

Genomic alterations that underlie colorectal cancer progression affect the main pathways involved in cell proliferation, migration and survival (Fig.4).

The Wnt signaling exerts a major role in developmental processes, influencing cell proliferation, differentiation and polarity. Under basal conditions, the cytosolic protein β-catenin (CTNNB1) binds to a destruction complex formed by APC, Glycogen Synthase Kinase3β (GSK3β), axin, casein kinase 1 (CK1) and is consequently phosphorylated, ubiquitinated and destroyed in the proteasome. Following Wnt binding to the Lipoprotein Receptor-related Protein (LRP) and Frizzled, the cytosolic Disheveled (DSL) protein is activated and can consequently inhibit β-catenin phosphorylation and degradation. CTNNB1 accumulates in the cytosol and translocates in the nucleus, where it activates the transcription of target genes involved in processes of tissue development and homeostasis[33]. Excessive binding of Wnt

ligands with Fz receptors and their coreceptors or malfunction of the destruction complex causes aberrant accumulation of free β-catenin in the cytoplasm and translocation in the nucleus, where it targets oncogenes related to invasive growth like c-MYC, CD44 and uPAR. The most common alterations of this pathway are inactivating mutations of APC and activating mutations of CTNNB1, which confer a selective advantage to transformed cells[34]. Recently a renewed interest has raised about the involvement of the Wnt inhibitor Notum and its related molecules glypicans in the modulation of Wnt signaling. Our group demonstrated for the first time Notum over-expression in early and late lesions of the AOM/DSS murine model of sporadic CRC and in human colorectal adenocarcinomas. Notum expression levels were correlated to β-catenin abnormal distribution, indicating that Notum expression is associated with canonical Wnt signal modulation in CRC pathogenesis. Moreover, Glypican-1 and Glypican-3 dysregulation were related to Notum and β-catenin alterations[35].

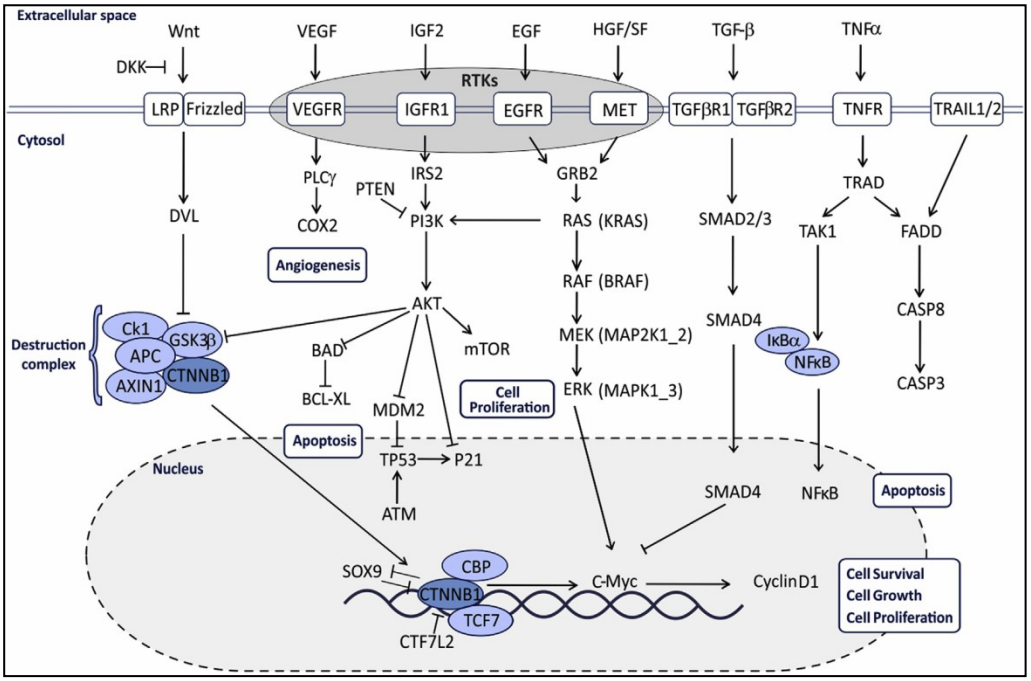

Figure 4: Main Pathways affected in Colorectal Cancer. Palma S *From Molecular Biology to Clinical Trials:Toward Personalized Colorectal Cancer Therapy* Clinical Colorectal Cancer, Vol. 15, No. 2, 104-15

The p53 protein, encoded by the tumor suppressor gene TP53, leads a downstream pathway that plays a crucial role in regulating cell cycle and apoptosis. DNA damage and oncogenic stress activate p53 signaling to either induce cell cycle arrest through p21, facilitate DNA damage reparation, or promote apoptosis through PUMA, Bax, Bak and Bcl-2, among others[36]. Under physiological conditions, p53 signaling pathway inhibits tumor formation through modulating DNA reparation, cell cycle and apoptosis[37]. Accumulating evidence has indicated that p53 signaling is frequently dysregulated in CRC progression and the aberrant signaling is associated with poor prognosis. Indeed inactivating mutations in TP53 gene or conformational alterations in p53 protein cause the loss of the tumor suppressive activity, promoting tumorigenesis and progression[38].

COX, also named prostaglandin hyperoxide synthase, is the key enzyme of the pathway that regulates the metabolism of eicosanoids: it catalyzes the conversion of arachidonic acid to PGH2 which finally converts in PGs and thromboxane A2. The COX-1 isoform of the enzyme is expressed in a numbers of cells and tissues in their physiological activity; whereas the COX-2 isoform is inducible by cytokines, growth factors ant tumor promoters. COX-2 signaling regulates angiogenesis, apoptosis and invasion: increased COX-2 expression is related to advanced stages and reduced survival rate in CRC according to clinical retrospective trails. COX-2 is also an independent prognostic CRC metastasis[39].

TGF-β/Smad signaling pathway is triggered by two serine–threonine kinase receptors, TGF-βR I and TGF-βR II and has been found to be implicated in CRC carcinogenesis, acting as a tumor suppressor in the early stage, and as a metastasis promoter in the later stage[40]. The activated receptors promote the phosphorylation of the Smad2/3 dimers, that dissociate from the receptors and together with Smad4 form Smad2/3/4 complex. The complex translocates into the nucleus to modulate the transcription of multiple target genes, leading to cell growth inhibition. TGFBR2 mutation and loss of SMAD2 and SMAD4 are frequent aberration in colorectal cancer to remove the antitumoral effect of TGF-β signaling[41].

Tyrosine Kinases Receptors (RTKs) are cell membrane proteins with intrinsic enzyme activity. Physiologically, they regulate a wide variety of

cellular processes including cell proliferation, apoptosis and migration. An enhanced activity of RTKs has been linked to development and progression of various types of cancer.

Between the more than 20 different RTK families, the most described include hepatocyte growth factor receptor (HGFR, or MET), ErbB receptors, insulin receptor, insulin-like growth factor receptors (IGF-R), platelet-derived growth factor receptors (PDGFR), fibroblast growth factor receptors (FGFR), vascular endothelial growth factor receptor (VEGFR) and Eph-receptors.

Insulin Growth Factor-1 Receptor (IGF1-R) has been found to be overexpressed in CRC: receptor activation by ligands such IGF2 leads to activation of PI3K-AKT pathway with increased cell growth and proliferation[42].

Vascular Endothelial Growth Factor (VEGF) is the main promoter of tumor neo-angiogenesis, a crucial mechanism in cancer development to sustain the rapid and uncontrolled growth of cancer cells. Five VEGF family members have been identified in mammals, including VEGFA, VEGFB, VEGFC, VEGFD (FIGF) and placenta growth factor (PIGF or PGF). VEGF receptor tyrosine kinases include three high-affinity receptors named VEGFR1 (FLT1), VEGFR2 (FLK1/KDR) and VEGFR3 (FLT4), and two coreceptors, neuropilin 1, NP1 (NRP1) and 2 NP2 (NRP2). The binding of VEGF ligands to the different VEGFRs activates distinct downstream signaling pathways, including MAPK and PI3K-AKT, that regulate different cellular functions from proliferation to cytoskeletal reorganization and migration, all contributing to the angiogenetic process. VEGF upregulation has been associated with CRC progression and survival[43].

Activation of EGFR and ErbB2 are early events during colon carcinogenesis. EGFR belongs to the ErbB family of related cell membrane receptors whose members include HER1 (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). EGFR is also known as HER1. A multiplicity of ligands binds these receptors, including EGF, TGF, amphiregulin, epiregulin, betacellulin, heparin-binding EGF and epigen. The receptor-ligand bound, following the recruitment of the PTPN12-regulated adaptor protein SHC, activates a complex multilayered network generated by receptor cross-talk and lateral signaling that converge on the classical MAPK and PI3K routes of signal transduction, which trigger transcription factors like ATF2 to express genes that maintain cell division, proliferation, differentiation and migration[44].

Although their diverse functions, all the RTKs share common signaling cascades triggered by adaptor proteins such SHC, that are often deregulated in the malignant progression.

The PI3K-AKT-mTOR cascade is one of the most studied in tumor biology. Following by the activation by RTKs, AKT is phosphorylated and activated by PI3K, PDK and MTORC2. Direct consequences of AKT activations are: inhibition of the pro-apoptotic activity of BCL2, degradation of p53 by MDM2, activation of mTOR, that lead to increased cell growth, survival and proliferation. Activating mutations of PI3KA, inactivation of the suppressor PTEN and overexpression of AKT are commonly found in CRC[45,46].

MAPK cascade is another crucial way of the complex RTKs network. It starts with RAS activation by SOS, complexed with a docking protein to the activated tyrosine kinase receptor, that displaces guanosine diphosphate (GDP) molecules from RAS and thus allowing guanosine triphosphate (GTP) molecules to bind and activate it. Active GTP-RAS recruits and removes the constitutive inhibition from the RAF proteins, which are then capable of binding and activating the KSR1 enzyme. KSR1 enzyme phosphorylates and activates MEK which in turn phosphorylates and activates ERK that enters the cell nucleus to activate a range of transcription factors, with the consequent expression of genes involved in cell proliferation. Hyperactivation of this signaling pathway is one of the most common aberrations in colorectal cancer[47].

1.1.6 MiRNAs in colorectal cancer

MicroRNAs (miRNAs) are non-coding RNAs which regulate the gene expression at a post-translational level. Their biogenesis begins in the nucleus, with the enzymatic activity of RNA polymerase II, which transcribes genes located in intragenic regions, and RNA polymerase III, which transcribes the rare miRNA genes surrounded by repetitive DNA sequences. The transcription product is named pri-miRNA (primary-miRNA), a molecule of hundreds of nucleotides which includes a 5’ cap and a polyadenylated tail. Pri-miRNAs are still processed in the nucleus by the RNase DROSHA complexed with its cofactor DGCR8 to produce a hairpin molecule of 70 nucleotides termed pre-miRNA (precursor-miRNA). The product is exported in the cytoplasm to be

processed by the endonuclease Dicer in a double stranded RNA of 18-25 nucleotides, which includes a leading strand (miR) and a passenger strand (miR*). One of these strands is destroyed by argonaute proteins (AGO) while the other is incorporated in the RNA-induced silencing complex (RISC) to exert its silencing activity[48].

Target identification is based on complementary base-pairing between the miRNA and a region usually located in the 3'UTR of the mRNA, named "seed sequence" and is followed by target degradation, in case of perfect complementarity, or steric translational repression, in case of imperfect complementarity. MiRNAs' pathway of expression is strictly ruled: the huge power of this class of molecules resides in the fact that a single miRNA is able to inhibit a number of targets, exerting its function on a wide range of physiological and pathological processes.

MiRNAs that play a key role in cancer, targeting genes involved in development, apoptosis, differentiation and cell proliferations, are named oncomirs. These molecules are usually located in genomic regions subject to deletion, duplications or mutations and are often deregulated during carcinogenesis, influencing hundreds of genes and pathways. They can act as tumor promoter, if their target is a tumor suppressor gene, or as tumor suppressors, if their target is an oncogene[49].

Particularly, in colorectal cancer miRNAs control the main pathway involved in CRC development and progression (Fig.5).

WNT signals are crucial for the regulation of the stem cell activity at the base of intestinal crypt and for epithelial cells renewal. Incongruous activation of this pathway leads to development of gastrointestinal polyps and adenocarcinoma, as described above. MiR-135a/b targets the tumor suppressor APC, enhancing WNT pathway activation and consequent premalignant colorectal adenoma development[50]. MYC, a downstream transcript of this pathway, exerts its oncogenic function also through the upregulation of the expression of the cluster miR17-92 (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, miR-92-1), whose most important target is E2F1, a cell cycle transcription factor involved in a pro-apoptotic pathway[51]. Mir-26b inhibits LEF1, a member of the transcription complex activated by CTNNB1, a key player of WNT[52]. TCF/LEF complex is repressed also by members of mir-34 family, whose transcription is activated by TP53, linking these two oncogenic

signaling[53]. CTNNB1 function is also perturbed by mir-143 and -145, which target CTNNG1, acting on CTNNB1 translocation[54], and are usually downregulated in CRC.

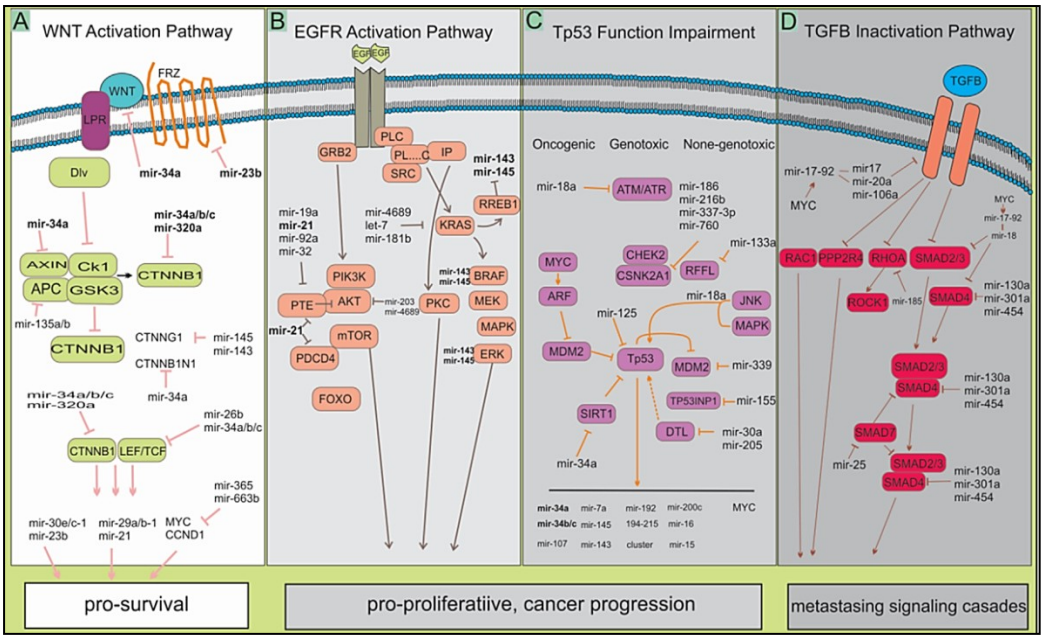

Figure 5: miRNA and CRC pathways. An overview of WNT, EGFR, TP53 and TGFb signaling pathways in CRC and the regulation of their key molecules by miRNAs. Edited from Mohammadi A *The role of microRNAs in colorectal cancer* Biomed & Pharmacoth 84 (2016) 705–713

MiRNAs are also involved in the complex network of p53. This protein is a tumor suppressor that responds to diverse stress signals by directing specific cellular responses including senescence, cell cycle arrest, apoptosis, invasion and metastasis. Its fundamental role in tumor suppression has been extensively reviewed and comprises also CRC[55]. TP53 is able to regulate directly miRNA transcription: p53 binding site has been identified in the promoters of let-7i, miR-20a, miR-21, miR-25, miR-34a/b/c, miR-145, miR-181b, miR-183, miR-195, miR-215, miR-451[56]. Particularly, mir-34 is a well-known p53 inducible miRNA[57] with a role in the inhibition of genes involved in cell cycle regulation, cell proliferation, apoptosis and DNA repair, like CDK4/6 (Cyclin-dependent kinase 4/6), Cyclin E2, E2F5, BIRC3 (Baculoviral IAP Repeat-Containing 3) e Bcl-2. A positive feedback loop is generated when mir-34 silences the TP53 inhibitor SIRT1 (Silent mating Type Information Regulation 2

homolog 1). Also miR-192, 194, 195, whose expression is regulated by p53, activate a positive feedback loop with a complex network of miRNAs that induce p53 accumulation[58].

EGFR signaling pathway is involved in the most important mechanisms altered in the process of carcinogenesis, including cell proliferation, survival and migration. The most common alterations perturbing this pathway are EGFR mutations and KRAS mutations, described in 30-60% of CRCs[59]. Mir-143 targets KRAS and is often downregulated in CRC[60]. Also the let-7 family of miRNA targets KRAS, which in turn is able to regulate the expression of this cluster, removing its inhibiting activity and promoting carcinogenesis[61].

The MAPK cascade is also affected by epigenetic silencing by miR-26b, which targets and inhibits the transcription factor ATF[62]: its downregulation is very common in CRC tumor development.

Also PI3K/AKT signaling is targeted by epigenetic silencing by the activity of miR-520a and -525a, which inhibits PI3KA subunit. However a point mutation the 3'UTR of PI3KA gene escapes miRNA silencing and promotes the oncogenic activity of this signaling[63]. Moreover, the expression of mir-126 and mir-30a that respectively target PIK3R2 and PI3KCD is usually reduced in CRC[64,65]; while mir-19- miR-21, miR-32 and miR-92-1-5p activates PI3K/AKT signaling targeting the negative regulator PTEN[66-68].

A number of miRNAs, including miR-17-5p, miR-20a, miR-21, miR-23b, miR-106a and miR-301a, have been reported to target the transcript of TGF-β, a growth factor that in colon cells controls a wide spectrum of cellular functions including proliferation, differentiation, apoptosis and migration, with a role in CRC carcinogenesis suppression. In particular miR-21 is activated by WNT pathway and is involved in stemness regulation[69]. Another connection with WNT signaling involves miR-17-92 cluster that is modulated by MYC and inhibits SMAD and TGF-β[70]. MiR-106a/363 and miR-106b/25 clusters are involved in the inhibition of TGFBR2 and SMAD2/SMAD4[71]. Also miR-130a, miR-301a, and miR-454 target SMAD4 and are commonly upregulated in CRC[72]. Mir-25 on the contrary is able to inhibit SMAD7, a well-known negative regulator of TGF-β signaling, and its expression is preferentially decreased in colon carcinogenesis[73].

MicroRNAs are also involved in metastasis, targeting the principal genes involved in the process. ZEB1, an EMT (Epithelial to Mesenchymal Transition) inducer, downregulates miR-200 miRNA family members (miR-200a, miR-200b, miR-200c, miR-141, miR-429), known MET (Mesenchymal to Epithelial Transition) promoters that, in turn, target TGF-β2 and ZEB1, triggering a feed-forward loop[74]. The oncogene MET (Mesenchymal-Epithelial Transition factor) induces tumor growth, angiogenesis and metastasis and is negatively regulated by miR-133b and miR-1[75]. Finally, COX-2, which promotes the apoptosis, angiogenesis and tumor invasion, is target of miR-101, a microRNA commonly downmodulated in CRC[76].

1.2 EPHA2 and EPHB2

Studies on Eph receptors and their ephrin ligands have significantly improved in the last years. This rapid development is not only because the Ephs are the largest group of Receptor Tyrosine Kinases (RTK), but also because detailed biochemical studies have revealed them as very attractive drug targets and diagnostic biomarkers. EphA2 and EphB2, in particular, seem to play a key role in CRC initiation and progression and are currently under observation for their therapeutic value.

1.2.1 Structure and signaling of EPHA2 and EPHB2

The Eph (erythropoietin-producing human hepatocellular receptors) superfamily is the largest group among tyrosine kinase receptor families. Eph family comprises 16 receptors classified in two subclasses, EphA or EphB, depending on their sequence homology and their binding affinity for their ephrin ligands[77]. Although Eph receptors preferentially bind ligand of the same class, cross-binding has been shown for EphA4, which can also bind to ephrin-B ligands[77], and EphB2 which can bind to ephrinA5[78]. All Eph receptors contain an extracellular region, with a conserved N-terminal globular ligand-binding domain (LBD), a cysteine-rich domain which comprises a Sushi and an epidermal growth factor (EGF)-like domain and two fibronectin type-III repeats (FN1 and FN2). The intracellular region contains a juxtamembrane region (JM), a tyrosine kinase domain, a sterile alpha motif (SAM) domain,

and a (PDZ) domain-binding motif[79-81]. The ectodomain and the intracellular domain are linked by a transmembrane helix (TM) (Fig.6).

Ephrins (Eph receptor interacting proteins) are also divided into EphrinA and EphrinB subclasses[79,80]. EphrinA proteins (A1-A6) are anchored to the plasma membrane via a glycosylphosphatidylinositol (GPI) linkage while ephrinB members (B1-B3) are transmembrane proteins containing a cytoplasmic domain with several conserved Tyrosine residues and a terminal PDZ-binding motif (Fig. 6).

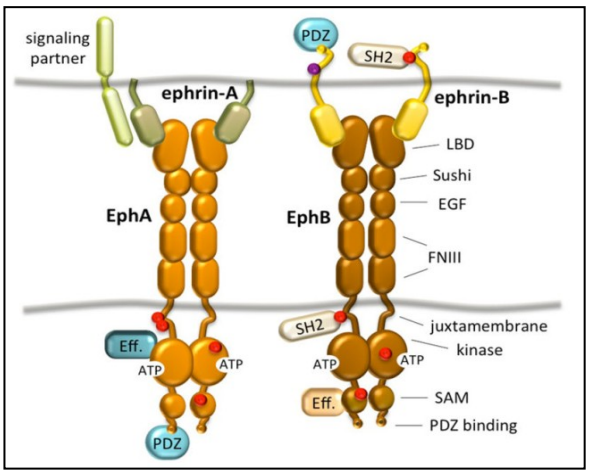

Figure 6: Schematic representation of Eph/ephrin structure. Edited from Barquilla A *Eph receptors and ephrins: therapeutic opportunities* Annu Rev Pharmacol Toxicol. Epub 2014 Oct 3.

Eph–ephrin binding occurs on the surface of the same cell (in cis) or at the site of contact of two opposing cells (in trans) and results in bidirectional signaling into both the receptor cell (‘‘forward signaling’’) and the ligand cell (‘‘reverse signaling’’)[82,83]. The trans bound requires cell-cell direct contact and it results in cell repulsion or adhesion, depending on a complex interaction of factors. Signal transduction by the Eph family is a multistep process leading to the assembly of higher-order signaling clusters in the interacting cells[79].

The first step in the formation of Eph-ephrin cluster is the binding 1:1 between Eph receptor and an ephrin ligand on opposing cell surface that leads to the formation of an ephrin-Eph dimer that aggregates successively in a heterotetramer. Eph and ephrin complexes aggregate into larger clusters through the recruitment of ephrin-bound Eph receptors and additional Eph receptor in an ephrin-independent lateral mode. Only the association of

signaling/adaptor proteins elicit Eph receptor signaling, the strength of which correlates with the size and composition of the clusters[84], and which partly explain the myriad of cellular response that are elicited by Eph activation.

Effects induced by Eph/ephrin binding involve their interaction with specific intracellular proteins, including phosphoinositide 3-kinase (PI3K), Src family kinases, Vav2, Vav3 and ephexin which coupling to Rho GTPases trigger cytoskeleton modulation[85,86].

Forward signaling involves autophosphorylation of Eph receptor and successively activation of the tyrosine kinase intracellular domain through Src family kinase–mediated phosphorylation[87]. Phosphorylation of downstream substrates is mediated by adaptors like proteins containing Src-homoloy 2 (SH2) domains and then by a number of Rho guanidine nucleotide exchange factors (GEFs), e.g. Vav2, Tiam, Kalirin, and Intersectin[88].

Eph signaling interplays with several molecules and signaling pathway, including members of Rho family of GTPases (RhoA, Cdc42, and Rac)[88], focal adhesion kinase (FAK), the PI3 kinase pathway and Jak/Stat pathway[89] (Fig. 7).

In particular, EphA2 is preferentially expressed on the membrane of epithelial cells, including small intestine, and colon where it regulates tissue development and maintains epithelial tissue homeostasis[90,91].

Unlike other receptor tyrosine kinases, EphA2 receptor does not require ligand binding for some of its activities, and can directly activate GTPases of the Rho family through the GEF Ephexin[92]. EphA2 has diametrically opposite roles in regulating cell migration and invasion, depending on its ligand dependent or independent activity: if activation of EphA2 with its ligand ephrin-A1 inhibits chemotactic migration, EphA2 overexpression promotes migration in a ligand-independent manner. EphA2 ligand independent activity requires receptor phosphorylation on serine 897 by Akt. Ephrin-A1 stimulation of EphA2, on the contrary, inhibits Akt activation with a negative feedback mechanism and causes EphA2 dephosphorylation on the serine residue, as demonstrated by Miao and colleagues in human astrocytoma[93].

Co-clustering with other RTKs and cross-class transphosphorylation of Eph receptors have also been reported, with the contribution of either receptor-type to the signaling outcome depending on relative abundance of the two receptors.

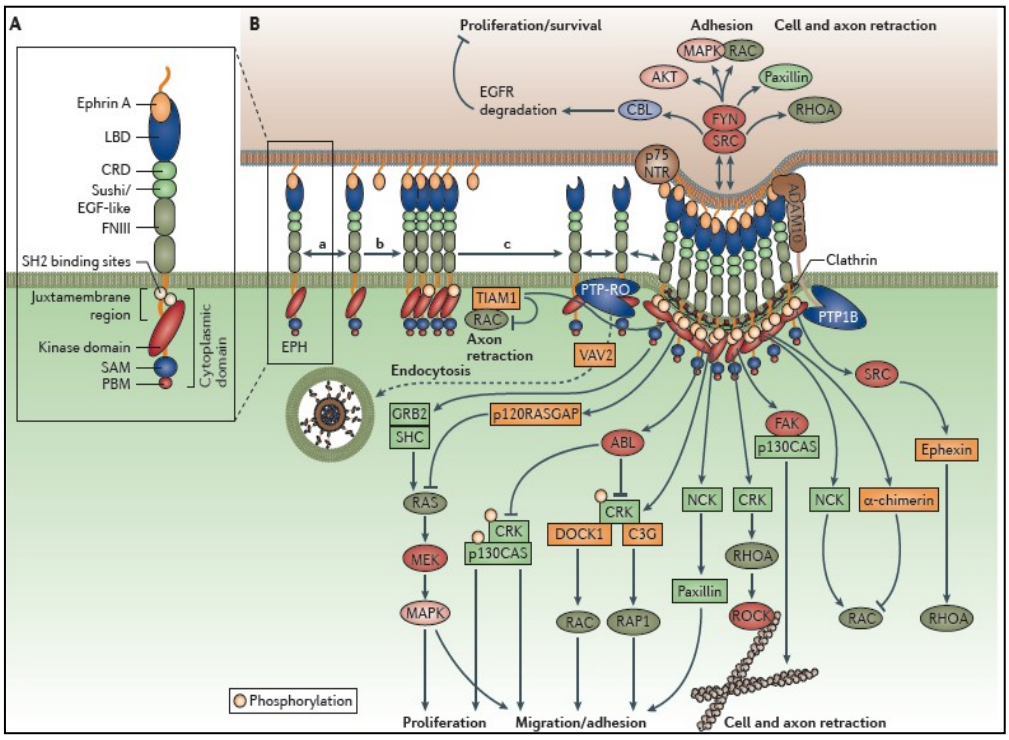

Figure 7: Eph signaling. Boyd AW *Therapeutic targeting of EPH receptors and their ligands*. Nat Rev Drug Discov. 2014 Jan;13(1):39-62.

EphB2 receptor forward signaling is mediated by specific GEF, intersectin and kalirin which regulate the EphB2-mediated cytoskeleton reorganization, mesenchymal invasion and migration[94,95].

The most important and best described EphB2 forward signaling influences the regulation of cell positioning and cell proliferation, activated by PI3K and Abl pathways, respectively[96]. In addition, EphB activation can also reduce cells adhesion through a negative modulation of the MAPK pathway via R-Ras[97].

1.2.2 EphA2 and EphB2 in the intestinal epithelium

In the intestine, Eph/ephrin signaling regulates a number of biological processes including cell proliferation, differentiation, migration and tissue morphogenesis. Eph receptors play a key role in tissue organization, particularly in maintaining the appropriate structure and preventing cell intermingling.

EphA2 and its ligand ephrinA1 are mainly involved in maintenance of intestinal barrier and in colon epithelial homeostasis with functions in stress response. Eph/ephrin system co-works with junctional molecules to accomplish cell sorting processes and modulate epithelial integrity.

Several members of EphA family are largely expressed in intestinal epithelium. High expression of EphA1, EphA4 and Eph7 are found at the crypt basis, in cell with high proliferative activity; whilst EphA2, EphA5 and ephrinA1 are highly expressed in differentiated cells in the crypt top[98].

EPHA2 and E-cadherin co-localize along the lateral membrane at site of cell-cell contact and ensure morphologic maintenance of epithelial cells. A reciprocal regulatory positive loop between EPH receptors and E-cadherin has been demonstrated. E-cadherin regulates phosphorylation and localization of EphA2[99] and stabilizes cell-cell contacts facilitating EphA2 association with its ligands. Moreover, ligand-mediated activation of EphA2 promotes E-cadherin–based cell-cell adhesion[100] (Fig. 8).

As described above, in the intestine context, stem cells localize at the bottom of crypt, where they divide and give rise to progenitor cells, which continue to divide as they migrate up the crypt axis. As cells leave the crypt, they also abandon their cycling activity and start to differentiate. Wnt signaling is a pivotal mitogenic regulator for intestinal stem cells and it also transcriptionally regulates the expression of EphB receptors and negatively regulates expression of their ligands[101,102]. EphB receptors and their ephrin-B ligands are expressed in counter gradients along the crypt-villus axis in the intestine, where EphB2 and EphB3 are present at high levels in stem cells at the bottom of the crypt and ephrin-B1 and ephrin-B2 are predominantly expressed by differentiating cells in the upper portion of the crypt[103]. In differentiating cells low levels of EphB2 favor their migration up to the gradient of ephrin-B1 expressing cell, in contrast up regulation of ephrin-B1 drives cells down the EphB2 gradient. In this way Eph/ephrin system controls the correct positioning of cells in the intestine through a unidirectional flow mediated by repulsive mechanism[101](Fig. 8).

EphB receptors (EphB2 and EphB3) regulate proliferation and migration in the intestinal stem cell niche by two independent EphB signaling[103]. Proliferation is mediated by tyrosine kinase dependent signaling, via Abl and

cyclin-D1, whereas cell positioning is mediated via PI3K in a kinase independent fashion[96]. Studies on null mice showed that Ephrin-B1 null mice displays distorted cell positioning[104], whilst EphB2- and EphB3-null mice result in decreased stem/progenitor cell proliferation and in distorted migration[101,103].

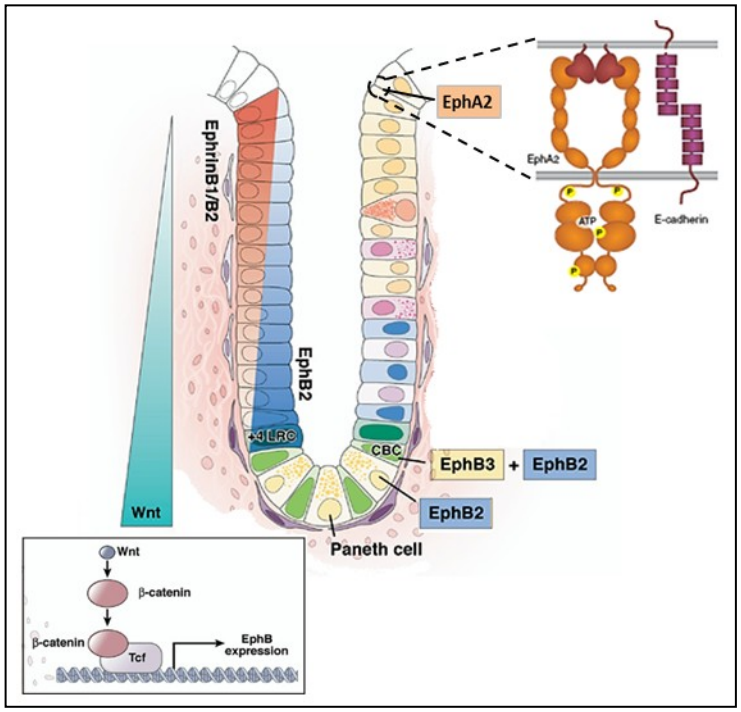

Figure 8: EphA2 and EphB2 in the intestinal crypt. Edited from Scoville *DH Current View: Intestinal Stem Cells and Signaling* GASTROENTEROLOGY 2008;134:849–864

1.2.3 EphA2 and EphB2 in Colorectal Cancer

Eph receptors and their ligands have emerged as integral players in the pathogenesis of cancer: aberrant expression of Eph and ephrin genes have been identified in a wide range of human tumors such as neuroblastoma, carcinomas of the breast, lung, gastric, prostate, ovarian, melanoma and colon.

Ephs and ephrins were thought to play an oncogenic role in human cancer, as initially demonstrated by their first isolation from a hepatocellular carcinoma cell line, where they were found to be at least 10-fold overexpressed compared with non-malignant tissue. However, recent

evidence demonstrates a tumor suppressive role for Ephs in some instances, suggesting that the role of Ephs and ephrins is far more complex than first assumed[105].

As evidence, their downstream signaling pathways control processes such as cell growth, proliferation, organization of the cytoskeleton, cell-matrix and cell-cell attachment, the dysregulated function of which contributes to an invasive and metastatic tumor phenotype.

- **EphA2 in Colorectal Cancer**

In particular, EphA2 overexpression has been documented at mRNA and protein level in a number of human malignancies such as lung, breast, liver, gastric, renal, prostate, ovary, esophagus, bladder, pancreas, cervical, melanoma, glioblastoma, SCCHN and colon[106]. Moreover, in CRC genetic ablation of EphA2 in ApcMin/+ mice has been found to result in significant reduction in number and size of intestinal tumors[107]. The pro-oncogenic role of EphA2 resides in its ligand independent activity: a number of studies have documented low levels of EphA2 phosphorylation in malignant cells compared to normal cells despite its overexpression[108]. A crucial initial step of colorectal carcinogenesis is reduction of E-cadherin expression and function, resulting in decreased cell-cell adhesion and destabilization of the epithelial architecture with loss of cell-cell attachment. As a consequence, the interaction between EphA2 and ephrinA1 on neighboring cells is inhibited, abolishing its tumor suppressing function mediated by Tyr phosphorylation, internalization and degradation of EphA2 receptor[109]. Moreover, the ephrinA1 inhibiting activity on AKT is removed and EphA2 ligand-independent effect is switched on by AKT-mediated phosphorylation on Serine897. This signaling promotes cancer cell migration, by means of the association between EphA2 and Focal Adhesion Kinase (FAK), a tyrosine kinase involved in EphA2/Integrins crosstalk: FAK phosphorylation results in active conformation of integrins and triggering of integrin-mediated adhesion, cell spreading and migration[110]. Unligated EphA2 is also able to destabilize adherent junctions via Rho-GTP activation: on one hand, it enhances the low molecular weight phosphotyrosine phosphatase (LMW-PTP) activity that in turns hinders p190 RhoGAP, a Rho-GTP inhibitor[111],

on the other hand it interacts with Ephexin4, one of guanine nucleotide exchange factors for RhoG, and activates RhoG[109](Fig. 9).

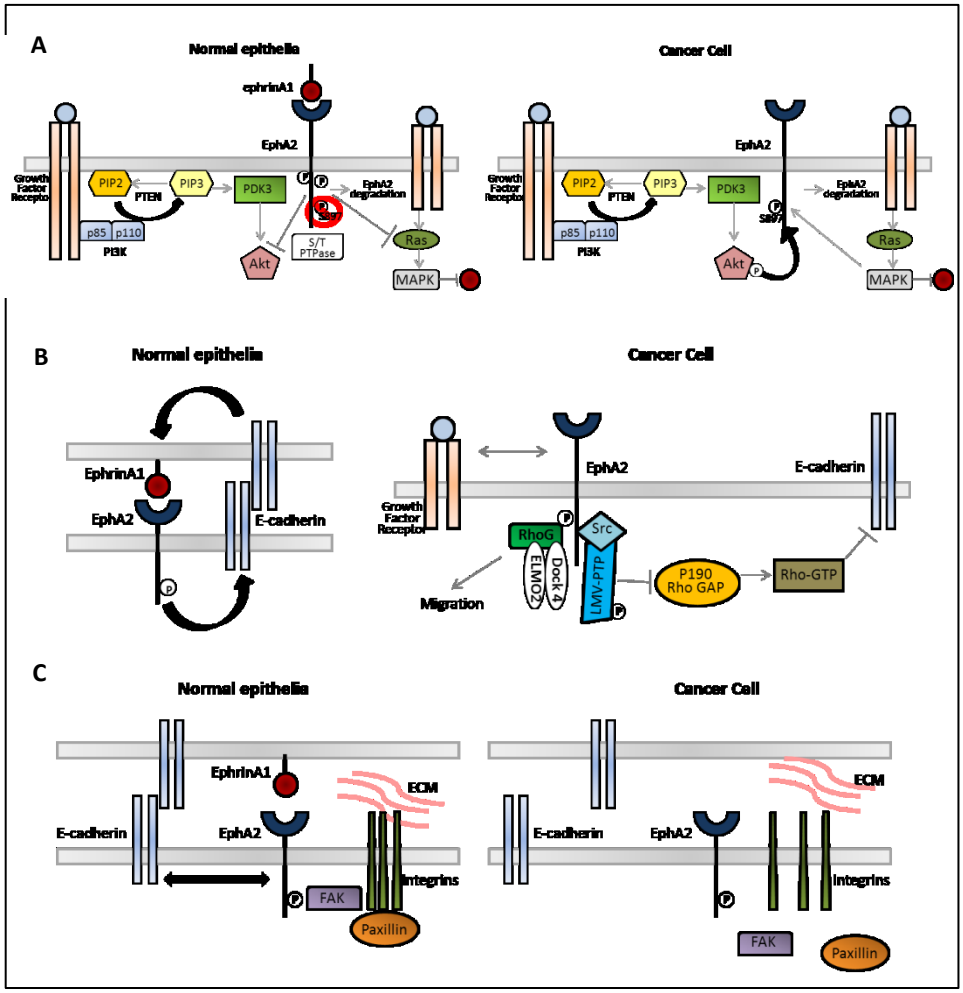

Figure 9: EphA2 molecular pathways in normal and cancer cells. (A) AKT and MAPK**; (B)** GAP and cadherins**; (C)** integrins**.** Edited from Beauchamp A *Ephs and Ephrins in Cancer: Ephrin-A1 Signaling* Semin Cell Dev Biol. 2012 23(1): 109–115.

When ephrinA1-EphA2 bound is broken, the negative feedback loops existing between EphA2 and Ras/MAPK and PI3K/Akt pathways are removed, so EphA2 can exerts its pro-oncogenic activity also through direct crosstalk with EGFR network stimulating cancer cell proliferation and survival. In turn, EphA2 expression is upregulated in response to cell adhesion by EGFR, MEK and SRC family kinases[112](Fig. 10).

Recently, EPHA2 receptor has been found to play an important role in many aspects of EMT, including induction of a mesenchymal-like phenotype,

inhibition of epithelial characteristics and crosstalk with EMT-related signal transduction pathways such as the previously described E-cadherin, RAS/MAPK and Akt/mTOR networks.

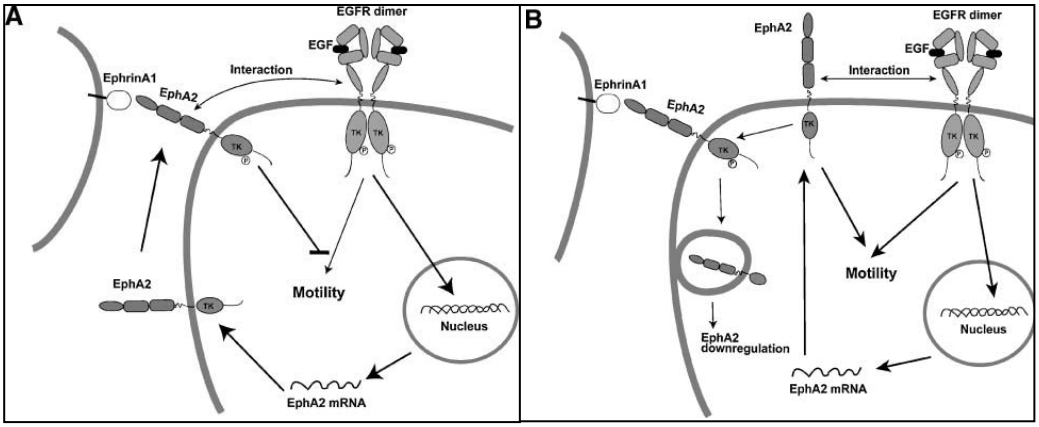

Figure 10: EphA2 and EGFR crosstalk. Schematic representation of the crosstalk existing between EphA2 and EGFR in (A) normal and (B) cancer cells**.** Edited from Larsen AB *Activation of the EGFR Gene Target EphA2 Inhibits Epidermal Growth Factor-Induced Cancer Cell Motility* Mol Cancer Res 2007;5:283-293.

The role of EphA2 in tumor metastasis has been widely investigated in a number of tumors including melanoma, ovarian, lung, renal, prostate[179], but less is known about colon cancer. An immunohistochemical study of Saito et al. found a direct relation between EphA2/E-cadherin expression and colorectal cancer lymph node metastases[131].

EphA2 is also involved in tumor cell-extrinsic, microenvironmental mechanisms of tumor progression: EphA2 and ephrin-A1 expression were correlated with MVD in human CRC samples, suggesting they might regulate neovascularization as well as tumorigenesis. These clinical observations are consistent with data derived from cell culture and animal studies[113]: EphA2-ephrinA1 system has been demonstrated playing a key role in tumor angiogenesis with a clearly distinct mechanism from which this system plays in affecting the behavior of tumor cells. Indeed EphA2 expressed on cancer cell is not the principal actor, but rather the EphA2 receptor localized on the endothelial cells that, stimulated by the tumor-derived ephrin-A1, is able to induce expression of VEGF and subsequently activate distant host endothelial cells, leading to angiogenesis and metastasis[114]. Moreover recent evidences found that EphA2 expressing cells participate to the process of vasculogenic

mimicry, where aggressive and dedifferentiated tumor cells form fluid-conducting channels not lined by endothelial cells[115].

- **EphB2 in Colorectal Cancer**

Deregulated mRNA and protein expression of EphB2 have also been reported in human colon cancer. Although increased EphB RTK expression was detected in the initial phases of CRC, subsequent expression analyses coupled with genetically engineered mouse models suggest tumor suppressive functions for EphB receptors: a number of studies report a direct correlation between loss of EphB2 with CRC progression[116,117] and EphB2 overexpression with prolonged survival[118].

So, if EphA2 has a clear pro-oncogenic function in colorectal carcinogenesis, the role of EphB2 seems to be dual, or biphasic. In the first phases of CRC progression, EphB2 expression is upregulated and the receptor acts as tumor-promoter. As the cancer evolves from adenoma to carcinoma, EphB2 expression is gradually lost, due to the prevalence of its onco-suppressor function. The EphB2 tumor suppressor activity was demonstrated in a mouse model of adenomatous polyposis (APCMin/+) where an invasive adenocarcinoma developed when EphB2 signaling was inhibited[116].

This duality resides in two distinct pathways in which EphB2 is involved: proliferation and positioning of intestinal stem cells. The principal regulator of proliferation in the intestine is Wnt, through the activation of c-Myc and inhibition of p21. Recently EphB2 has been identified as direct transcriptional target of TCF/β-catenin complex and a third mediator of Wnt proliferating effect. The Wnt pathway is over-activated in the 70% of CRCs, which show homozygous inactivation of Adenomatous Polyposis Coli (APC) tumor suppressor gene that inhibits β-catenin nuclear translocation. When β-catenin/Tcf complex migrates to the nucleus it switches on EphB2 transcription in the stem cells located at the base of the colonic crypt. EphB2 expression and kinase function activate a signaling cascade that involves Abl and CyclinD1, direct effector of cell cycle regulation. As the tumor progresses from adenoma to carcinoma, the proliferating kinase-dependent function of EphB2 becomes less relevant, to the advantage of the kinase-independent function of cell-positioning. At the same time, CyclinD1 expression becomes independent from EphB signaling, keeping on exerting its mitogenic activity.

The second activity of EphB2 receptor consists in the regulation of cell positioning along the crypt axis. Its kinase-independent signaling inhibits PI3K, suppressing migration and invasion of cancer cells. Moreover EphB-ephrinB1 bound regulates the formation of E-cadherin-based adhesions thanks to the interaction with the metalloproteinase ADAM10[96].

Battle and colleagues hypothesizes a model for EphB2 activity during colorectal cancer progression: in the first phases, APC-mutant cells populate the stem cell niche at the bottom of the crypt forming the so-called dysplastic niche. Cancer cells proliferate laterally, in strict contact with adjacent normal crypts, where the exposed ephrinB1 can bind the EphB2 overexpressed on tumor cells in response to constitutively activated Wnt signaling. This bound compartmentalizes EphB2+ cells expansion in the niche context and activates CyclinD1 pathway of proliferation, resulting in in situ adenoma growth. At the transition from adenoma to carcinoma EphB2 expression in lost, so the cancer cell is free to exit the crypt and invade the surrounding tissue. This model is coherent with a study of 2005 where reduction or loss of EphB2 and EphB4 expression correlated with the shift from adenoma to invasive carcinoma in a panel of 108 human CRC samples[116]. Moreover, a positive correlation between EphB2 expression and better overall and recurrence-free survival in human CRC patients has been identified in three independent studies[111] and reduced EphB2 expression has been associated with metastasis both in CRC metastatic cell lines and in human colorectal cancer[132,133] (Fig. 11).

$EphB2_{high}$ cell population at the bottom of normal colon crypt represents a small percentage of the overall amount of intestinal cells, expressing the classical stemness markers like Ascl2 and Lgr5. In colorectal cancer this proportion is maintained, leading to ascribe a less important role to this population in cancer development. However a study of Suarez et al. demonstrated a high tumorigenic activity for a mouse colorectal cancer FACS sorted $EphB2_{high}$ cell population: these cells were able to form organoids when cultured in vitro and to generate xenograft when injected into NOD/Scid mice in histological patterns that largely resembled the primary tumor, demonstrating to hold high tumor-initiating potential as well as long-term self-renewal and differentiation capacity[120]. Also Feng et al. validated the CSCs identity of human CRC cells overexpressing EphB2 by the mean of a sphere formation assay[121].

So we could speculate that $EphB2_{high}$ cell population in CRC constitutes the cancer stem cell niche that represents the reservoir of the tumor itself, fueling its expansion and invasion.

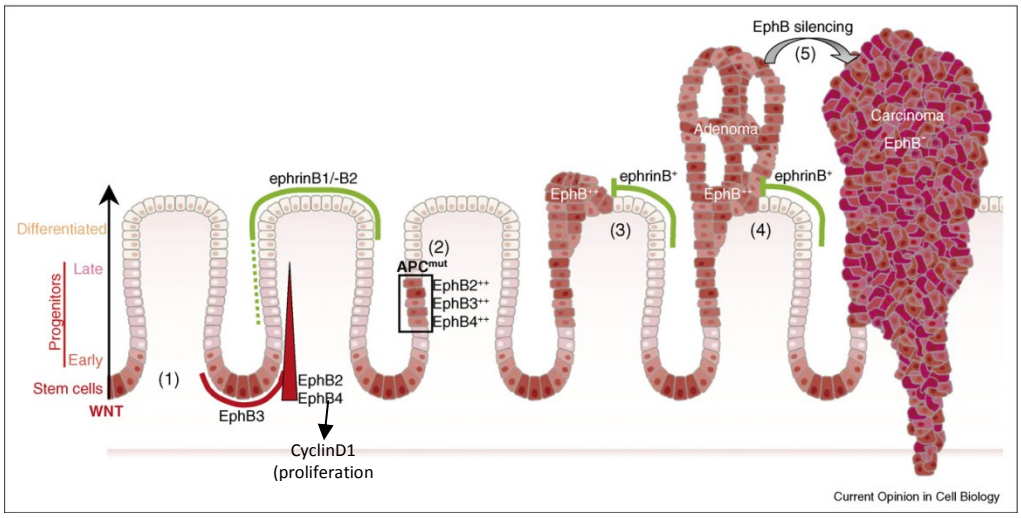

Figure 11: EphB–ephrinB interactions during CRC progression. Edited from Merlos-Suárez A *Eph-ephrin signalling in adult tissues and cancer.* Curr Opin Cell Biol. 2008

➢ EphA2 and EphB2 role in metastasis

Circulating tumor cells (CTCs) are cancer-derived cells that are able to detach from the tumor, enter the circulation, reach and invade the target organ and form metastasis[122]. To gain these competences, CTCs undergo epithelial to mesenchymal transition, a reversible process in which cells decrease expression of epithelial markers and acquire mesenchymal features. Once completed the metastatic process, cells undergo mesenchymal to epithelial transition, resembling again primary tumor genetically and phenotypically. Circulating tumor cells have been found in the peripheral blood of patients with a wide range of solid tumors, including colorectal cancer, and their detection in liquid biopsy could represent a less invasive sampling for tumor diagnosis and follow up[123].

The association of EphA2 with cancer, its exclusive overexpression on tumor cells and its involvement in EMT make it a potential surface marker to isolate CTCs. In 2008 Scarberry and colleagues successfully used magnetic nanoparticle conjugated with the EphA2-specific peptide YSA to target and remove metastatic ovarian cancer cells from the fluid of the abdominal cavity

or circulatory system[124]. EphA2 was also found on the surface of CTCs isolated from diverse stage III-IV tumor types including colorectal cancer[123].

The role of EphB2 in CTCs is less investigated: only recently Hamilton et al. identified EphB2 expression on a CTC cell line expanded ex vivo from human small cell lung cancers[125].

Exosomes are microvesicles containing an array of proteins, DNA, mRNAs and microRNAs that are normally released from many cell types in the microenvironment to influence target cells with their content. Cancer cells secrete higher concentration of exosomes in order to suppress the immune system, modulate the angiogenesis and condition the metastatic niche, to generate a pro-tumor environment for the adhesion and growth of distant tumors[126]. Researchers' efforts are focused not only on the cargo of these microvesicles, but also on the recognition molecules they express to selectively target recipient cells.

A number of studies identified different types of Eph receptors on exosomes' surface, inducing tumor promotion or suppression with different effects from the classical bidirectional signaling. Sun and colleagues[127] recently recognized an essential bound between ephrinA2 expressed on osteoclast-derived exosomes' surface and EphA2 expressed on osteoblast to permit osteoclasts/osteoblast crosstalk. Moreover, EphA2 seems to be crucial for sorting molecules in multivesicular bodies and so in exosomes[128]. Tauro et al.[129]revealed in colorectal cancer-derived exosomes the expression of EphA2 and ephrin-B1 and Gong[130] found also EphB2 in vesicles extracted from HEK293 and HeLa cells overexpressing EphB2 and from U251 glioma cells and cultured primary cortical neurons expressing endogenous EphB2.

Both EphA2 and EphB2 seem to exert a key role in the metastatic process: on the one hand, EphA2 has been demonstrated to be involved in neoangiogenesis and epithelial to mesenchymal transition and its presence on CTCs' and exosomes' surface could be crucial to target the metastatic niche and establish distant tumors; on the other hand, EphB2 is a validated marker of intestinal stem cells and its stemness function is maintained during tumor progression, so its expression could probably characterize circulating tumor cells committed to form metastasis, even if not yet demonstrated as in exosomes. So, circulating tumor cell committed to metastasize could have an

intermediate EphA2/EphB2 phenotype, showing in part epithelial and in part mesenchymal features fine-tuned in the ongoing process of epithelial to mesenchymal transition.

1.3 Colorectal Cancer therapies

In CRC management tumor-related features, including number and localization of metastases, tumor progression, presence or absence of biochemical markers, and patient-related factors, like co-morbidity and prognosis, influence the choice of the first-line treatment. According to this, CRC patients have been classified in four distinct risk groups to match the best treatment strategy[135]. Group 0 includes patients with no metastatic disease or with resectable liver or lung metastases and lack of poor prognostic signs and is treated with surgical resection of the metastasis. Chemotherapy has not been found to provide a great advantage in the overall survival of this group. Group 1 includes patients initially treated with induction chemotherapy to reduce the number and size of the metastases and enable subsequent surgical resection. Recommended chemotherapy for these cases comprises combinations of cytotoxic agents with targeted therapy. Group 2 includes patients with disseminated unresectable disease. Treatment is palliative rather than curative and should induce metastatic regression in a short time to reduce the symptoms, aggressiveness and extension of the disease: the preferred option comprises a cytotoxic doublet in combination with a targeted agent (anti-VEGF or anti-EGFR strategies). In oligometastatic patients who respond to treatment, additional ablative methods may be considered to increase the progression-free interval. If ablative methods cannot be used, de-escalation or discontinuation of the initial combination should be studied as a maintenance treatment. Group 3 includes patients with unresectable disease. In this case the purpose of the treatment will be to prevent tumor progression and increase treatment-free life: the most commonly used strategies comprise a fluoropyrimidine as cytotoxic agent combined, or not, with a biological targeted agent (Fig. 12).

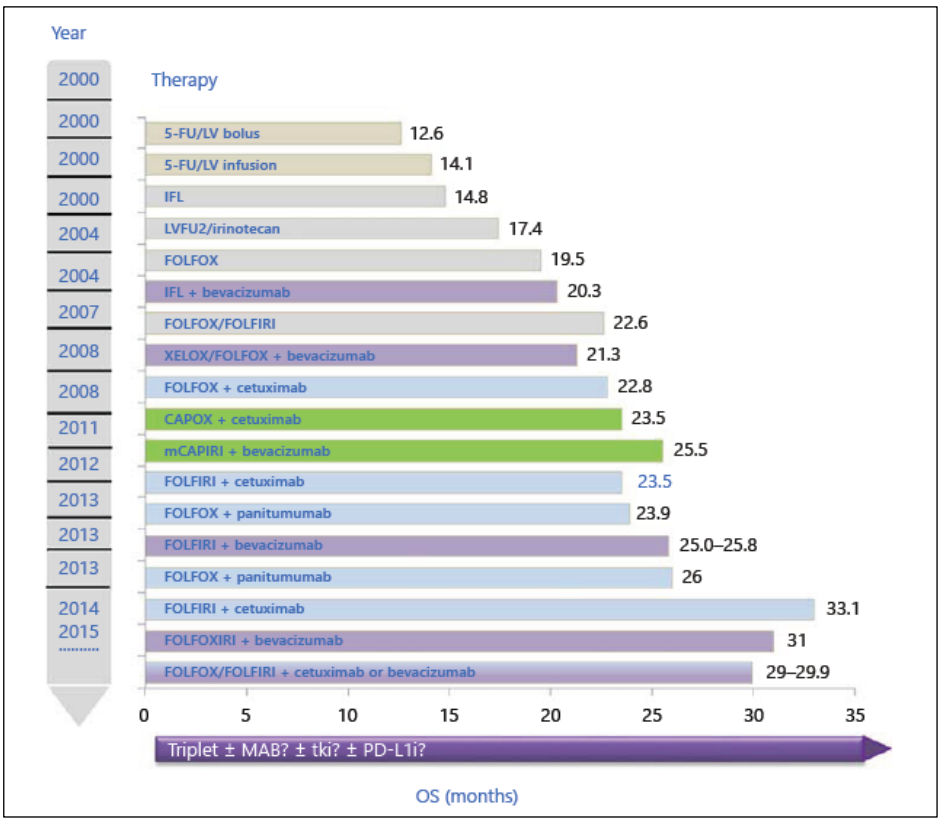

Figure 12: Improvement of OS in stage IV mCRC. Examples of phase II/III studies between 2000 and today with improvement of OS in the therapy of mCRC * Phase II studies. Edited from Pohl M *Therapeutic Strategies in Diseases of the Digestive Tract – 2015 and Beyond Targeted Therapies in Colon Cancer Today and Tomorrow* Dig Dis 2016;34:574–579

1.3.1 Chemotherapeutic agents

Fluoropyrimidines are anti-metabolite agents whose main mechanism of action consists in the inhibition of thymidylate synthase activity. 5-fluorouracil (5-FU), developed in 1957, was the first studied compound: it inhibits tumor cell division by blocking the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (thymidylate). 5-FU is commonly given either as a bolus injection with leucovorin (folinic acid) or a continuous infusion, but if 5-FU bolus treatment favors RNA damage, continuous treatment with 5-FU favors DNA damage[136].

5-FU activity can be potentiated by reduced folate[137]: since Ullman reported that leucovorin (5-formyl tetrahydrofolate [THF]) enhances 5-FU cytotoxicity in cultured leukemia cells, a number of clinical trials have been started, as the one conducted by Poon in 1989 in which Response Rate of 5-FU/Leucovorin was reported at 23%[138].

In 2000s the topoisomerase I inhibitor Irinotecan and the diaminocyclohexane platinum compound Oxaliplatin were included in anti-CRC therapies. Irinotecan is a semisynthetic derivative of the natural alkaloid camptothecin that is converted by liver carboxylesterases in the active metabolite SN-38[139]. Oxaliplatin acts by impairing DNA replication and inducing cellular apoptosis[140]. In the Intergroup trial N9741[141], the efficacy of FOLFOX (5-FU/leucovorin with oxaliplatin) was significantly better than that of IFL (5-FU/leucovorin with irinotecan) with regard to overall survival (OS), time to progression (TTP) and response rate (RR). A phase III study conducted by Gruppo Oncologico Nord Ovest (GONO) on 244 untreated metastatic CRC patients comparing fluorouracil, leucovorin, oxaliplatin and irinotecan (FOLFOXIRI) with infusional fluorouracil, leucovorin and irinotecan (FOLFIRI) associated FOLFOXIRI regimen with a significantly higher RR, progression-free survival (PFS) and OS compared to the FOLFIRI regimen[142]. However regimens containing combinations of daily bolus of 5-FU/LV and oxaliplatin or irinotecan showed severe gastrointestinal toxicity and high mortality rates [143].

1.3.2 Angiogenesis inhibitors

The fact that tumor growth is sustained by new blood vessel formation led to the formulation of an anti-angiogenetic strategy by the mean of the monoclonal antibody bevacizumab. This therapeutic agent inhibits the protein that most of all stimulates blood vessel development, the Vascular Endothelial Growth Factor (VEGF). In 2004 the pivotal bevacizumab/Fluorouracil 2107 phase III trial[144] evaluated bevacizumab efficacy in patients randomized to IFL with bevacizumab or IFL alone. The addition of bevacizumab significantly improved OS, PFS and RR compared with IFL alone.

The Eastern Cooperative Oncology Group 3200 study enrolled patients previously treated with IFL and found that OS, PFS, and RR were all

significantly improved with bevacizumab and FOLFOX treatment compared with FOLFOX alone[145].

The recombinant fusion protein Aflibercept, which blocks VEGF-A, VEGF-B and placental growth factors, is another in-use anti-angiogenetic agent[135].

1.3.3 EGFR inhibitors

Epidermal growth factor receptor (EGFR) is a 170kD transmembrane glycoprotein member of the tyrosine kinase receptor family ErbB involved in cell proliferation and survival that is frequently overexpressed in CRC patients. Based on this observation in 1984 Mendelson and Sato first proposed this receptor as anti-cancer therapeutic target[146]. Cetuximab and Panitumumab were the first therapeutic agents targeting a specific molecular pathology approved to treat patients with chemorefractory metastatic colorectal cancer in 2004 and 2006 respectively: EGFR-positive tumors expressing wild type KRAS (Kirsten rat sarcoma viral oncogene homolog)[147]. In the CRYSTAL (Cetuximab Combined with Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer) study, patients with EGFR-positive tumors were randomized to receive FOLFIRI alone or FOLFIRI with cetuximab. FOLFIRI with cetuximab marginally improved PFS compared with FOLFIRI alone, but the OS, PFS and RR significantly improved in a subset analysis of patients with wild type KRAS[148].

In the UK Medical Research Council Continuous Chemotherapy Plus Cetuximab or Intermittent Chemotherapy trial patients were randomized to receive continuous FOLFOX, continuous FOLFOX with cetuximab, or intermittent FOLFOX alone. FOLFOX with cetuximab increased RR compared with FOLFOX alone but there was no evidence of improved PFS or OS in patients with wild type KRAS[149].

In the PRIME (Panitumumab Randomized Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy) trial, patients were randomized to treatment with FOLFOX with or without panitumumab. In the subset with wild type KRAS, panitumumab with FOLFOX significantly improved PFS compared with FOLFOX alone but did not lead to a significant improvement in OS[150].

➢ Mechanisms of resistance to EGFR inhibitors in CRC

From the last decade's studies it becomes clear that only the 10-20% of patients benefits from anti-EGFR monoclonal antibodies[151]. The fundamental reason of this limited success of targeted therapy resides in tumor heterogeneity: more than a half of CRCs holds mutations in EGFR pathway-related genes that negatively affect response to mAbs directed against EGFR itself[152] (Fig. 13) .

However, EGFR expression level and somatic mutations are not correlated with the response rate[151]. On the contrary, increasing in EGFR gene copy number, independently by its translation, seems to improve the response rate of CRC patients, even if with a weak statistical correlation[153]. Also high levels of the EGFR ligands amphiregulin and epiregulin have been demonstrated to be linked to cetuximab monotherapy sensitivity with a weak statistical correlation[154].

The molecular mechanisms involved in the resistance to EGFR moAbs involve a constitutive activation of the two principal axis of its complex pathway: MAPK cascade and PI3K signaling.

The KRAS-RAF-MAPK axis controls cell growth, differentiation and apoptosis. KRAS belongs to the family of HRAS and NRAS encoding guanosine di/tri-phosphate binding proteins, often mutated in human tumors. When activated, KRAS recruits the serine protein BRAF and triggers the cytoplasmic cascade of MAPK that leads to transcription of target genes in the nucleus[155]. KRAS activating mutations in codon 12 and 13 are today the major negative predictor of response to cetuximab therapy in CRC patients and are used as a test to restrict the use of this moAb in combination with chemotherapy[156]. The National Cancer Institute of Canada Clinical Trials Group (NCIC-CTG) monotherapy study conducted in relapsed/refractory patients definitively established KRAS mutations as a negative outcome predictor of cetuximab-based therapies in mCRC[157].

However, not all the patients negative for KRAS mutations benefit from cetuximab therapy, even if presence of RAS mutations accounts for 35-45% of non-responsive patients [158].

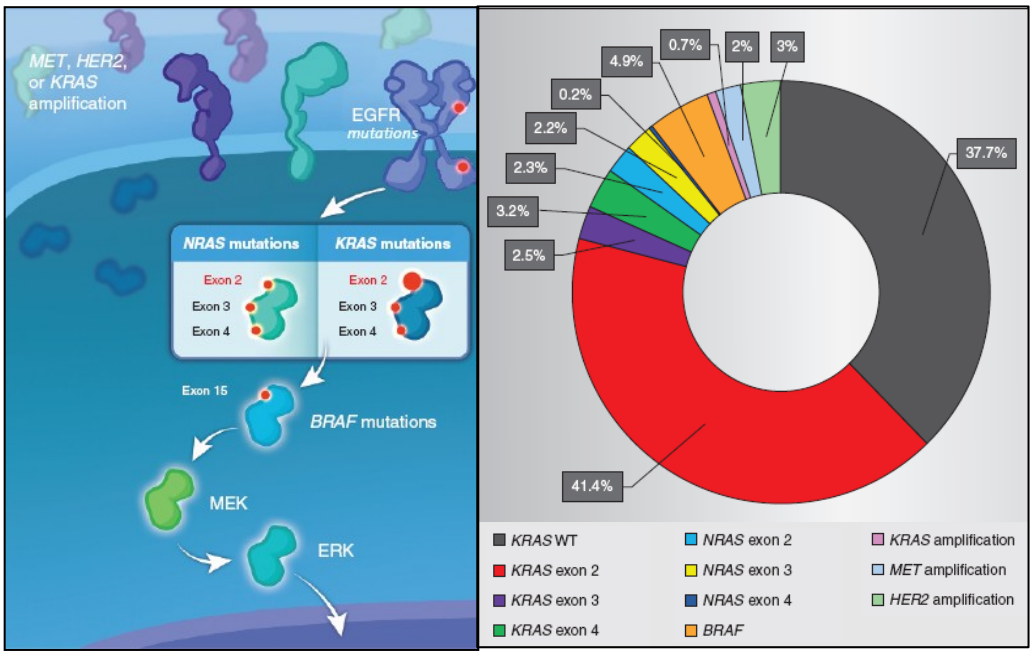

Figure 13: Genetic alterations associated with *de novo* resistance to anti-EGFR therapies in mCRC. Edited from Misale S *Resistance to Anti-EGFR Therapy in Colorectal Cancer: From Heterogeneity to Convergent Evolution* Cancer Discov 7 2014

BRAF mutations occur in 5-8% of the cases and are mutually exclusive with KRAS mutations in colorectal cancer[159]. It is important to note that mutated BRAF is also associated with poor prognosis. Drugs to inhibit BRAF kinase activity are clinically approved or under development: studies considering a concomitant treatment of patients carrying the BRAF mutation with BRAF inhibitors and cetuximab are now in progress [160].

The PI3K axis involves AKT activation and can be deregulated with mutations occurring in the PI3KCA p110 subunit or epigenetic or genetic silencing of the pathway inhibitor PTEN. These alterations are not mutually exclusive with KRAS or BRAF mutations and the correlation with cetuximab treated CRC patients' outcome is not strong enough to be applied as negative predictive markers[161].

Additional genetic mechanisms have been proposed to be involved in cetuximab resistance that activate parallel pathways to EGFR network, as the

amplification of MET and ERBB2 genes that together cover the 5% of the total alterations[162,163].

The aberrations described above do not cover the totality of patients who show clinical resistance to anti-EGFR drugs. Indeed in the 10% of cases the genetic alteration that confers *de novo* resistance is unknown[161].

This variety of escape mechanisms reflects the high level of molecular heterogeneity of human CRC but is entirely aimed to reactivate EGFR signaling pathway. So an integrated therapy that blocks at the same time the signaling nodes known to be involved in anti-EGFR therapy resistance would be far more effective than a treatment with cetuximab alone.

1.3.4 EphA2 and EphB2 based targeted therapy

Cancer targeted therapy's intent is to destroy tumor cells and preserve normal tissues, taking advantage of cancer molecular markers. Eph receptors, in particular EphA2 and EphB2, are ideal targets for their involvement in the progression of a large range of metastatic cancers and for their preferential expression in tumor rather than in normal tissues: their altered expression has been modulated to suppress their pro-oncogenic and enhance their tumor-suppressor activities (Fig.14, Tab. 1).

- **Ligand-based**

One of the approaches of the Eph/ephrin based targeted therapy to influence Eph activity in cancer is the ligand-based one, where Eph, ephrins or surrogates are delivered to the tumor to bind the respective receptor and activate or suppress the forward or the reverse signaling.

Recombinant ephrins extracellular domains (ECDs) bind receptors with high affinity, have long in vivo half-life and act on multiple family members, with the double consequence of increase the efficacy and develop unwanted effects. EphA2–ephrin A1 signaling it's of main interest for tumor treatment, for its prominent role in proliferation, neovascularization, migration and invasion and has been targeted with soluble EphA2–Fc and ephrinA1–Fc proteins in a number of studies. The ephrinA1 bound is followed by the downregulation and internalization of cancer cells' EphA2 receptor, with the suppression of its pro oncogenic activity[108]. Noblitt et al. promoted the

degradation of EphA2 with the overexpression of human ephrinA1-Fc from a human adenoviral type 5 vector in a breast cancer cell line, decreasing tumor cell activity[165]. However, attention must be payed to the role of ephrinA1 in the stimulation of endothelial EphA2 pro-angiogenetic activity: EphA2 and EphA3 Fc, for example, can function as anti-cancer agents in mouse models competing with ephrin-A1 bound and so inhibiting EphA2 forward signaling in the tumor vasculature[166].

Pasquale and colleagues[167] identified by phage display a series of dodecapeptides that can selectively target the ephrin-binding pocket of individual Eph receptors and antagonize ephrin binding, such as the EphA2-directed YSA and SWL and the EphB2-directed SNEW.

- **Small molecules**

The receptor/ligand interface is also the target of small molecules able to interfere with the receptors bound, as polyphenols, salicylic acid derivatives, bile acid derivatives, and doxazosin.

Norberini et al. demonstrated that polyphenols from green tea are able to interfere with EphA2/ephrin-A1 interaction, but with low specificity; salicylic acid derivatives, exemplified by 4-(2,5- dimethyl-1H-pyrrol-1-yl)-2-hydroxybenzoic acid (Compound 1), inhibit ligand binding of the EphA2 and EphA4 receptor sub-types with IC50 around 10uM[167]; lithocholic acid (LCA) was identified at the University of Parma[168] as a potent antagonist of the EphA2–ephrin-A1 interaction, with the derivative UniPR126 reaching an IC50 of 2mM (LCA). Petty et al. identified a quinazoline-based compound named doxazosin that behaves as an agonist of Eph signaling. Its activity mimics the ephrinA1's effect: it is able to inhibit downstream EphA2 pathway mediators such Akt and ERK, reducing migration of cancer cell and prolonging tumor survival in a mouse cancer model[169].

Moreover, Azurin, a Pseudomonas aeruginosa electron transfer protein, has been found to block ephrin binding to EPHB2 and has shown cytotoxic activity in EPHB2+ prostate carcinoma cells[170]. However the efficacy, specificity, stability and bioavailability of these compounds needs to be better verified to use them as therapeutic agents.

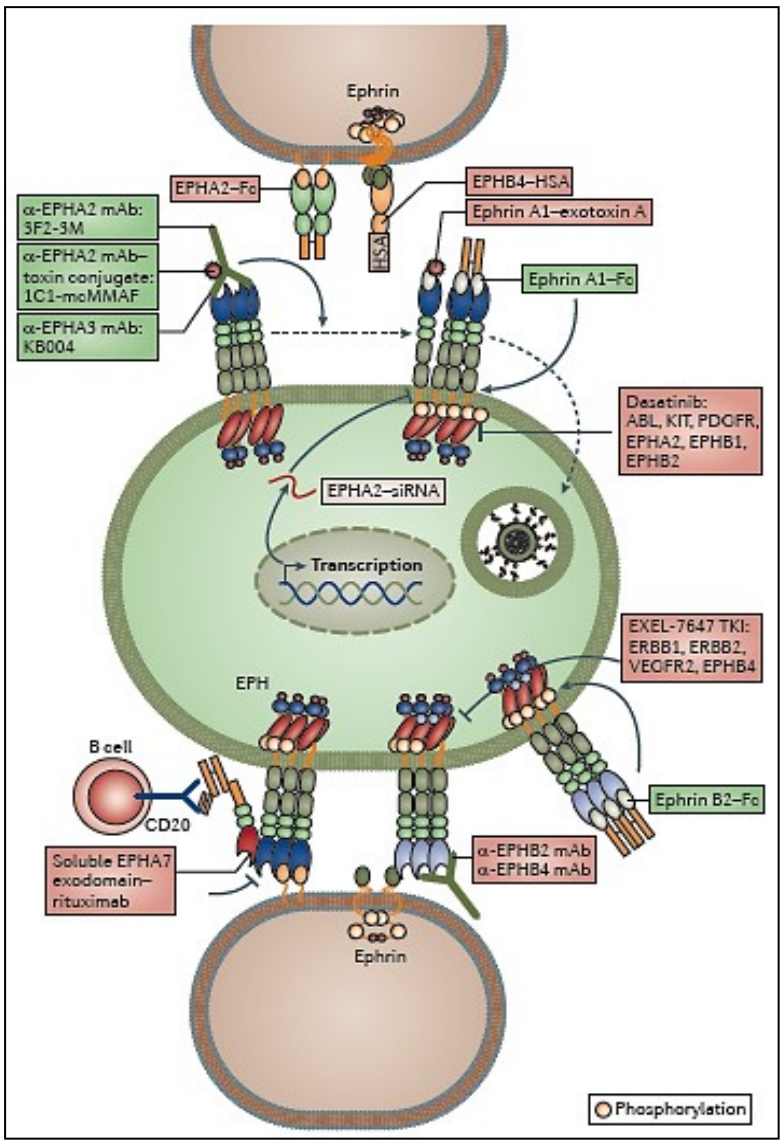

Figure 14: Strategies for therapeutic targeting of EPH and ephrin functions. Boyd AW *Therapeutic targeting of EPH receptors and their ligands.* Nat Rev Drug Discov. 2014 Jan;13(1):39-62.

- **Tyrosine Kinase Inhibitors**

Another strategy to block Eph/ephrin system oncogenic function consists in inhibiting the tyrosine kinase activity, receptor autophosphorylation and consequent activation of the downstream signaling. Between the multi-targeted tyrosine kinase inhibitors (TKI) evaluated for their potential effects on

oncogenic EPH function, Dasatinib (BMS‑354825), targeting Src, BCR-ABL, c-Kit and platelet-derived growth factor-beta receptor has been demonstrated to inhibit both EphA2 and EphB2[171-173] kinase activity and, only recently, also EphA2 kinase independent activity, in an indirect way[174]. A number of studies demonstrated its potent antitumor activity in both in vitro and in vivo tumor xenograft models[175-177] and FDA approved its use in the treatment of imatinib-resistant chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia.

For these reasons dasatinib is now object of several clinical trials: a Phase II trial in stage III and IV melanoma (ClinicalTrials.gov identifier: NCT00436605) revealed minimal clinical response and poor tolerability; a pilot study on stage III, IV or recurrent endometrial carcinoma is assessing the combined effect of carboplatin, paclitaxel and dasatinib on tumor cell proliferation, cell division, motility, oncogenic signaling and EPHA2 expression (ClinicalTrials.gov identifier:NCT01440998). Moreover, two Phase I/II clinical trials evaluated the correlation between EPHA2 expression and response to dasatinib treatment of squamous cell carcinoma (ClinicalTrials.gov identifier: NCT00563290).

In vitro inhibitory activity has also been demonstrated for Nilotinib, a BCR-ABL inhibitor, which binds and inactivates EphB2 and the ABL and LYN inhibitor Bafetinib, which acts against EphA2. Moreover, ALW-II-49–7 was reported to inhibit EphB2 tyrosine kinase activity[178].

- **Monoclonal antibodies**

Monoclonal antibodies with their high binding affinity, specificity and long in vivo half-life, are an alternative and effective approach for their agonistic and antagonistic activity versus EphA2 and EphB2 receptors. EphA2-directed agonistic antibodies stimulate forward signaling to induce receptor degradation and negatively regulate tumor cell growth. Between the diverse EphA2 monoclonal antibodies, mAb EA5 reduced microvascular density and tumor growth and increased survival in ovarian cancer xenografts, and inhibited breast cancer xenograft growth and metastasis[179]; the humanized version of mAb B233 with improved FcγRIII binding capacity (3F2‑3M) exerts antibody-dependent cell-mediated cytotoxicity (ADCC)-mediated antitumor effects in ovarian, lung and breast cancer xenografts. Interestingly, treatment

with 3F2‑3MmAbs restored drug sensitivity in trastuzumab-resistant tumor cell lines by targeting the oncogenic ERBB2–EPHA2 crosstalk[178].

However, EphA2-directed monoclonal antibodies have shown discordant results in mouse preclinical models[180]. This could be due to the combination of different effects on cancer cells and the tumor microenvironment: agonistic mAbs could promote tumor angiogenesis activating the EphA2 receptor expressed by endothelial cells. However in combination therapy with tamoxifen, paclitaxel and docetaxel[181] they have been demonstrated to enhance treatment efficacy. Recently an antibody, named D2, directed to the ligand binding domain of EphA2 to block ephrinA1 binding, was generated by phage display and it seems able to induce apoptosis in COS-7 cells[182].

- **Conjugates**

Besides inhibiting Eph activity in cancer cells, monoclonal antibodies and peptides could target Eph receptors to deliver cytotoxic agents to tumor-specific cells, regardless the function that the bound activates. A number of recent studies have provided insight into drug/toxin-conjugated Eph antibodies and peptides capable of killing tumor cells that overexpress Eph receptors. For example, the agonistic anti-EphA2 antibody 1C1 (Medimmune) derived from phage display didn't show cytotoxic activity in EphA2 overexpressing cells, but stimulated receptor phosphorylation and degradation, and inhibition of Ras/MAP kinase and Akt pathways. To enhance its efficacy, the antibody was conjugated with the microtubule inhibitor auristatine (1C1-mcMMAF) to effectively deliver the cytotoxic agent to cancer cells[182]. Unfortunately, the clinical trial for 1C1-mcMMAF has been interrupted for adverse side-effect such as bleeding and coagulation (ClinicalTrials.gov identifier: NCT00796055)

This cytotoxic agent was also conjugated with the antagonistic anti-EphB2 monoclonal antibody 2H9 (Genentech), which antagonizes EPHB2–ephrin B1 interactions and causes internalization of non-phosphorylated EPHB2. Even if the antibody alone didn't affect tumor progression, the conjugated compound showed antitumor activity in human colon cancer xenografted mice, with less efficiency than 1C1-mcMMAF. However, clinical development has not yet been reported[178]. Another anti-EphA2 antibody, selected between antibodies that

internalize through micropinocytosis, was endowed with the cytotoxic drug saporin and was able to specifically destroy EphA2-positive prostate cancer cells[182].

EphrinA1- conjugated agents were also investigated to specifically kill EphA2- expressing cancer cell. EphrinA1-PE38QQR conjugate, for example, induced caspase-dependent apoptosis of prostate cancer cell lines through the cytotoxic activity of the Pseudomonas endotoxinA derivative[155].

Also PEG-coated nanoshells were conjugated to EphrinA1 to target PC-3 cells overexpressing EphA2. Upon treatment with a NIR laser; targeted cells were thermal destructed, but not EphA2-deficient human dermal fibroblast (HDF) cells.

The EphA2-specific YSA agonistic peptide was used to deliver paclitaxel in mouse xenografts, doxycyclin-containing liposomes in a rat choroidal neovascularization model and siRNAs and nanoparticles to cancer cells in culture. Core/shell hydrogel nanoparticles (nanogels) encapsulating anti-EGFR siRNAs were conjugated with YSA to target EGFR expression via EphA2 receptor in Hey cells[179].

- **Small interfering RNAs**

A highly sensitive strategy to downregulate Eph/ephrin expression is the use of antisense oligonucleotides (siRNAs), even if their in vivo delivery could be inefficient. Duxbury and colleagues treated a pancreatic cancer xenograft model with EphA2 siRNAs, which inhibited tumor growth and metastasis suppressing EphA2 expression, cellular invasiveness, anoikis resistance and FAK phosphorylation in vitro. Moreover, EphA2 siRNA has also been tested in combination with siRNA targeting FAK or Src tyrosine kinases[181]. For a more efficient delivery, Landen et al. encapsulated EphA2 siRNAs into the neutral liposome 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC). This compound decreased tumor cell proliferation and tumor growth in an orthotopic mouse model of ovarian cancer particularly when combined with delivery of siRNA silencing focal adhesion kinase (FAK) or with paclitaxel chemotherapy.

- **Immunotherapy**

Immunotherapy versus Eph receptor has been showed to be an effective anti-tumor strategy: their almost exclusive presence on tumor cells makes them suitable targets for anticancer vaccines.

EphA2 peptides have also been loaded to dendritic cells in a murine colon cancer model. This particular kind of vaccine suppressed MC38 tumor-derived cells overexpressing EphA2 and "instructed" splenocytes to recognize and kill MC38 cells, developing and anti-tumor immunity[183].

Chen and colleagues used the peptide EphA2883–891 as vaccine therapy for human malignant gliomas to induce an antigen-specific cytotoxic T-lymphocyte response[181].

In addition, bispecific antibodies (BiTe constructs) were synthetized with the fusion of the scFv derived from the anti-EphA2 to the scFv of an anti-CD3 antibody in order to target EphA2 overexpression on cancer cells and direct T cell cytotoxicity[182]. In human colon carcinoma-derived SW480 cells, the EphA2/CD3 bispecific antibody demonstrated potent cytotoxicity in the presence of unstimulated CD3+ cells in an in vitro and in a human xenograft nude mice model[179].

Therapeutic (company)	Target or patient population	Targeting strategy or observation	Trial design and stage	ClinicalTrials.gov identifier, refs
Cancer treatment: mAbs targeting various EPH⁺ tumours				
MEDI-547, human α-EPHA2 antibody–drug conjugate 1C1–mcMMAF[140,142,303] (MedImmune)	Patients with relapsed or refractory solid EPHA2⁺ tumours	EPHA2 activation and degradation; delivery of a cytotoxic drug	Phase I dose escalation study: terminated owing to dose-limiting toxicities	NCT00796055, REF. 143
KB004, humaneered α-EPHA3 mAb[150,151] (KaloBios)	Patients with haematological malignancies (AML, ALL, CML, MDS, MPN)	EPH activation on leukaemic blasts and stem cells, tumour vasculature and tumour stroma; direct apoptosis and ADCC-mediated tumour cell killing	Phase I dose escalation study: recruiting	NCT01211691, REF. 114
Cancer treatment: soluble EPH fusion proteins targeting solid tumours				
Soluble EPHB4–HSA[153,154] (University of Southern California, Norris Comprehensive Cancer Center)	Patients with advanced (metastatic or recurrent) solid tumours	Competitive inhibition of EPHB4 signalling blocking tumour cell growth and tumour angiogenesis	Phase I dose escalation safety study: recruiting	NCT01642342
Cancer treatment: small molecule EPH kinase inhibitors				
XL647 (Kadmon Corporation)	Histologically confirmed metastatic or unresectable tumours	Inhibition of multiple receptor tyrosine kinases: EGFR, VEGFR2, ERBB2 and EPHB4	Phase I dose escalation safety study: completed, no results published	NCT00336765
XL647 (Kadmon Corporation)	Previously untreated patients with NSCLC	Inhibition of multiple receptor tyrosine kinases: EGFR, VEGFR2, ERBB2 and EPHB4	Phase II interventional study: completed, no results published	NCT00364780
XL647 (Kadmon Corporation)	XL647 versus erlotinib, stage IIIB–IV NSCLC with progression after first- or second-line chemotherapy	Inhibition of multiple receptor tyrosine kinases: EGFR, VEGFR2, ERBB2 and EPHB4	Phase III treatment study: recruiting	NCT01487174
JI-101 (Jubilant Innovation)	Advanced solid tumours	Inhibition of VEGFR2, PDGFR and EPHB4	Phase I treatment study: completed, no results published	NCT00842335
Dasatinib, carboplatin, paclitaxel (MD Anderson Cancer Center; NCI)	Advanced stage and recurrent endometrial cancer	Effect of combined inhibition of proliferation (carboplatin), cell division (paclitaxel) and oncogenic kinases (dasatinib): BCR–ABL, SRC, KIT and EPHA2	Exploratory treatment study: recruiting	NCT01440998
Dasatinib (NCI)	Patients with unresectable or metastatic squamous carcinoma or stage 0–I CLL	Inhibition of BCR–ABL, SRC, KIT and EPHA2 kinases	Phase II treatment study: active, not recruiting	NCT00563290
Dasatinib, radiotherapy and temozolamide (MD Anderson Cancer Center; Bristol-Myers Squibb)	Patients with newly diagnosed glioblastoma	Exploratory objective: tumour expression of dasatinib targets: SRC, EPHA2, KIT and PDGFR	Phase I/II treatment study: active, not recruiting	NCT00895960
Dasatinib and type I-polarized autologous DC vaccines targeting tumour blood vessel antigens[304] (University of Pittsburgh Cancer Institute; NCI)	Patients with unresectable stage IIIB/C or stage IV metastatic melanoma	Exploratory objective: level of EPHA2 expression in tumour biopsies	Phase II safety and efficacy study: active, not yet recruiting	NCT01876212
Cancer treatment: dendritic-cell based vaccines				
$EPHA2_{883-891}$ as part of a type-1-polarized DC vaccine loaded with tumour-related peptide antigens (University of Pittsburgh Cancer Institute; NCI)	Patients with stage III/IV melanoma	Natural killer, CD4⁺ and CD8⁺ T cell response to either α-type-1 DC-based or cDC (non-polarized DC)-based intra-lymphatic vaccines	Phase I treatment study: completed, no results published	NCT00390338
Cancer treatment: siRNA				
siRNA–EPHA2–DOPC (MD Anderson Cancer Center; Ovarian Cancer Research Fund)	Patients with advanced solid tumours	Targeting of *EPHA2* gene expression using liposomal siRNA delivery	Open label Phase I treatment study to evaluate maximum tolerated dose of 2× weekly 0.45 mg/m² i.v. injection of siRNA–EPHA2–DOPC: not yet recruiting	NCT01591356
Observational: effects of ephrin B1 expression and mutation				
Effect of ephrin B1 mutation on craniofacial (CFNS) development[16,17] (National Human Genome Research Institute)	Patients with CFNS	Determine whether all patients with CFNS harbour mutations in the ephrin B1 (*EFNB1*) gene in Xq12	Observational study: completed, no results published	NCT00339846
Ephrin B1 expression in heart disease (ephrin B1 regulation in human right appendage)[305] (University Hospital, Toulouse)	Dilated cardiomyopathy	Determine whether ephrin B1 expression is regulated in human heart and correlates with heart rate variability	Observational study: completed, no results published	NCT01080781

ADCC, antibody-dependent cell-mediated cytotoxicity; ALL, acute lymphocytic leukaemia; AML, acute myeloid leukaemia; BCR, breakpoint cluster region protein; CFNS, craniofrontonasal syndrome; CLL, chronic lymphocytic leukaemia; CML, chronic myeloid leukaemia; DC, dendritic cell; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; EGFR, epidermal growth factor receptor; EPH, EPH receptor; i.v., intravenous; mAb, monoclonal antibody; mcMMAF, maleimidocaproyl-auristatin phenylalanine; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasms; NCI, US National Cancer Institute; NSCLC, non-small cell lung cancer; PDGFR, platelet-derived growth factor receptor; siRNA, small interfering RNA; VEGFR, vascular endothelial growth factor receptor.

Table 1: Clinical trials that involve Eph-ephrins as therapeutic targets. Boyd AW *Therapeutic targeting of EPH receptors and their ligands*. Nat Rev Drug Discov. 2014 Jan;13(1):39-62.

1.4 Experimental models of colorectal cancer: the AOM/DSS mouse model

To reproduce the etiology and pathobiology of human CRC different *in vivo* and *in vitro* models have been developed[184].

Despite the *in vitro* models allow the analysis of particular molecular pathways or pharmacological effects of specific agents they exhibit some important limitations, including the restriction of the study to the tumor phase from which the cell line has been isolated and the lack of information about the tissue context and the relative tumor microenvironment.

The *in vivo* models surpassed these limitations, allowing the study of all the distinct phases of cancer development, considering also the fundamental role of the microenvironment. Even if they cannot replace human clinical trials, mouse models are useful to study CRC development and pathogenesis and to test new therapeutic strategies, with the aim to set well targeted diagnostic and therapeutic trials.

A number of mouse models have been developed, each with its peculiarity and scientific interest, distinguished by three different methods of tumor induction: genetic induction, xenograft induction, chemical induction.

The genetic induction generates mice with a heterozygous mutation in APC (Min/ΔAPC-mouse) which corresponds to the alteration found in FAP patients. These models are useful to study APC protein and its domains, together with environmental and nutritional risk factors. The most important difference with human FAP neoplasia is the localization of tumor lesions in the small intestine, whereas human polyps affect mostly colon and rectum.

Xenograft induction is driven by cancer cells' ability to give tumor following an endovenous or subcutaneous injection in immunodeficient mice (nude, SCID or bg/nu/xid). This is the only model able to reproduce tumor growth including metastasis. Even if its easy induction procedure made this model highly eligible for the in vivo study of therapeutic cytostatic compounds, it ignores the complex process of carcinogenesis and the tumor-microenvironment interaction. To bypass this limitation and to create a more reliable CRC model orthotopic xenografts in caecum and rectum have been developed.

Mouse model generated by chemical induction by the mean of a carcinogenic compound are able to recapitulate all the single phases of human

tumor initiation and progression and consequently result more suitable to individuate risk factors and chemopreventive pharmacological agents. These models are highly reproducible and can be tested on animals with different genetic background.

There are four different families of chemical carcinogenetic compounds: heterocyclic amines, aromatic amines, alkylnitrosammines, dimethylhydrazine and azoxymetan.

The most used heterocyclic amines in CRC models are the 2-Amino-3,4-dimethylimidazo [4,5-f] quinoline (IQ) and the 2-Amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP), both undergoing hepatic activation before combining with the DNA molecule. IQ and PhIP administration for 52 weeks results in a minor tumor incidence (5%-28%) respect to the 104 weeks administration (43%-55%).

Aromatic amines like 3,2'-dimethyl-4-aminobiphenyl (DMBA) retain a less potent tumorigenic activity respect to the other carcinogenetic compounds: they deserve multiple injections and give rise to lesions in tissues different from the target.

Alkylnitrosammines including methylnitrosurea (MNU) are alkylating carcinogenetic compounds that don't deserve biochemical activation, representing ideal inductors of a localized carcinogenesis. However their administration requires laborious intra-rectal injections.

Azoxymetan (AOM) is a derivative of dimethylhydrazine that is activated in multiple steps: following a N-oxydation, the hydroxylation forms the reactive methylazoxymethanol (MAM) that alkylates hepatic and colonic macromolecules adding methyl groups in O6 or N7 of DNA guanine. The most common genetic alterations arising in AOM induced tumors are the ones involving KRAS and β-Catenin. Rarely do we find microsatellite instability, APC or p53 mutations and almost never metastatic activity. The major strenghtnesses of AOM induction are its reproducibility, effectiveness, simple administration, stability in suspension and low cost.

One of the most advantageous chemically-induced CRC model was published in 2003 by Tanaka et al.[185]: the AOM/DSS mouse model shows high effectiveness, reproducibility and short time of treatment. The induction-promotion protocol includes the tumor induction via AOM (10mg/kg weight)

administration with a single intraperitoneal injection and a single cycle of the proinflammatory agent Dextran Sodium Solphate (DSS) for 7 days in drinking water. This protocol allows the rapid emergence of multiple tumor lesions within 12 weeks.

DSS is the proinflammatory promoting agent and expresses its toxicity on colon epithelium, developing a transient colitis. The administration of this agent reduces the latency period to only 10 weeks.

AOM/DSS combination allows a reliable reproduction of colorectal carcinogenesis: the initial acute inflammation is followed by a latency period and subsequently by the emergence of tumor lesions mostly in the distal colon. Tumor lesions' development follows the single steps of human carcinogenesis: normal crypts evolve in aberrant crypt foci (ACF) that proliferate by fission forming microadenoma and then macroadenoma, adenomatous polyps and adenocarcinoma. However this cancer model has no tendency to invade and metastatize.

The molecular features of the AOM/DSS model reproduce the alterations of the human CRC. They involve the perturbation of the pathways of APC/β-catenin, which includes c-myc, ciclyn D1 and Cdk4, K-Ras, COX-2 and iNOS.

Recent studies investigated also the epigenetic match between this model and human CRC, revealing a similar general hypomethylation pattern, even if the frequency of hypermethylated genes is reduced in mouse cancers.

2. AIM OF THE PROJECT

Tumor heterogeneity and the presence of stem-like cells have been identified as key features for resistance to anticancer treatments including targeted therapy.

Elevated EphA2 expression is frequently found in colorectal cancer and plays a role in tumor progression, metastasis and angiogenesis. Indeed EphA2 is involved in extensive crosstalk with the major tumor signaling networks that control cell survival, migration and differentiation, including EGFR, FAK and VEGF pathways. In particular, the overlapping between EphA2 and EGFR pathways at the level of RAS/MAPK and PI3K/AKT signaling is of crucial interest to investigate mechanisms of resistance to the targeted therapeutic agents directed against TK receptors, like cetuximab.

The aim of this study was to isolate and characterize homogeneous $EphA2_{high}$ and $EphB2_{high}$ cell subpopulations from colorectal tumors to investigate their role in carcinogenesis and tumor progression, with a focus on the molecular crosstalk and microRNAs modulation of EphA2 and EGFR pathways to elucidate new molecular processes contributing to CRC pathogenesis and drug resistance and explore the role of EphA2/EGFR pathway mediators as prognostic factors in colorectal cancer.

With this aim we articulated our experimental design in four phases:

1. set up of the AOM/DSS murine model of sporadic colon carcinogenesis, selected for its high reproducibility and ability to recapitulate, within a predictable time line, colorectal lesions distinctive of human CRC development;
2. purification from murine CRC and normal colon mucosa of representative cell subpopulations with stem/differentiation-like features based on the differential expression of EphB2 and EphA2 receptors;
3. characterization in the $EphA2_{high}$ subpopulation of the expression levels of relevant EphA2/EGFR targets to investigate the crosstalk existing between EphA2 and EGFR pathway;
4. assessment in public datasets of genomic data derived from multiple cohorts of CRC patients of the prognostic role and predictive value for responsiveness to cetuximab for the EphA2/EGFR obtained gene signature.

3. MATERIALS AND METHODS

3.1 Achievement and characterization of the AOM/DSS murine model

The AOM/DSS model was induced in 7-week-old Balb/c male mice following the protocol proposed for the first time by Tanaka. It consists of a single intraperitoneal injection of AOM (10 mg/kg of body weight) followed after a week by and a single cycle of 2% DSS for seven days in drinking water. All animal procedures were performed in accordance with institutional guidelines for laboratory animal care and in adherence with ethical standards[186]. The study was approved by the Italian Ministry of Health according to the decree n. 336/2013-B. Animals were housed in the Plaisant animal facility in Castel Romano (RM) and their health status was checked daily monitoring lethargy, ruffled fur, dyspnea, dehydration, weight loss, presence of porphyrin around nose and eyes, paralysis of limbs, diarrhea, dermatitis, anemia, bleeding, cachexia, self-harm, prolonged hypo- or hyper-thermia. The 60 animals in study were organized in two groups: the 40 mice belonging to the group 1 were treated with the AOM/DSS protocol; the 20 mice belonging to the group 2 (control group) were treated with an intraperitoneal injection of a saline solution and drinking water.

To obtain samples at the distinct phases of tumor development, mice were euthanized by CO_2 exposure at different time points, following the experimental scheme below:

Time point	Group 1	Group 2
Week 5	6	3
Week 6	6	3
Week 8	7	3
Week 10	7	3
Week 12	7	4
Week 20	7	4

Animals were weighted, intracardiac blood was collected and colons were recovered in necroscopy. Colons were flushed in PBS, measured, cut longitudinally, opened, cleaned from fecal residues and divided in 4 sections. Sections were collected in plastic devices for formalin fixation or in cryovials

for -80° storage. Colon tissues obtained from animals at the 20th week after the start of the treatment were collected and treated for the cytofluorimetric analyses.

Histological analyses

Colons were fixed in 10% formalin for at least 48 hours and then dehydrated in solutions of progressive ethanol concentration (70%, 95%, 100%). Samples were included in paraffin blocks and cut with the microtome in 2 µm thick sections. Sections were stained following the Hematoxylin-Eosin protocol:

- thermal deparaffinization in dry oven at 60°C for 30';
- chemical deparaffinization in Xilene for 20';
- hydration in solutions of regressive ethanol concentration (100%, 95%, 70%) and deionized water;
- Hematoxylin staining for 5' and washing in running water;
- removal of the Hematoxylin excess with Acid Alcohol (70% Ethanol + HCl);
- Eosin staining for 2'-3' and washing in running water;
- dehydration in solutions of progressive ethanol concentration (70%, 95%, 100%) and Xylene;
- air drying of the slides and mounting with Eukitt® (O Kindler GmbH & Co.).

Tissue sections were observed under a microscope to detect microscopic lesions that were classified according to the histopathological criteria described by Boivin et al. [187]:

a) Normal mucosa: structure well-organized in separate crypts formed by epithelial cells intermingled with globular mucus cells. In transversal section the crypts appear as regular circles formed by the cells that line the crypt lumen.

b) ACF: foci of epithelial cells with high nuclear / cytoplasmic ratio, hyperchromatic nuclei, basophilic cytoplasm, appreciable mitotic spindles. These foci consist of 2 to 10 crypts with altered lumen diameter, thick epithelium, greater width of the adjacent crypts, progressive cellular crowding and decreased mucus.

c) Microadenoma: preneoplastic lesion smaller than 1 mm composed by more than 10 crypts at different degrees of dysplasia.

d) Adenoma: consists of several crypts with different degrees of dysplasia. Crypts with moderate degree of dysplasia are constituted by cells with elongated nuclei, bundled and pseudostratified, with well-preserved cell polarity and normal or slightly reduced number of globular cells. Crypts with intermediate degree of dysplasia are constituted by cells with elongated nuclei, that result more crowded and pseudostratified, but with still right polarity. It is instead reduced the number of mucus cells. Crypts with severe dysplasia present cells with enlarged circular or ovoid nuclei, with prominent nucleoli. Cell polarity is partially lost and the number of mucus cells is dramatically reduced.

e) Adenocarcinoma: consists of crypts with a high degree of dysplasia that have completely lost their original architecture. Nuclei are enlarged, pseudostratified, hyperchromatic, dotted and oval, with moderate or marked anisokaryosis. It also can be noted a severe depletion of mucus cells and accumulation of necrotic debris.

3.2 Isolation of EphA2 and EphB2 cell populations in AOM/DSS murine model

Flow cytometry is a cytometric technique that allows the analysis of the physical and chemical characteristics of particles in a fluid as it passes through at least one laser. Cell components are fluorescently labelled and then excited by the laser to emit light at varying wavelengths. The flow cytometer offers high-throughput automated quantification and separates and isolates particles having specified properties. It consists of a flow cell of liquid stream (sheath fluid), which aligns the cells so that they pass one by one through the light beam; a measuring system, which converts analog measurements of forward-scattered light (FSC) and side-scattered light (SSC) as well as dye-specific fluorescence signals into digital signals that can be processed by a binary computer; an amplification system and a computer supported by a software for the acquisition and analysis of the signals. The data generated by flow-cytometers can be plotted in two-dimensional dot graphs in which regions with different fluorescence intensity can be sequentially separated by creating a series of "gates" and analyzed using a dedicated software.

Fluorescence-activated cell sorting (FACS) is a particular application of flow cytometry. It provides a method by which cells stained using fluorophore-

conjugated antibodies can be separated from one another depending on which fluorophore they have been stained with. The cell suspension passes through a narrow, rapidly flowing stream of liquid that grossly separates cells according to their diameter. A vibrating mechanism breaks the stream into individual droplets each containing one single cell. Just before the stream breaks into droplets, a laser measures the fluorescence of each cell of interest and a charge is consequently assigned to each drop. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge.

According to our experimental design, FACS sorting of CD45-EpCAM+EphA2$_{high/low}$ and CD45-EpCAM+EphB2$_{high/low}$ cell subpopulations was performed on colonic normal mucosa and tumors of mice euthanized at the end of the 20th week after the start of the treatment (AOM administration). Colons were removed from each mouse, cut longitudinally and flushed with cold PBS. Normal mucosa and adenocarcinomas were disaggregated and incubated in 8 mM EDTA in HBSS for 20 min on ice. Samples were then vigorously shaken to obtain a supernatant enriched for crypts. Isolated crypts were then enzymatically disaggregated (0.4 mg/mL Dispase, 0.8 U/µL DNAse I in HBSS) for 30 min at 37ºC with orbital shaking, in order to obtain single cell suspensions. After disaggregation, 5% FBS was added and cells were sequentially passed through 100, 70 and 40 µm mesh filters. Cells were then centrifuged (1200 rpm for 5 min at 4ºC) and resuspended in staining buffer (SB; 5% FBS in HBSS). Up to 10^7 cells were used for the staining with the following mix of antibodies: rat anti-EpCAM-PE (eBioscience, Mab G8.8), rat anti-mouse CD45-FITC (eBioscience, Mab 30-F11), rat anti-mouse EphA2-APC (R&D System, Mab 233720), rat anti-mouse EphB2-APC (R&D Systems, Mab 512012) or appropriate isotype controls. Fixable viability dye eFluor 780 (eBioscience, San Diego, CA) was added to identify dead cells and debris.

Stained cells were sorted in a FACS Aria 2.0 (Becton Dickinson, Franklin Lakes, NJ) with the support of the BD FacsDIVA software version 6.1.3 (BD Biosciences, Erembodegem, Belgium).

The following selection gates were applied to live cells: first, lymphoid cells were discarded by removing the CD45+ cell population; then, epithelial cells were included by selecting for EpCAM+ staining.

Then, different intestinal epithelial cells were selected according to graded EphA2 and EphB2 surface levels. Normal and tumor CD45-EpCAM+ $EphA2_{high/low}$ and CD45-EpCAM+ $EphA2_{high/low}$ cell subpopulations were sorted and collected in DMEM medium. The percentages of $EphA2_{high/low}$ or EphB2high/low positive cells were defined on the base of the Fluorescence Minus One (FMO) control stain strategy necessary to accurately identify expressing cells in the fully stained sample[188]. Briefly, we prepared a sample with all reagents except for those of interest (EphA2 and EphB2). Sorted cells were centrifuged and cell pellets were resuspended in Trizol® Reagent (Thermo Fisher Scientific, Waltham, MA) and stored at -80°C for RNA extraction.

Authentication of cell subpopulations was performed by qPCR analysis in order to test the gene expression levels of EphA2 and EphB2 and stemness/differentiation genes (Lgr5, Ascl2, and Krt20).

3.3 Total RNA extraction and molecular analysis in murine sorted cells

RNA was isolated using Trizol® Reagent according to the manufacturer's instructions:

- cells suspended in Trizol® were thawed, vortexed and incubated for 5' at room temperature (RT);
- 200 µl of Chlorophorm were added, tubes were vortexed for 15''and incubated for 3' at RT;
- tubes were centrifuged for 15' at 12.000 RCF at 4°C;
- aqueous phase was collected and transferred in a new tube;
- 500 µl of Isopropanol were added and tubes were shaken by hand;
- tubes were centrifuged for 10' at 12.000 RCF at 4°C;
- supernatant was removed and pellets were washed with 1 ml of 75% Ethanol;
- tubes were centrifuged for 5' at 7.500 RCF at 4°C;
- supernatant was removed and tubes were centrifuged for 2' at 7500 RCF at 4°C;
- last drops of Ethanol were removed and the pellet was air dried;
- pellet was resuspended in 20-40 µl of RNase free water;
- RNA was stored at -80°C.

RNA concentration and purity were evaluated with Nanodrop (Thermo Scientific) spectrophotometer. A260/A280 values between 1,8 and 2,1 defined pure samples.

Gene and miRNA expression analyses were performed starting from the retrotranscription of the extracted RNA in cDNA and the selective amplification of the genes or miRNAs of interest with Real Time PCR.

RNA retrotranscription was performed with TaqMan® High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) for gene expression and TaqMan® microRNA Reverse Transcription Kit (Applied Biosystems) for miRNA expression, following manufacturer's instruction.

RT master mix was prepared on ice with the following reagents.

TaqMan® High Capacity cDNA Reverse Transcription Kit

Component	**Volume**
10X RT Buffer	2.0 µL
25X dNTP Mix (100 mM)	0.8 µL
10X RT Random Primers	2.0 µL
MultiScribe™ Reverse Transcriptase	1.0 µL
RNase Inhibitor	1.0 µL
Nuclease-free H2O	3.2 µL
Total per reaction	10.0 µL

TaqMan® microRNA Reverse Transcription Kit

Component	**Volume**
10X RT Buffer	1.5 µL
dNTP Mix (100 mM)	0.15 µL
5X RT primer	3.0 µL
MultiScribe™ Reverse Transcriptase	1.0 µL
RNase Inhibitor	0.19 µL
Nuclease-free H2O	4.16 µL
Total per reaction	10.0 µL

The reverse transcription reactions were prepared:

- 10 µL of RT master mix were pipetted into the tube;
- 10 µL (for gene expression) or 5 µL (for miRNA expression) of RNA sample were added into each well and the solution was mixed;

- tubes were centrifuged to spin down the contents and to eliminate any air bubbles;
- tubes were place on ice until the thermal cycler was ready;
- thermal cycler conditions were programmed using the conditions below:

TaqMan® High Capacity cDNA Reverse Transcription Kit

Settings	Step 1	Step 2	Step 3	Step 4
Temp.	25°C	37°C	85°C	4°C
Time	10 minutes	120 minutes	5 minutes	∞

TaqMan® microRNA Reverse Transcription Kit

Settings	Step 1	Step 2	Step 3	Step 4
Temp.	16°C	42°C	85°C	4°C
Time	30 minutes	30 minutes	5 minutes	∞

Then Real Time PCR was performed with TaqMan® gene expression Assay (Applied Biosystems) for gene analysis and TaqMan® small RNA Assay (Applied Biosystems) for miRNAs analysis and run in ABI Prism 7900HT Fast (Applied Biosystems), following manufacturer's instructions.

The mix was prepared on ice with the following reagents.

TaqMan® gene expression Assay

Component	Volume
20X TaqMan® Gene Expression Assay	1.0 µL
2X TaqMan® Gene Expression Master Mix	10 µL
cDNA template (1 to 100 ng)	4.0 µL
RNase-free water	5.0 µL
Total per reaction	20.0 µL

TaqMan® small RNA Assay

Component	Volume
20X TaqMan® small RNA Assay	1.0 µL
2X TaqMan® Universal PCR Master Mix	10 µL
cDNA template	1.33 µL
RNase-free water	7.67 µL
Total per reaction	20.0 µL

- 20 µL of PCR reaction mix were transferred into each well of a 384-well reaction plate;
- the plate was sealed with the appropriate cover, centrifuged and load into the instrument;
- PCR reaction was launched with SDS software.

Step	**Temperature**	**Time**
Hold	95 °C	10 min
Cycle (40 cycles)	95 °C	15 sec
	60 °C	1 min

Data were analyzed using SDS software 2.3 (Applied Biosystems). Relative expression was calculated according to the method of Fold Change ($2^{-\Delta\Delta Ct}$). The housekeeping genes *Hprt1* and *Hmbs* gave comparable normalized results, similarly for *U6snRNA* and *SnoRNA202* normalized data of microRNAs. Student-T test was used to analyze the Q-PCR results.

$2^{-\Delta\Delta Ct}$, or fold change, indicates how many times the expression of a given sample is greater or lower than the calibrator. The analysis was performed following the passages below.

ΔCt sample = Ct sample – Ct calibrator

ΔCt normal mucosa = Ct normal mucosa – Ct calibrator

ΔΔCt = ΔCt sample – ΔCt normal mucosa

Ct cutoff value was established at 35: genes or miRNAs associated with Ct<35 have been considered as “non-amplified”.

3.4 Immunohistochemistry of murine tissue samples

Part of the tumor masses and normal colon mucosae were analyzed with immunohistochemistry.

Immunohistochemistry (IHC) is a technique that identifies discrete tissue components by the interaction of target antigens with specific antibodies tagged with a visible label. IHC allows to visualize the distribution and localization of specific cellular components within cells and in the proper tissue context.

To prepare the samples to the staining assay, after they have been formalin-fixed and paraffin embedded they are cut in 4-5µm thick slices, that

are collected on poly-L-lysine treated glass slides which avoid section detachment during the treatments. To recover the epitope, sections need to be deparaffinized and treated either by heat (heat-induced epitope retrieval; HIER) or enzymatic degradation (proteolytic-induced epitope retrieval; PIER) to unmask the antigen crosslinked with methylene bridges generated by formaldehyde fixation. Chemically quenching of the endogenous forms of peroxidases is also required for the enzymatic detection of target antigens to prevent false positive and high background detection.

The sample labelling is a multistep process that requires optimization at every level to maximize the signal detection. In the context of antibody-mediated antigen detection, it is important to avoid antibody binding to sites on nonspecific proteins that might cause high background staining and mask the detection of the target antigen. To this purpose, samples are incubated with a buffer (commonly normal serum) that blocks the reactive sites to which the primary or secondary antibodies may otherwise bind. Primary antibodies are raised against an antigen of interest and are typically unconjugated (unlabelled), while secondary antibodies are raised against immunoglobulins of the primary antibody species. The secondary antibody is usually conjugated to a linker molecule, such as biotin, that then recruits reporter molecules, or the secondary antibody itself is directly bound to the reporter molecule. IHC target antigens are detected through either chromogenic or fluorescent means, with the type of readout depending on the experimental design. For fluorescent detection, the primary or secondary antibody is conjugated to a fluorophore that is detected by fluorescent microscopy. Chromogenic detection is based on the activities of enzymes, most often horseradish peroxidase (HRP) or alkaline phosphatase (AP), which form colored, insoluble precipitates upon the addition of substrate, such as DAB and NTP/BCIP, respectively.

In our case, immunohistochemistry was performed by the means of the Avidin-Biotin Complex (ABC) staining method taking advantage of the ABC Staining kit (SantaCruz, California, USA) following manufacturer's instructions:

- collection of 4-µm-thick FFPE tissue sections on poly-Lysine coated slides;
- thermal deparaffinization in dry oven at 60°C for 15';
- chemical deparaffinization in Xilene (3 incubation of 5' each);

- hydration in solutions of regressive ethanol concentration (100%, 95% , 80%) and deionized water;
- thermal-induced antigen retrieval with 10mM Sodium Citrate Buffer pH 6.0 for 1hr at 95-100°C.

In a humidified chamber:

- 2 washes with PBS Tween 20 0,05% for 2'each;
- endogenous peroxidase inhibition with 0,5% H2O2-PBS for 10';
- 2 washes with PBS Tween 20 for 5' each;
- blocking of aspecific binding sites with 1,5% blocking serum in PBS for 1 hr;
- overnight incubation with primary antibody at 4°C;
- 3 washes with PBS Tween 20 for 5' each;
- Incubation with biotinylated secondary antibody for 30'
- 3 washes with PBS Tween 20 for 5' each;
- Incubation with AB enzyme reagent;
- 3 washes with PBS Tween 20 for 5' each;
- chromogenic signal development with DAB exposure;
- 2 washes with dH2O for 5'.

On the bench:

- counterstaining with hematoxylin for 5-10'' and washing in running water;
- dehydratation in solutions of progressive ethanol concentration (95%, 100%) and Xylene;
- air drying of the slides and mounting with Organo/Limonene Mount (Sigma-Aldrich).

Goat anti-mouse Krt20 and Lgr5, rabbit anti-mouse EphA2 and EphB2 (Santa Cruz Biotechnology, Santa Cruz, CA, 1:50) were used. The immunostained slides were observed under a microscope, and the image data were analyzed using NIS FreeWare 2.10 software (Nikon, Japan).

3.5 Selection of CRC patient cohorts and genomic data from TCGA and GEO datasets

To assess the prognostic and predictive value of the EphA2/EphB2 molecular signature we made use of bioinformatics analysis on public microarray databases.

A microarray database is a repository that makes study data available to other applications for analysis and interpretation. Microarray databases as Gene Expression Omnibus (GEO) from NCBI or The Cancer Genome Atlas (TCGA) are peer reviewed, public repository that adheres to academic or industry standards and are designed to be used by many analysis applications and groups. Conversely, specialized branded repositories associated to an application suite, a topic, or an analysis method, whether it is commercial, non-profit, or academic require a subscription or license to gain full access and need to be reprocessed for standard applications or analysis.

The analysis of the genes and microRNAs of interest was carried out on a multi-study microarray database of CRC expression profiles (total n = 1171) based on the Affymetrix U133 Gene Chip microarray platform. According to Lee et al.[189], five different CRC cohorts were assembled in the database and microarray data and clinical annotations were obtained from the GEO public data repository.

Cohort 1 - patients with stage I–III CRC (n = 226). GEO accession number GSE14333[190].

Cohort 2 - patients with stage II–III CRC (n = 130). GEO accession number GSE37892[191].

Cohort 3 - patients with stage I–IV CRC (n = 566). GEO accession number GSE39582[192]. This cohort allowed us to calculate the Disease Free Survival (DFS), meant as the difference between the time of surgery and the time of the first occurrence of death or of cancer recurrence[190,191].

Cohort 4 - we considered only patients at stage I-III of the disease (n = 125) as done by Lee et al.[189]. GEO accession number GSE41258[193]. We considered the “death” event only if related to cancer disease (Cancer Specific Survival, CSS). All the other causes of deaths, i.e., for other or unknown causes, and alive patients were considered “censored” events.

Cohort 5 - patients with refractory metastatic CRC (n = 80) that received cetuximab monotherapy in a clinical trial. GEO accession number GSE5851[194]. In the study of this cohort, patient characteristics were available, and the progression-free survival (PFS) duration was defined as the time from study enrollment to disease progression or death[193]. Further, KRAS mutation status in cohort 5 was available (exon 2 genomic region)[194].

Gene expression data for a sixth cohort were downloaded from The Cancer Genome Atlas (TCGA; http://cancergenome.nih.gov)[195]- patients with stage I–IV CRC (n = 130). We excluded patients having Mucinous Adenocarcinoma. For this study the Overall Survival (OS) is available, i.e. the time from study enrolment to death.

3.6 Bioinformatic and statistical analysis

Analysis of gene expression data and other statistical analyses were performed in R ver. 3.1.3 (http://www.r-project.org). Raw data from GEO were downloaded by *GEOquery* and *Biobase* tools. Patients were dichotomized through *maxstat* R package, in order to obtain a significant difference between survival values. Prognostic significance was estimated by log-rank tests and plotted as Kaplan–Meier curves. Multivariate Cox proportional hazards regression analysis was used to evaluate the effect of EphA2, Efna1, EGFR, Ptpn12, Pi3k, Akt and Atf2 signatures on survival, independently of other clinical parameters. When coupled with other gene signatures (e.g., Efna1high/low), the threshold value between EphA2high and EphA2low groups of samples was set to the median expression value of EphA2, because of the extremely unbalanced sample sizes obtained with the maxstat R package. In cohort 5, differences in response of CRC to treatment of cetuximab were verified using the Fisher's-exact test. Differences of expression between class members were detected by Student T-test. P values less than 0.05 were considered statistically significant.

4. RESULTS

4.1 Histopathological analysis of the AOM/DSS model

We confirmed the high reproducibility of the murine AOM/DSS model observed in previous works. Microscopic observation and histopathologic analysis of the tissues detected the 100% of the lesions in the distal-rectal region of the colon with a nature and timing overlapping the expected sequence ACF – microadenome – adenoma –carcinoma (Tab.2, Fig.15):

- In the first five weeks we detected the typical preneoplastic lesions (ACF), about 7 per colon and microadenoma, about 3 per colon;
- In the eighth week we observed about 4 adenoma per colon and the first adenocarcinoma, about 2 per colon;
- In the twelfth week the number of adenoma per colon was reduced while increased adenocarcinoma size and numerousness (about 3 and 4 respectively);
- In the twentieth week we observed exclusively large adenocarcinoma (2-3mm), about 6 per colon.

Week	ACF	Microadenoma	Adenoma	Adenocarcinoma
V	6.5±1.7	2.5±1.1		
VIII			4.1±1.6	2±0.5 (1mm)
XII			3±1.1	3.5±2.1 (1-2mm)
XX				5.6±2.4 (2-3mm)

Table 2: Type and number of lesions detected at each time point. Arithmetical averages and standard deviation of the total number of lesions observed in about 4-5 animals analyzed at each time point.

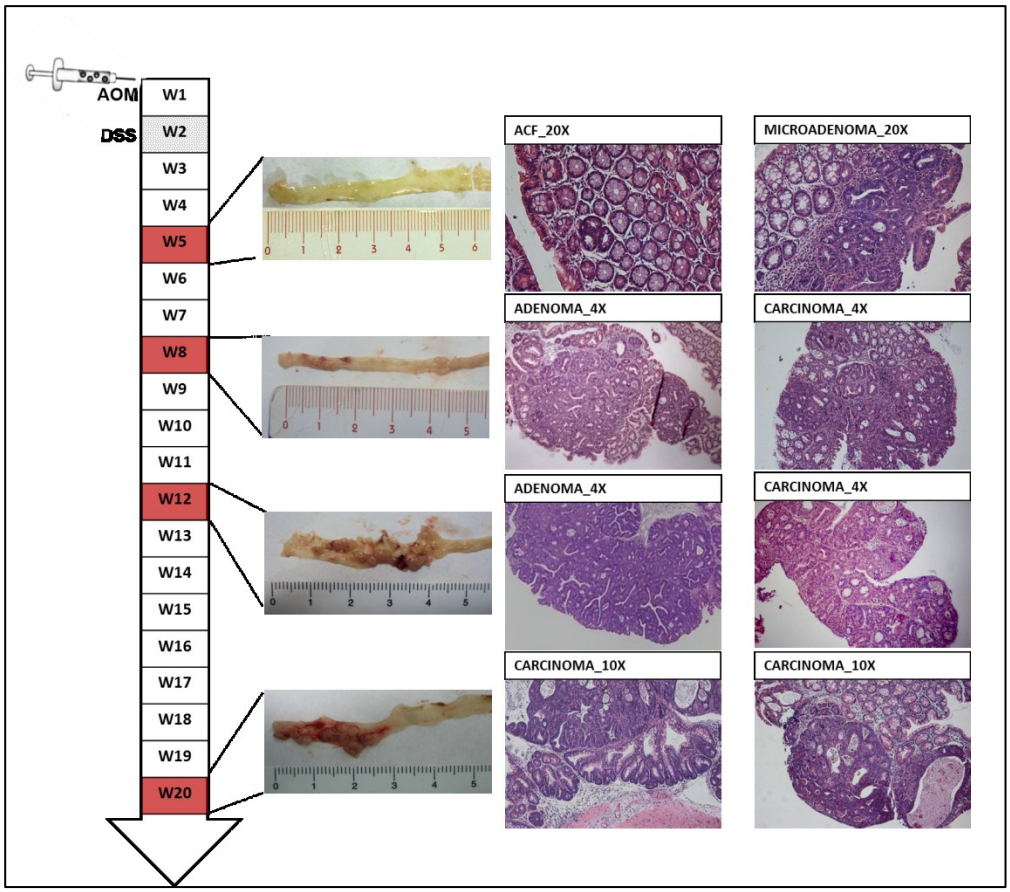

Figure 15: Experimental scheme and histopathological analysis of colon sections of the AOM/DSS model. (H-E staining; magnification: 5X, 10X, 20X).

4.2 Molecular characterization of murine CRC EphA2 and EphB2 cell subpopulations

Our first interest was to characterize two homogeneous cell subpopulations present in the heterogeneous landscape of normal and tumoral mouse intestinal epithelium. To this aim we firstly localized and compared the expression of EphA2 and EphB2 receptors on tissue sections by the mean of immunohistochemistry. Then a cytofluorimetric analysis precisely quantified the presence of $EphA2_{high}$ and $EphB2_{high}$ subpopulations in normal and cancer tissues. Finally with a gene expression analysis we characterized at the molecular level the cell subpopulations of interest isolated with FACS.

In the IHC assay, in the normal colon mucosa EphB2 presented an expression pattern characterized by a decreasing gradient from the crypt base to the top (Fig.16)[24]. Crypt base columnar cells (ISCs) showed the highest expression of membrane EphB2 (Fig.16 right, black arrowhead), whereas the transient amplifying cells progressively decreased EphB2 protein levels as they migrated toward the top of crypts. Apical differentiated cells in the villi were negative for EphB2 expression (Fig.16 right, white arrowhead). Conversely, maximum EphA2 expression was observed in the most differentiated crypt apical cells of the normal colon and a weak staining was shown at the crypt basal level (Fig.16 left, black and white arrowhead, respectively). Tumor cells displayed a highly heterogeneous and not gradient disposed staining for both anti-EphA2 and anti-EphB2 antibodies (Fig.16).

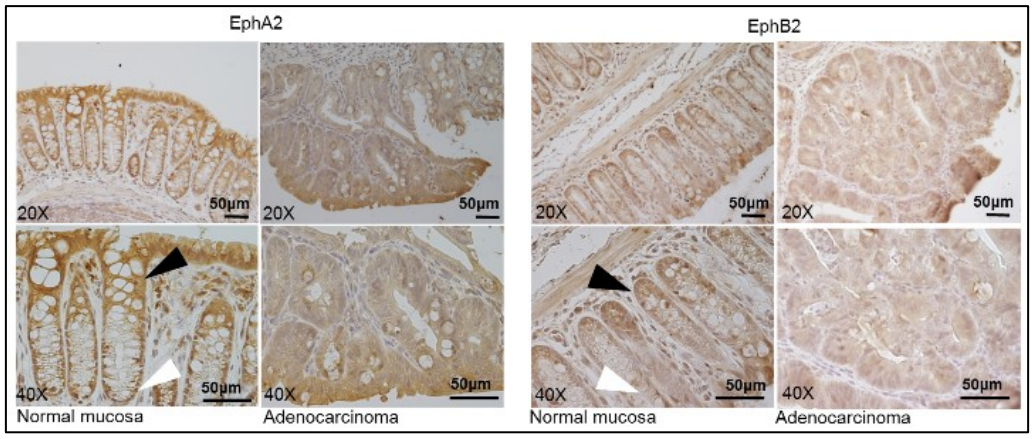

Figure 16: IHC analysis. Normal colorectal tissue of control untreated mice demonstrated maximum EphA2 and EphB2 expression in crypt apical columnar cells (white arrowhead) and basal crypt compartment (black arrowhead), respectively; adenocarcinoma shows a diffuse staining for both EphA2 and EphB2 (20X and 40X magnification).

The cytofluorimetric analysis showed a change in the cellular density of both EphA2 and EphB2 cell populations between the adenocarcinoma and the normal colon mucosa (Fig.17A). Specifically, an increase of $EphA2_{high}$ cell fraction was measured in adenocarcinoma (17.18%) comparing to normal mucosa (0.71%). Differently, $EphB2_{high}$ cells resulted poorly represented both in the adenocarcinoma (4.76%) and in normal colon mucosa (0.27%).

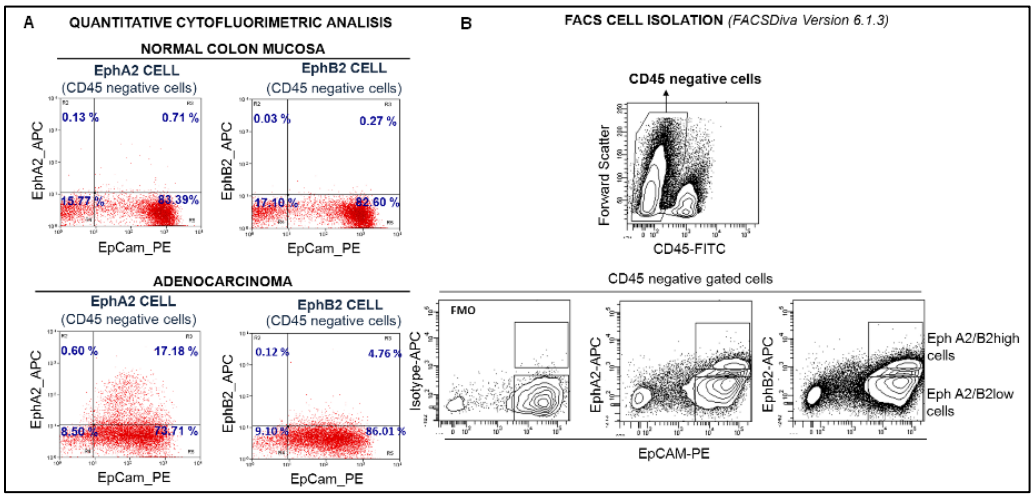

Figure 17: (A) Flow cytometry. Analysis of crypt cells stained for EphA2 revealed an increase of EphA2high cell subpopulation in adenocarcinoma with respect to normal mucosa. EphB2high cells were poorly represented in normal mucosa and colon adenocarcinoma. **(B) Cell sorting strategy**. $EphA2_{high}$ and $EphA2_{low}$ cells as well as $EphB2_{high}$ and $EphB2_{low}$ subpopulations were sorted after gating for CD45- and EpCAM+ staining to ensure epithelial identity. Fluorescence Minus One (FMO) control stain strategy was used to accurately identify EphA2 and EphB2 expressing cells in the fully stained sample.

To confirm the identity of the FACS-sorted subpopulations, we performed a gene expression analysis with Real Time qPCR. The analysis conducted on $EphB2_{high}$ cell subpopulations obtained from adenocarcinoma as well as normal mucosa revealed an upregulation of the stemness-specific markers *Lgr5*[23,24] and *Ascl2*[24,31] ($p<0.001$ in normal mucosa; $p<0.01$ in adenocarcinoma), with a down-modulation of *Krt20*, a common differentiation marker[24] ($p<0.001$ in normal mucosa; p=ns (not significant) in adenocarcinoma) (Fig.18 right). Importantly, a different expression pattern resulted associated to the $EphA2_{high}$ cell population. In normal mucosa we observed a coherent down-modulation of stemness genes, *Lgr5* ($p<0.001$) and *Ascl2* ($p<0.001$) together with an up-modulation of *Krt20* expression level ($p<0.0001$), suggesting an enrichment of the $EphA2_{high}$ cell population with differentiated cells. In contrast in adenocarcinoma the $EphA2_{high}$ cells displayed a decreased expression levels both of *Krt20* ($p<0.0001$) and *Lgr5* ($p<0.01$) along with an increased expression of *Ascl2* ($p<0.0001$) (Fig.18 left).

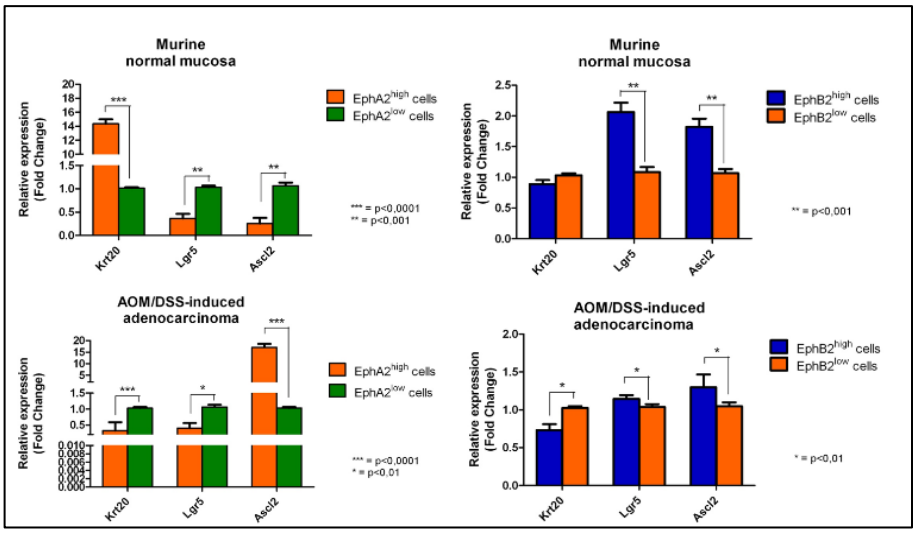

Figure 18: Q-PCR analysis. Differentiation (Krt20) and stem cell markers (Lgr5, Ascl2) were measured in $EphA2_{high/low}$ and $EphB2_{high/low}$ cell subpopulations purified from murine normal colon and colorectal adenocarcinoma. Data are represented as mean +/- SD. Statistically significant differences were calculated

IHC analysis further confirmed this expression pattern, showing an overlapping staining between Krt20 and EphA2 at the apical level of crypts in the normal mucosa samples and between Lgr5 and EphB2 cells at the basal level (Figg.16 and 19).

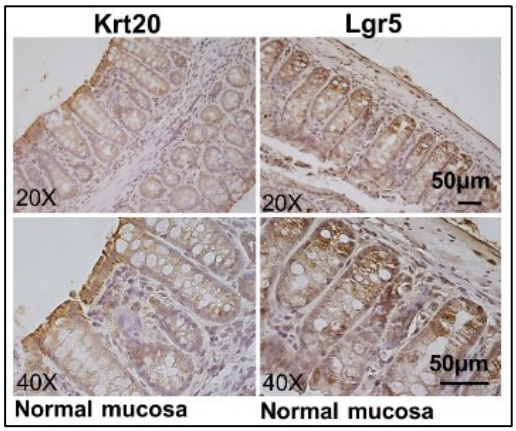

Figure 19: IHC analysis. Krt20 and Lgr5 protein were detected in normal murine colon. Left panels: cells on the top of the crypt were strongly stained for Krt20. Right panels: cells at the crypt bottom were strongly stained for Lgr5 (20X and 40X magnification).

4.3 EGFR/EphA2 related genes and miRNAs expression analysis of murine CRC EphA2$_{high}$ cell populations.

To investigate the crosstalk existing between EphA2 and EGFR a wider molecular characterization of EphA2$_{high}$ and EphB2$_{low}$ cells was performed with Real Time qPCR amplification of the principal genes and miRNAs involved in this complex pathway.

The molecular analysis in CRC EphA2$_{high}$ and EphB2$_{low}$ cells revealed a significant dysregulation of the expression levels of EphA2 and its ligand ephrinA1 (Efna1) as well as the perturbation of gene transcriptional levels of EGFR signaling downstream players in adenocarcinomas (Fig.20A,B). These results provide new evidences that the CRC EphA2 cell signaling involves the dysregulation of EGFR effectors. The analysis of the following genes of interest in EphA2$_{high}$ cells of adenocarcinoma versus normal colon mucosa showed a peculiar pattern of gene expression involving the downmodulation of *Efna1* ($p<0.0001$) as well as a slight over-expression of *Egfr* ($p<0.001$), a marked down-modulation of *Ptpn12* ($p<0.01$), *Akt* ($p<0.001$), and *Pi3k* ($p<0.0001$), and an upmodulation of *Atf2* ($p<0.0001$). The expression levels of *mir-200a* and *mir-26b* were both decreased ($p<0.0001$, and $p<0.0001$, respectively) (Fig.20C), with an inverse correlation respect to their target (*EphA2* and *Atf2*) gene expression levels.

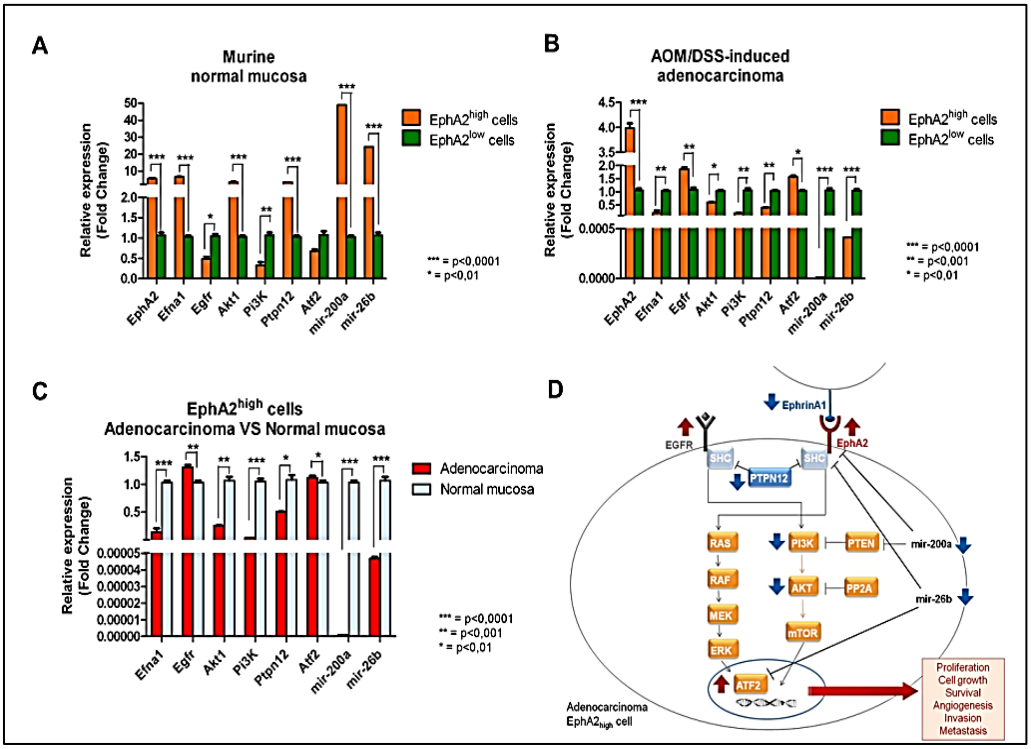

Figure 20: Q-PCR analysis. EGFR signaling effectors were measured in EphA2 cell subpopulations of murine CRC. Data are represented as mean +/- SD. Statistically significant differences were calculated using Student's t-test: *** p<0.0001; ** p<0.001; * p<0.01. Gene expression levels in EphA2high and EphA2low cell subpopulations of **(A)** normal mucosa and **(B)** adenocarcinoma. **(C)** Gene expression levels in $EphA2_{high}$ subpopulation of adenocarcinoma and EphA2high subpopulation of normal colic mucosa. **(D)** Schematic representation of the dysregulation of EphA2/EGFR pathways crosstalk in adenocarcinoma $EphA2_{high}$ cell.

4.4 Prognostic significance of EphA2 and EphA2/EGFR downstream genes in CRC patients

The expression pattern of genes belonging to EphA2 and EGFR pathways (Fig. 20 D) obtained with the described molecular analysis was subsequently investigated in clinical sample cohorts to assess an association with CRC disease.

We examined the correlation of EphA2 gene expression with the clinical characteristics of CRC patients included in six cohorts of public microarray dataset (Tab.3).

	Cohort 1 GSE14333 (N=226)	Cohort 2 GSE37892 (N=130)	Cohort 3 GSE39582 (N=566)	Cohort 4 GSE41258 (N=125)	Cohort 5 GSE5851 (N=80)	Cohort 6 TCGA (N=130)
Gender						
Female	106 (46.9%)	61 (46.9%)	256 (45.2%)	62 (49.6%)	36 (45%)	61 (46.9%)
Male	120 (53.1%)	69 (53.1%)	310 (54.8%)	63 (50.4%)	44 (55%)	69 (53.1%)
Age, years						
Median (range)	67 (26-92)	68 (22-97)	68.1 (22-97)	68 (23-87)	60.5 (25-89)	71.5 (36-90)
Location						
Proximal	101 (44.7%)	57 (43.9%)	224 (39.6%)	50 (40%)	NA	62 (47.7%)
Distal	93 (41.5%)	72 (55.4%)	342 (60.4%)	65 (52%)	NA	68 (52.3%)
Rectum	30 (13.3%)	0 (0%)	0 (0%)	10 (8%)	NA	0 (0%)
Unknown	2 (0.9%)	1 (0.8%)	0 (0%)	0 (0%)	NA	0 (0%)
Stage						
I	41 (18.1%)	0 (0%)	37 (6.5%)	28 (22.4%)	0 (0%)	23 (17.7%)
II	94 (41.6%)	73 (56.2%)	264 (46.6%)	48 (38.4%)	0 (0%)	53 (40.8%)
III	91 (40.3%)	57 (43.9%)	205 (36.2%)	49 (39.2%)	0 (0%)	33 (25.4%)
IV	0 (0%)	0 (0%)	60 (10.6%)	0 (0%)	80 (100%)	20 (15.4%)
Unknown	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0.8%)
Adjuvant chemoth						
Done	87 (38.5%)	NA	233 (41.1%)	NA	NA	NA
Undone	139 (61.5%)	NA	316 (55.8%)	NA	NA	NA
Unknown	0 (0%)	NA	17 (3%)	NA	NA	NA
KRAS status						
WT	NA	NA	328 (57.9%)	NA	43 (53.8%)	1 (0.8%)
Mutant	NA	NA	217 (38.3%)	NA	27 (33.8%)	1 (0.8%)
Unknown	NA	NA	21 (3.7%)	NA	10 (12.5%)	128 (98.5%)
EphA2 Expression						
Low	193 (85.4%)	117 (90%)	388 (68.6%)	66 (52.8%)	64 (80%)	95 (73.1%)
High	33 (14.6%)	13 (10%)	178 (31.5%)	59 (47.2%)	16 (20%)	35 (26.9%)

Table 3: Patient characteristics in 6 cohorts analyzed. Abbreviations: NA, not available.

We found that 10% to 47.2% of the patients in the six cohorts had a high expression of EphA2 gene. Also we analyzed the correlation of clinical characteristics of patients with the $EphA_{high}$ gene expression level (Tab.4). We excluded cohort 5 since it consisted of patients with only stage IV CRC.

Although $EphA2_{high}$ patients apparently had a more advanced disease than did $EphA2_{low}$ patients in cohort 1 and cohort 4 (p=ns), we did not see a clear difference in stage distribution between the two groups of patients in the other cohorts. Interestingly, in the cohort 3 we observed a slightly higher percentage of *KRAS* wild type (WT) in $EphA2_{low}$ patients than in $EphA2_{high}$ patients (p=0.02). Finally, we found no differences in other clinical variables between $EphA2_{high}$ and $EphA2_{low}$ patients groups (Tab.4).

Cohort 1 GSE14333 (N = 226)			
	EphA2 low N= 193 (85.4%)	**EphA2 high N= 33 (14.6%)**	**p-value**
Gender[a]			0.9933
Male	103 (53.37%)	17 (51.51%)	
Female	90 (46.63%)	16 (48.48%)	
Age (mean)[b]	67 (62-74)	67 (65-70.6)	0.7187
Stage[a]			0.0777
I	39 (20.21%)	2 (6.06%)	
II	81 (41.97%)	13 (39.39%)	
III	73 (37.82%)	18 (54.54%)	
Location[a]			0.0696
Proximal R	80 (41.45%)	21 (63.63%)	
Distal L	82 (42.49%)	11 (33.33%)	
Rectum	29 (15.03%)	1 (3.03%)	
Unknown	2 (1.03%)	0 (0%)	
Cohort 2 GSE37892 (N = 130)			
	EphA2 low N= 117 (90%)	**EphA2 high N= 13 (10%)**	**p-value**
Gender[a]			0.8147
Male	62 (52.99%)	7 (53.85%)	
Female	55 (47%)	6 (46.15%)	
Age (mean)[b]	68 (64-71)	73 (59.53-78)	0.3623

Stage[a]			0.1949
I			
II	63 (53.85%)	10 (76.92%)	
III	54 (46.15%)	3 (23.08%)	
Location[a]			0.1485
Proximal R	48 (41.03%)	9 (69.23%)	
Distal L	68 (58.12%)	4 (30.77%)	
Rectum	-	-	
Unknown	1 (0.85%)	0 (0%)	
Cohort 3 GSE39582 (N=566)			
	EphA2 low N= 388 (68.55%)	**EphA2 high N= 178 (31.45%)**	**p-value**
Gender[a]			0.364
Male	218 (56.19%)	92 (51.69%)	
Female	170 (43.81%)	86 (48.31%)	
Age (mean)[b]			0.8083
	68 (67-70)	69 (67-71)	
Stage[a]			0.225
I	30 (3.13%)	7 (3.93%)	
II	185 (38.58%)	79 (44.38%)	
III	133 (41.61%)	72 (40.45%)	
IV	40 (16.68%)	20 (11.24%)	
Location[a]			0.0002
Distal R	133 (34.28%)	91 (51.12%)	
Distal L	255 (65.72%)	87 (48.88%)	
Rectum	-	-	
KRAS status[a]			0.0216
Wild type	238 (61.34%)	90 (50.56%)	
Mutant	134 (34.54%)	83 (46.63%)	
Unknown	16 (4.12%)	5 (2.81%)	
Cohort 4 GSE41258 (N=125)			

	EphA2 low N= 66 (52.8%)	EphA2 high N= 59 (47.2%)	p-value
Gender[a]			0.6578
Male	35 (53.03%)	28 (47.46%)	
Female	31 (46.97%)	31 (52.54%)	
Age (mean)[b]	66.5 (62-69)	70 (63.5-74)	0.1889
Stage[a]			0.4928
I	17 (25.75%)	11 (18.64%)	
II	26 (39.39%)	22 (32.29%)	
III	23 (34.48%)	26 (44.07%)	
IV	-	-	
Location[a]			0.8926
Proximal R	26 (39.39%)	24 (40.68%)	
Distal L	34 (51.51%)	31 (52.54%)	
Rectum	6 (9.09%)	4 (6.78%)	
Unknown	-	-	

Cohort 5 GSE5851 (N = 80)			
	EphA2 low **N= 64 (80%)**	**EphA2 high** **N= 16 (20%)**	**p-value**
Gender[a]			0.6578
Male	38 (59.4%)	6 (37.5%)	
Female	26 (40.6%)	10 (62.5%)	
Age (mean)[b]	60.21 (53.27-67.16)	61 (57.61-64.39)	0.8419
KRAS status[a]			0.2798
WT	37 (57.8%)	6 (37.5%)	
Mutant	19 (29.7%)	8 (50%)	
Unknown	8 (12.5%)	2 (12.5%)	

Cohort 6 TCGA (N=130)			
	EphA2 low **N= 95 (73.1%)**	**EphA2 high** **N= 35 (26.9%)**	**p-value**

Gender[a]			0.6696
Male	52 (54.74%)	17 (48.57%)	
Female	43 (45.26%)	18 (51.14%)	
Age (mean)[b]	70 (68-72.5)	61 (57.61-64.39)	0.1666
Stage[a]			0.3552
I	18 (18.95%)	5 (14.29%)	
II	34 (36.84%)	19 (54.29%)	
III	25 (26.32%)	8 (22.86%)	
IV	17 (17.89%)	3 (8.57%)	
Unknown	1 (1.05%)	-	
KRAS status[a]			0.6879
WT	1 (1.05%)	0 (0%)	
Mutant	1 (1.05%)	0 (0%)	
Unknown	93 (97.89%)	35 (100%)	

Table 4: Clinical characteristics of $EphA2_{high}$ and $EphA2_{low}$ patients in cohort 1 to 6. [a] χ^2-test; [b] t-test

We then investigated the prognostic impact of EphA2 gene upregulation analyzing data of patients with stage I-III CRC (cohort 1 and 3) (Fig.21). Tumor recurrence and DFS data were available for these two cohorts. We also analyzed CSS data for cohort 4 since DFS data were not available for this group. Kaplan-Meier curves significantly showed much worse survival durations in $EphA2_{high}$ patients than in $EphA2_{low}$ patients (Fig.21 A), indicating that the upregulation of EphA2 gene expression is related to poor prognosis for CRC. This result was also confirmed in cohort 5 and 6.

Additionally, down-modulation of Efna1 had a prognostic impact evaluating both all patients and $EphA2_{high}$ CRC patients (Fig. 21 B,C).

Moreover, Kaplan-Meier curves for $EphA2_{high}$ patients showed a possible prognostic role also for Ptpn12, Pi3k, and Atf2 (Fig.21 E,F,G). The down-modulation of Akt gene expression in $EphA2_{high}$ CRC patients did not show a significant prognostic role for such gene (data not shown).

Kaplan-Meier curves for mir-200a and mir-26b were calculated considering all patients of TCGA dataset, not stratified for EphA2 gene expression levels, because gene and microRNA expression data were not available for the same

set of subjects. Coherently to what has been shown previously in this analysis we confirmed the prognostic impact of mir-200a in CRC. Noteworthy a reduced expression of mir-26b was related to a decreased OS in patient with CRC (Fig.21 D).

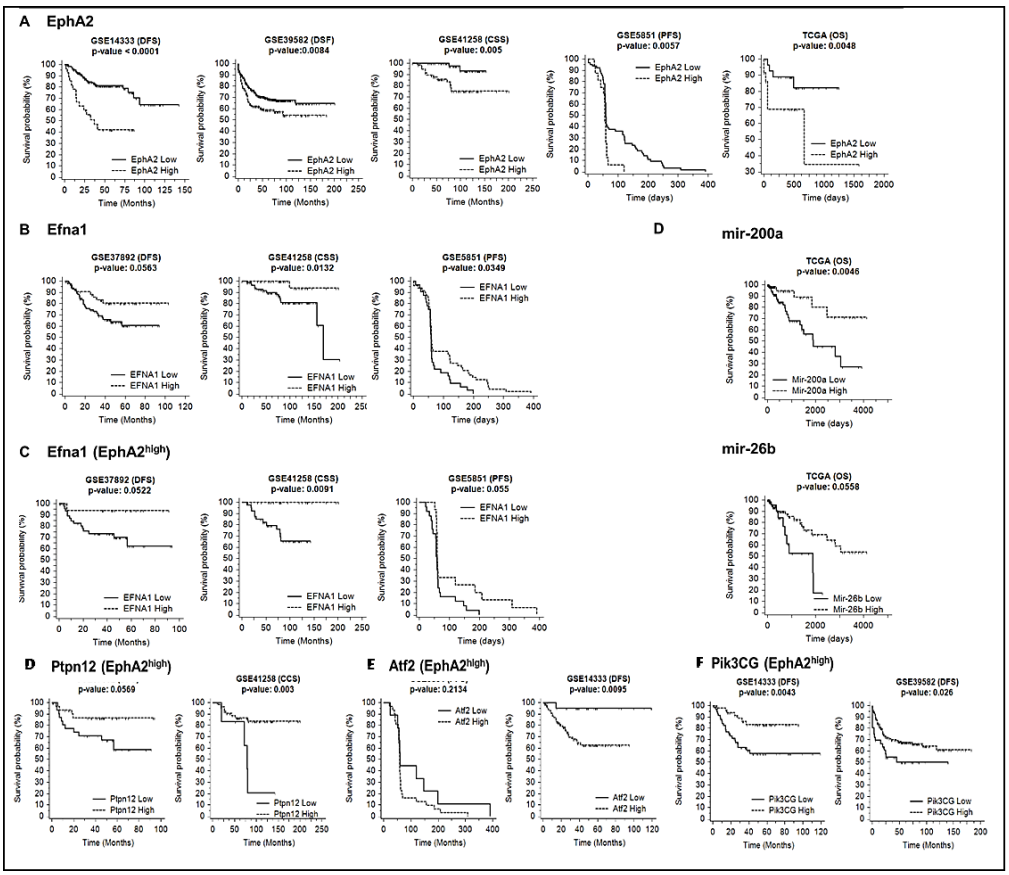

Figure 21: Kaplan-Meier survival curves (A) $EphA2_{high}$ (dashed line) versus $EphA2_{low}$ (solid line) for cohort 1, 3, 4, 5 and 6 **(B)** $Efna1_{high}$ (dashed line) versus $Efna1_{low}$ (solid line) for cohort 2, 4 and 5. **(C)** Analysis of Efna1 conducted only for patients belonging to EphA2high group for the same cohorts of **B**. **(D)** Kaplan-Meier survival curves on TCGA dataset of mir-200a_{high} (dashed line) versus mir-200a_{low} (solid line) and mir-26b_{high} (dashed line) versus mir-26b_{low} (solid line). **(D)** Ptpn12 (high versus low for $EphA2_{high}$ group) **(E**) Atf2 (high versus low for $EphA2_{high}$ group) and **(F)** Pik3CG (high versus low for $EphA2_{high}$ group) for cohorts in which results were significant. P-values were calculated using log-rank tests. Expression value thresholds for determining high and low groups were determined through maxstat R package. Tick marks represent censored data.

Interestingly, a significantly worse survival duration (DFS) was associated with elevated EGFR gene expression for all patients and for patients stratified

for EphA2 high expression level (Fig.22). The hazard ratio (HR) values resulted statistically significant for the cohorts 1 [HR, 2.7152; 95% confidence interval (CI), 1.26-5.84] and 3 [HR, 2.0696; 95% CI, 1.02-4.19], meaning that patients with high expressions of EGFR and EphA2 die at twice (and more) the rate per month as the $EphA2_{high}$ patients with $EGFR_{low}$.

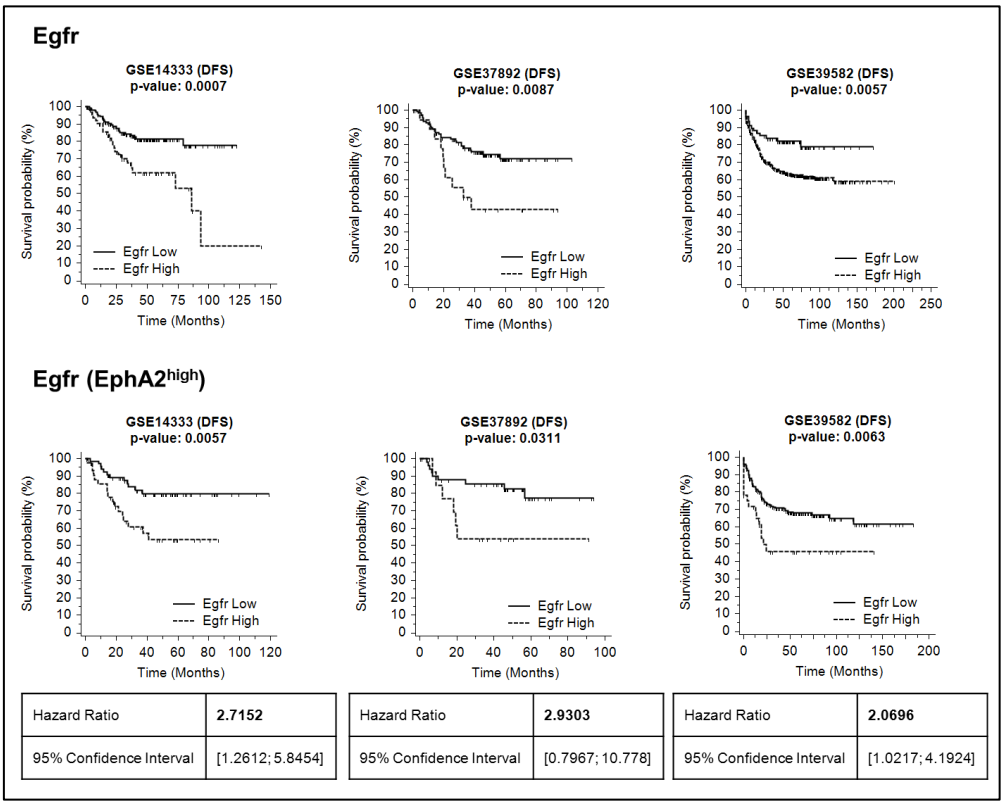

Figure 22: Kaplan-Meier survival curves of $EGFR_{high}$ (dashed line) versus $EGFR_{low}$ (solid line) for cohort 1, 2 and 3. P-values were calculated using log-rank tests. Tick marks represent censored data. Expression value thresholds for determining high and low groups were found through *maxstat* R package. For the analysis conducted only for patients belonging to $EphA2_{high}$ group (below figures) Hazard Ratio and 95% Confidence Intervals are reported below each cohort analyzed.

We conducted further analyses to determine whether the prognostic impact of the EphA2 gene expression pattern is independent of other clinical variables. We pooled the patients in cohorts 1, 2 and 3 with available DFS data (n = 853) for univariate and multivariate analyses of factors affecting DFS (Tab.5). In the multivariate analysis, $EphA2_{high}$ status was related to worse DFS

rates than was $EphA2_{low}$ [HR, 1.47; 95% CI 1.10-1.96; p=0.0095] independently of other clinical variables.

		Univariate analysis[a]		Multivariate analysis[b]		
Variables	**N**	**5-Years DFS**	**P-value**	**HR**	**95% CI**	**P-value**
Age[c]						
<70 y	447	68.50%	–	–	–	–
>= 70 y	405	74.60%	0.1518	–	–	–
Gender						
Female	388	75.20%	0.0758	0.7539	0.58 – 0.98	0.0386
Male	465	68%	–	1	–	–
Location			0.5376			
Left	462	69.30%	–	–	–	–
Rectum	30	77.50%	–	–	–	–
Right	358	73.30%	–	–	–	–
Unknown	3	–	–	–	–	–
Adjuvant chemotherapy			0.0002			
Done	289	64.40%	–	0.9553	0.69 – 1.32	0.7838
Undone	433	77.30%	–	1	–	–
Unknown	131	67.90%	–	1.0719	0.72 – 1.6	0.7734
Stage			<0.0001			
I	77	95.40%	–	0.2092	0.07 – 0.66	0.00081
II	427	79.10%	–	1	–	–
III	349	57.10%	–	2.5309	1.86 – 3.44	<0.0001
EphA2 Expression						
High	196	63.70%	0.0041	1.4697	1.01 – 1.96	0.0095
Low	657	73.60%	–	1	–	–

Table 5: Univariate and multivariate analysis of factors affecting DFS in stage I-III patients (patients' data from the cohorts 1 to 3 were pooled together. N = 853). [a] In univariate analyses, log-rank tests were conducted; [b] In the multivariate Cox proportional hazard model, only variables with P < 0.15 in univariate analysis were included and the "enter method" was applied; [c] Data on age of one patient were missing.

Furthermore, the univariate analysis only in CRC patients with $EphA2_{high}$ status, belonging to cohorts 1, 2 and 3, showed a significant statistical association with the disease stage ($p<0.0001$) and the adjuvant chemotherapy ($p=0.042$). Moreover, the percentage of up/down-expression of EGFR, Ptpn12 and Atf2 associated to $EphA2_{high}$ status followed the same trend of our preclinical expression results, although Atf2 did not reach statistical significance (Tab.6). Additionally, the multivariate analysis showed that $EGFR_{high}$ is related to worse DFS rates than was $EGFR_{low}$ [HR, 1.81; 95% CI 1.24-2.66; p=0.0024], while opposite results were observed for Pik3CG, i.e. the lower Pik3CG, the worse the DFS [HR, 1.68; 95% CI 1.15-2.47; p=0.0083]. In this regard, this conclusion was reached by the analysis of only cohort 1 and 3, because the second cohort did not profile this gene. Efna1 and Ptpn12 resulted not significant by multivariate analyses (Tab.6).

		Univariate analysis[a]	
Variables	**N**	**5-Years DFS**	**P-value**
Age[c]			
<70 y	224	63.30%	–
>= 70 y	230	72%	0.0644
Gender			
Female	229	70.50%	–
Male	226	64.40%	0.2993
Location			0.633
Left	237	65.40%	
Rectum	13	83.90%	
Right	204	69.20%	
Unknown	1	100.00%	
Adjuvant chemotherapy			0.042
Done	151	62.80%	
Undone	229	72.30%	
Unknown	75	62.10%	
Stage			<0.0001

I	27	95.70%	
II	224	80.10%	
III	172	55.10%	
IV	32	27.20%	
Atf2 Expression			
High	311	69.30%	0.2593
Low	144	63.80%	–
Efna1 Expression			
High	248	64.00%	0.0252
Low	207	71.80%	–
Ptpn12 Expression			
High	79	61.20%	0.062
Low	376	69.10%	–
Egfr Expression			
High	91	50.90%	0.0002
Low	364	71.60%	–
Pik3CG Expression[d]			
High	294	70.500%	0.0095
Low	96	55.00%	–

	Multivariate analysis[b]		
Variables	**HR**	**95% CI**	**P-value**
Age			
<70 y	1.2272	0.8575 to 1.7563	0.2654
>= 70 y	1	–	–
Adjuvant chemotherapy			
Done	0.7933	0.5248 to 1.1992	0.2745
Undone	1	–	–
Unknown	1.5117	0.9282 to 2.4622	0.0985
Stage			

I	0.2370	0.0326 to 1.7212	0.1568
II	1	–	–
III	3.0104	1.9923 to 4.5487	<0.0001
IV	11.1993	6.3605 to 19.7192	<0.0001
Efna1 Expression			
High	1	–	<0.0001
Low	0.7146	0.4984 to 1.0244	0.0689

	Multivariate analysis[b]		
Variables	**HR**	**95% CI**	**P-value**
Age			
<70 y	1.2001	0.8385 to 1.7175	0.3212
>= 70 y	1	–	–
Adjuvant chemotherapy			
Done	0. 7908	0.5232 to 1.1954	0.268
Undone	1	–	–
Unknown	1. 2782	0.7537 to 2.1679	0. 3649
Stage			
I	0. 2168	0.0299 to 1.5716	0. 1323
II	1	–	–
III	2. 9680	1.9608 to 4.4926	<0.0001
IV	11.6472	6.6238 to 20.4802	<0.0001
Ptpn12 Expression			
High	1.1577	0.7246 to 1.8496	0.5424
Low	1	–	–

	Multivariate analysis[b]		
Variables	**HR**	**95% CI**	**P-value**
Age			
<70 y	1. 2115	0.8440 to 1.7391	0. 3007
>= 70 y	1	–	–
Adjuvant chemotherapy			
Done	0. 7732	0.5092 to 1.1740	0. 2297
Undone	1	–	–
Unknown	1. 2551	0.7755 to 2.0313	0. 3574
Stage			
I	0. 2093	0.0289 to 1.5167	0. 1236
II	1	–	–
III	2. 7874	1.8378 to 4.2277	<0.0001
IV	11. 9437	6.7925 to 21.0015	<0.0001
Egfr Expression			
High	1. 81340.	1.2377 to 2.6570	0. 0024
Low	1	–	–

	Multivariate analysis[b]		
Variables	**HR**	**95% CI**	**P-value**
Age			
<70 y	1	–	–
>= 70 y	0. 8736	0.5980 to 1.2760	0.4866
Adjuvant chemotherapy			
Done	0. 9015	0.5830 to 1.3939	0. 6426
Undone	1	–	–
Unknown	13.7540	4.5174 to 41.8762	<0.0001
Stage			
I	0. 1680	0.0232 to 1.2159	0.0788

II	1	–	–
III	2.3026	1.4607 to 3.6299	0.0004
IV	5.9874	2.9746 to 12.0514	<0.0001
Pik3CG Expression[d]			
High	1	. –	–
Low	1. 6827	1.1454 to 2.4721	0.0083

Table 6: Univariate and multivariate analyses of factors affecting DFS of patients with $EphA2_{high}$ from pooled cohorts 1, 2, and 3 (N = 455). [a] In univariate analyses, log-rank tests were conducted.; [b] In the multivariate Cox proportional hazard model, only variables with P < 0.15 in univariate analysis were included and the "enter method" was applied; [c] Data on age of one patient were missing. [d] Because of its unavailability on cohort 2, the analysis of Pik3CG was conducted by pooling cohorts 1 and 3.

These findings may suggest that the prognostic relevance of EphA2 (alone or in combination with Efna1, Ptpn12 and EGFR gene expression status) in CRC patients is maintained even when taking into account the classic clinical prognostic features.

4.5 Association between EphA2/Efna1/EGFR gene expression status and poor response to cetuximab treatment in CRC patients

Only the patients in cohort 5 (n=80) received cetuximab monotherapy. In the 70 patients of this cohort who had *KRAS* mutation status data available, we observed no difference in the *KRAS* mutation rates between the $EphA2_{high}$ and $EphA2_{low}$ patients groups (Tab.7A).

A. KRAS mutational status vs. EphA2 expression (N = 70; P = 0.133)

	EphA2 High (N=14)	EphA2 Low (N=56)
WT	6 (42.86%)	37 (66.07%)
Mutant	8 (57.14%)	19 (33.93%)

[*] Ten patients without KRAS data were excluded.

B. Response rate vs. EphA2 expression (N = 68; P = 0.33)

	EphA2 High (N=14)	EphA2 Low(N=54)
CR/PR	0 (0%)	6 (11.11%)
SD/PD	14 (100%)	48 (88.89%)

*Twelve patients without response data (N = 12) were excluded in this analysis.
#Abbreviation: CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease.

C. Disease control rate vs. EphA2 expression (N = 68; P = 0.012)

	EphA2 High (N=14)	EphA2 Low(N=54)
CR/PR/SD	1 (7.14%)	24 (44.44%)
PD	13 (92.86%)	30 (55.56%)

*Twelve patients without response data (N = 12) were excluded in this analysis.
#Abbreviation: CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease.

D. Response rate vs. EphA2 expression (In KRAS WT patients; N = 39; P = 0.574)

	EphA2 High (N=6)	EphA2 Low(N=33)
PR	0 (0%)	5 (15.15%)
SD/PD	6 (100%)	28 (84.85%)

* Among 70 patients with KRAS mutation data, 43 patients had KRAS WT. Four patients who had no response data (UTD) were excluded and no patients have CR (Complete Remission).
#Abbreviation: PR, partial remission; SD, stable disease; PD, progressive disease.

E. Disease control rate vs. EphA2 expression (In KRAS WT patients; N = 39; P = 0.008)

	EphA2 High (N=6)	EphA2 Low (N=33)
PR/SD	0 (0%)	20 (60.61%)
PD	6 (100%)	13 (39.39%)

* All patients (N = 80) were included in this analysis. Twelve patients had no response data (UTD) and no patients have CR (Complete Remission).
#Abbreviation: PR, partial remission; SD, stable disease; PD, progressive disease.

Table 7: correlation between EphA2 expression and clinical variables in KRAS WT e mutated patients.

However, we did notice differences in response to cetuximab between the two groups (Fig.23A). Specifically, complete remission or partial remission occurred only in the $EphA2_{low}$ group [response rate: 11.11% ($EphA2_{low}$) vs. 0.0% ($EphA2_{high}$); p=0.33], and the disease control rate was significantly higher in the $EphA2_{low}$ group than in the $EphA2_{high}$ group (44.44% vs. 7.14%; p=0.012) (Tab.7B and 7C). We then restricted our analysis to *WT KRAS* patients: partial remission occurred only in $EphA2_{low}$ group [response rate: 15.15% ($EphA2_{low}$) vs. 0.0% ($EphA2_{high}$); p=0.574] and also for the disease control rate only $EphA2_{low}$ patients showed partial remission or stable disease [disease control rate: 60.61% ($EphA2_{low}$) vs. 0.0% ($EphA2_{high}$); p=0.008] (Tab.7D and 7E). Patients with $EphA2_{high}$ status showed a shorter PFS duration than did $EphA2_{low}$ patients (p=0.0057) (Fig.23A). An inverse trend in PFS duration was displayed by $Efna1_{high/low}$ patients both in all patients (Fig.23A) and in $EphA2_{high}$ patients (Fig.23B) of cohort 5. Finally, it is worth noting that the cetuximab treated patients of the cohort 5 with increased expression of EGFR showed a statistically significant longer duration of PFS comparing to the patients with $EGFR_{low}$ status (Fig.23A). However, a marked inversion of the PFS duration trend was observed in patients $EGFR_{high}$ and $EphA2_{high}$ (Fig.23B), suggesting a possible role of EphA2 in bypassing the inhibition of EGFR pathway exerted by cetuximab.

4.6 Correlation between EphA2/Efna1/EGFR gene expression level and *KRAS* genetic status

We further investigated the correlation between EphA2 status and somatic mutations in *KRAS* gene in patient cohort 5. No significant differences in mutation rate for *KRAS* were exhibited in the univariate analysis of all patients (Tab.8) neither in only $EphA2_{high}$ patients of cohort 5 (Tab.9).

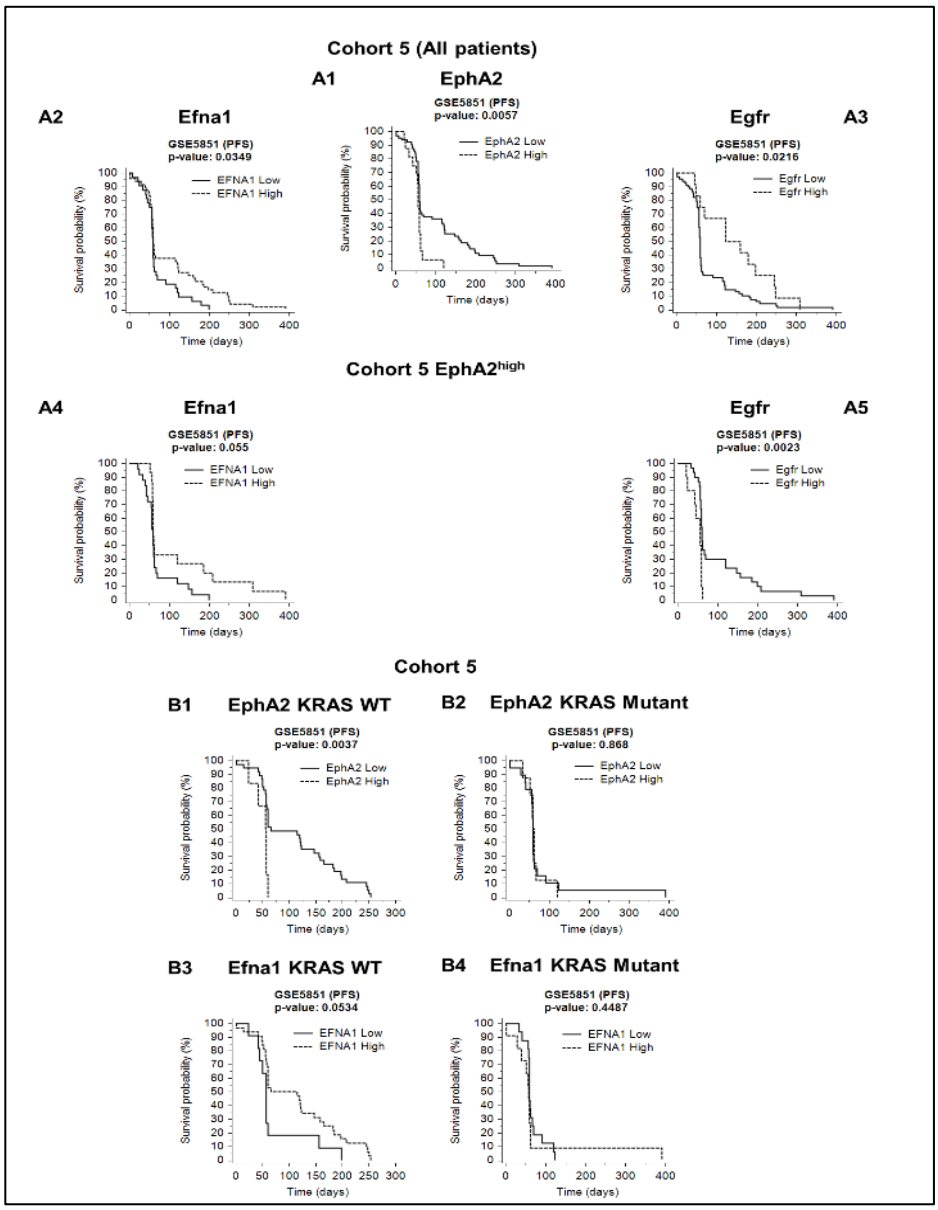

Figure 23: **Correlation between EphA2/Efna1/EGFR gene expression level and KRAS genetic status (A)** Kaplan-Meier survival curves of EphA2, Efna1 and EGFR for cohort 5. Survival curves of $EphA2_{high}$ (dashed line) versus $EphA2_{low}$ (solid line), $Efna1_{high}$ (dashed line) versus $Efna1_{low}$ (solid line) and $EGFR_{high}$ (dashed line) versus $EGFR_{low}$ (solid line) for all patients of the cohort. P-values were calculated using log-rank tests. Expression value thresholds for determining high and low groups were determined through *maxstat* R package. **(B)** Analysis of Efna1 and EGFR conducted only for patients belonging to EphA2high group. EphA2high group was determined with EphA2 median expression threshold. **(C)** Survival curves of EphA2 and Efna1 for patients with WT *KRAS*. **(D)** Survival curves of EphA2 and Efna1 for patients with mutant *KRAS*. P-values were calculated using log-rank tests.

	Univariate analysis[a]			Multivariate analysis[b]		
Variables	**N**	**PFS (median)**	**P-value**	**HR**	**95% CI**	**P-value**
Age[c]						
<70 y	54	59	–	–	–	–
>= 70 y	24	60	0.227	–	–	–
Gender						
Female	44	58	–	1.7653	1.04 – 2.99	0.035
Male	36	61	0.009	1	–	–
EphA2 Expression						
High	16	57	0.006	1.5101	0.75 – 3.04	0.2513
Low	64	60	–	1	–	–
KRAS Mutation[d]						
Mutant	27	59	–	1.3012	0.75 – 2.26	0.3521
WT	43	61	0.142	1	–	–

Table 8 - Univariate and multivariate analysis of factors affecting PFS in patients who received Cetuximab monotherapy (cohort 5) [a] In univariate analyses, log-rank tests were conducted. [b] In the multivariate Cox proportional hazard model, only variables with P < 0.15 in univariate analysis were included and the "enter method" was applied. [c] Data on age of 2 patients were missing. [d] Data on KRAS mutational status of 10 patients were missing.

EGFR, Ptpn12, and Pi3k were significant by univariate analyses, and exhibited a prognostic relevance when associated to gender (p=0.0036, 0.0493, 0.0584 respectively) (Tab.9). Moreover, PFS rate trends are comparable to those of cohorts 1, 2 and 3, described above. Considering the response to cetuximab treatment in the cohort 5, we observed, as expected, that patients with WT *KRAS* had a longer PFS duration than patients with *KRAS* mutations, although this correlation did not reach statistical significance (Tab.8). Furthermore, the PFS of patients with $EphA2_{high}$ status was short considering all patients of cohort 5 (p=0.0057; Fig.23 A) as well as for patients with WT *KRAS* (p=0.0037; Fig.23 C). On the contrary, for patients with mutant *KRAS*, no difference could be detected between the PFS of $EphA2_{high}$ and $EphA2_{low}$ status, although this correlation did not reach statistical significance (Fig.23D), suggesting that the role of EphA2 in the resistance to cetuximab treatment is independent from the *KRAS* mutations. Inversely to the trend of PFS observed for EphA2, $Efna1_{high}$ patients had a significantly longer PFS

duration than did Efna1$_{low}$ patients (p=0.0349; Fig.23A), more so in WT *KRAS* patients (p=0.0534; Fig.23C) than in *KRAS*-mutant patients (p=0.4487; Fig.23D) although this correlation did not reach statistical significance. Poor statistical significance of the results described above is due to the small number of patients remaining for the analysis after *KRAS* status and EphA2/Efna1-dependent stratification.

	Univariate analysis[a]		
Variables	**N**	**5-Years DFS**	**P-value**
Age[c]			
<70 y	23	43.50%	0.6662
>= 70 y	15	46.70%	–
Gender			
Female	19	31.60%	0.0829
Male	21	52.40%	–
KRAS Mutation[d]			
Mutant	16	50.00%	0.9498
WT	19	42.10%	–
Atf2 Expression			
High	31	38.70%	0.2134
Low	9	55.60%	–
Efna1 Expression			
High	15	46.70%	0.055
Low	25	40.00%	–
Ptpn12 Expression			
High	16	18.70%	0.0266
Low	24	58.30%	–
Egfr Expression			
High	10	10.00%	0.0023
Low	30	53.30%	–
Pik3CG Expression			
High	4	75.00%	0.0822
Low	36	38.90%	–

	Multivariate analysis[b]		
Variables	**HR**	**95% CI**	**P-value**
Gender			
Female	1. 4715	0.7436 – 2.9123	0. 2698
Male	1	–	–
Efna1 Expression			
High	0. 5917	0.2843 – 1.2315	0.1627
Low	1	–	

	Multivariate analysis[b]		
Variables	**HR**	**95% CI**	**P-value**
Gender			
Female	1. 6437	0.8566 – 3.1538	0. 137
Male	1	–	–
Ptpn12 Expression			
High	2.0218	1.0058 – 4.0640	0. 0493
Low	1	–	

	Multivariate analysis[b]		
Variables	**HR**	**95% CI**	**P-value**
Gender			
Female	1. 8426	0.9590 – 3.5403	0. 068
Male	1	–	–
Egfr Expression			
High	3.33	1.4886 – 7.4493	0. 0036
Low	1	–	

	Multivariate analysis[b]		
Variables	**HR**	**95% CI**	**P-value**
Gender			
Female	1. 977	1.0300 – 3.7947	0. 0.0415
Male	1	–	–

Pik3CG Expression			
High	0.3126	0.0943 – 1.0357	0.0584
Low	1	–	–

Table 9: Univariate and multivariate analyses of factors affecting PFS in patients who received cetuximab monotherapy belonging to EphA2$_{high}$ group (cohort 5, N=40). [a] In univariate analyses, log-rank tests were conducted; [b] In the multivariate Cox proportional hazard model, only variables with P < 0.15 in univariate analysis were included and the "enter method" was applied; [c] Data on age of 2 patients were missing, [d] Data on KRAS mutational status of 5 patients were missing.

5. DISCUSSION

This study explored the role of EphA2/EGFR pathway mediators as prognostic factors or predictors of cetuximab benefit in CRC patients, with the aim to translate potential novel prognostic biomarkers into clinical application.

EphB2 expression showed low relevance in the tumor bulk, even if $EphB2_{high}$ cancer cells retain stem-like signature. On the contrary, EphA2 plays a critical role in invasion, angiogenesis and metastasis in multiple crosstalks with other cellular molecular networks including FAK, VEGF and EGFR pathways[134,141,178].

With this assumption we isolated from a murine CRC model cell subpopulations that homogeneously expressed high or low level of EphA2 to study how the expression pattern of EphA2/EGFR downstream genes is perturbed in colorectal cancer modeled in the AOM/DSS mouse and in 6 independent public datasets of CRC clinical sample cohorts.

The IHC staining confirmed previous analyses[130]: in normal colon mucosa we observed a decreasing gradient of EphB2 from the crypt base to the top, whereas EphA2 expression was mostly detected in the differentiated compartment of crypt apical columnar cells; on the contrary, adenocarcinoma displayed a highly heterogeneous and not gradient-disposed staining for both EphB2 and EphA2. Moreover cytofluorimetric analysis of adenocarcinoma showed enrichment in $EphA2_{high}$ cell fraction, in line with the results of studies which showed marked EphA2 overexpression in different kinds of solid tumors, including colon cancer[196-200]. Also the reduction of the $EphB2_{high}$ tumor cell subpopulation is sustained by data reported elsewhere[146,149].

The interesting observation that adenocarcinoma $EphA2_{high}$ tumor cells showed low expression levels both of Krt20 and Lgr5 along with an increased expression of Ascl2 led us speculate that the $EphA2_{high}$ cell population in tumors could represent a fraction of cells that underwent dedifferentiation and likely acquired CSC-like properties as supported by other studies in CRC, NSCLC and glioblastoma[201-203]. This peculiarity of EphA2-marked cell subpopulation is coherent with the role that EphA2 has in epithelial to mesenchymal transition: tumor cells undergoing EMT display particular

characteristics such as resistance to cell death and senescence, evasion of immune surveillance and the acquisition of stem cell properties.

To validate the results of the gene expression analysis we performed an IHC assay, which confirmed the overlap between EphB2+ cells and Lgr5+ /Krt20- cells in normal mucosa. Similarly, normal EphA2+ cells were Lgr5- and Krt20+.

We focused our attention on the role of EphA2 receptor in CRC, described elsewhere as an important mediator of CRC cell migration/invasion[146], to investigate the signaling crosstalk existing between EphA2 and EGFR. The expression profiles of each molecule involved in EphA2/EGFR crosstalk in normal and tumoral cells resulted in reciprocal coherence with each other, supporting the general picture we defined as the basis of this study. $EphA2_{high}$ cells of murine adenocarcinoma showed a down-modulation of the ligand Efna1 as well as a slight over-expression of Egfr, a marked down-modulation of Ptpn12, and an up-modulation of the transcription factor Atf2. The upregulation of the expression of both the tyrosine kinase receptors EphA2 and Egfr and the downregulation of the ligand Efna1 suggest a higher activation of the downstream pathways, as confirmed by the overexpression of Atf2, a critical target of MAPK activities which are set downstream of EGFR and EphA2 receptor. Such transcriptional factor is responsible for the regulation of growth, survival or apoptosis in tumorigenesis[44]. The mechanisms that reside at the basis of this switch in molecular expression are mostly unknown and involved a transcriptional control of EphA2 expression by EGFR in its ligand-activated or constitutively active (EGFRvIII) status[141]. Moreover, Efna1 downregulation suggests the possibility of a ligand-independent mechanism of action of the receptor EphA2 in the $EphA2_{high}$ cells analyzed[122].

To deepen the particular perturbation of EGFR pathway, we also observed a down-modulation of the expression of the tumor suppressor Ptpn12, a tyrosine phosphatase that interacts with and inhibits multiple oncogenic tyrosine kinases, including EphA2 and EGFR[44]. Additionally, in cancer $EphA2_{high}$ cells we detected the down-modulation of two important downstream components of EGFR pathway: Pi3k and Akt. This could seem discordant with the well-known pro-oncogenic role of these two molecules in most cancers.

However, in this case, Pi3k and Akt functional hyper-activation in CRC is not dependent on transcriptional upregulation, but likely on genetic mutations of the respective genes[204]. It must be considered also the complex network of signals generated by a number of downstream components which interact at multiple levels with the Pi3k signaling pathway[205].

The gene expression pattern identified in preclinical setting was confirmed on a large number of CRC patients and it showed strong prognostic and predictive significance, considering different clinical endpoints (OS, DFS, CSS and PFS). In line with recent findings[196,197,200] investigating the oncogenic role of EphA2 in CRC and other tumors, we found a high expression of EPHA2 in 10% to 47.2% of the patients in microarray data of six public CRC datasets.

The ligand independent oncogenic activation of EphA2[122] was also suggested by the analysis of the available clinical outcome data derived from the public datasets. On the one hand $EphA2_{high}$ patients showed much worse survival durations (OS, DFS, CSS and PFS) than $EphA2_{low}$ patients, indicating a poor prognostic role of this receptor in CRC. On the other hand the down-modulation of the ligand Efna1 was associated to worse survival duration when all CRC patients or only $EphA2_{high}$ patients were evaluated.

In CRC patients with stage II/III the univariate and multivariate analysis confirmed that the prognostic role of EphA2 is independent of other clinical variables.

Also the role of EGFR in CRC outcome was evaluated. An increased EGFR gene expression was significantly associated with worse survival duration (DFS) for all patients and for $EphA2_{high}$ patients, with an increased HR values in $EphA2_{high}$ cases. This data suggest that patients with high expressions of both EGFR and EphA2 die at twice the rate per month as the $EphA2_{high}$ patients with low levels of EGFR.

The prognostic impact of downstream targets of the EGFR/EphA2 pathway such as Efna1, Ptpn12, Pi3k, Akt and Atf2 was also investigated in patients that overexpress EphA2: all these genes, except for Pi3k and Akt, are associated to a worse DFS when dysregulated with the trend observed in the $EphA2_{high}$ cells. Furthermore, the multivariate analysis showed that the prognostic relevance of EphA2 (alone or in combination with EGFR, Efna1, and Ptpn12 status) in CRC patients is independent from classic clinical prognostic features.

We moved our interest also on miRNAs known to target both EphA2 and EGFR pathways, to find a coordinated epigenetic control of this complex network. The molecular analysis was so extended to mir-200a and mir-26b: in $EphA2_{high}$ murine cells sorted from CRC the expression levels of mir-200a and mir-26b were both decreased and inversely correlated with the expression levels of their validated targets EphA2 and Atf2, suggesting an epigenetic regulation pattern coherent with the general expression framework object of our study[206,207]. We also evaluated the prognostic impact of mir-200a and mir-26b expression in CRC patients: mir-200a down-modulation was confirmed[208] to be associated with poor prognosis and for the first time also mir-26b decreased expression was significantly correlated with poor prognosis in patients with CRC.

Resistance to cetuximab remains one of the most critical issues to treat CRC as up to 40%-60% of patients with WT KRAS tumors do not respond to therapy. In this perspective we considered relevant to investigate in EphA2-stratified patients the relation between EphA2-EGFR overlapping downstream targets and the response to therapy, referring also to KRAS mutation status.

Particularly, between the patients treated with cetuximab the disease control rate was significantly higher in the $EphA2_{low}$ group than in the $EphA2_{high}$ group which also showed a shorter PFS duration: consistent with the picture outlined by our molecular results and survival analysis, $EphA2_{high}$ patients displayed a worse outcome.

Moreover, in line with other and well established evidences an increased expression of EGFR was significantly associated with a longer duration of PFS in patients treated with cetuximab, coherently with the role of EGFR as target of this drug[209]. It is worth noting that in patients with a simultaneous overexpression of EGFR and EphA2 the correlation of EGFR expression with clinical outcome (PFS) is inverted: this finding corroborates the hypothesis that the overexpression of EphA2 may be an escape route to cetuximab-dependent EGFR inhibition.

This observation is in line with other studies demonstrating that EphA2 overexpression is involved in the resistance to both EGFR tyrosine kinase inhibitors (TKI) such erlotinib (lung cancer)[201] and vemurafenib (melanoma)[210]

and moAbs as trastuzumab (breast cancer)[211]. Additionally the EphA2 blockade is proposed as a new strategy to restore the anti-EGFR sensitivity.

Collectively, our results and these studies demonstrate the promise and utility of targeting EphA2 to overcome the resistance to anti-EGFR therapy. The EPH is indeed a complex signaling system which impacts RAS–Pi3k–Akt and RAS–RAF-MAPK pathways.

Further in our study the predictive role of EphA2 expression level was not correlated to KRAS mutation status in patients treated with cetuximab. Indeed in the totality of patients, high levels EphA2 were associated with low PFS, as well as in the group of patients with WT KRAS, but not with mutant KRAS, suggesting that EphA2 may have a role in the resistance to cetuximab treatment independently from the KRAS mutations.

These results suggest the hypothesis that EphA2 can be linked to a novel mechanism of resistance to cetuximab therapy which can be considered alternative to KRAS mutations. It is known, indeed, that even in patients with WT KRAS, the efficacy of cetuximab therapy is restricted to a small subset of patients[98]. It becomes of outstanding relevance the necessity to define all the molecular features that identify between the metastatic CRC patients the best responders to cetuximab treatment.

6. CONCLUSIONS AND PERSPECTIVES

In conclusion, through a preclinical CRC model and retrospective studies on CRC patients, we identified novel potential prognostic and predictive targets in the molecular pattern composed by EphA2/Efna1/EGFR/Ptpn12/Atf2/mir-200a/mir-26b genes, which could be helpful in selecting CRC patients with poor prognosis and cetuximab resistance.

As EGFR signaling is one of the most druggable pathways, this study represents an important advance also for further development of more personalized targeted therapies against CRC which may take advantage of a chemosensitization approach through EphA2 blockade.

Since we applied our analysis to retrospective patients' cohorts, it would be of key interest to validate our results in prospective studies. Moreover, functional studies would elucidate the crosstalk of EphA2 with EGFR pathway effectors.

Integrating our results and literature data emerges a molecular pattern that we could name the "Eph paradox": in advanced CRC, EphA2 expression is significantly increased exerting a crucial role in migration and invasion, maintaining some stemness markers in its molecular signature; on the contrary, in advanced CRC, EphB2 expression is lost or significantly reduced in the tumor bulk, even if $EphB2_{high}$ cancer cells do persist and retain experimentally proven stem-like (CD44+Ascl2+Lgr5+) signature, *in vitro* organoid formation ability and *in vivo* high tumorigenic activity.

It is thus conceivable that gene expression signatures of EphB2, EphA2 and other tumor cell subpopulations might help characterize their functional roles in the contest of the progressive hierarchical organization of the tumor, throughout the different phases of colorectal carcinogenesis. Moving from animal models to clinical specimens might help assess whether and to what extent $EphA2_{high}$ and $EphB2_{high}$ cells contribute to CRC progression, in particular EMT and metastatic invasion.

This issue could be further tackled through the analysis of EphB2/EphA2 molecular signature and mesenchymal genes in liquid biopsy specimens of CRC

metastatic patients. Circulating tumor cells could be considered blood-born functional cancer stem cells, so although they are present in small numbers, their molecular characterization may provide a better understanding of the metastatic cascade, help with risk stratification and enable therapeutic selection and monitoring of progression for patients undergoing treatment.

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