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Review

Portrait of the PI3K/AKT pathway in colorectal cancer ☆

Stine Aske Danielsen [a,b,1], Peter Wold Eide [a,b,1], Arild Nesbakken [b,c], Tormod Guren [b,d], Edward Leithe [a,b], Ragnhild A. Lothe [a,b,*]

[a] Department of Cancer Prevention, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway
[b] K.G. Jebsen Colorectal Cancer Research Centre, Oslo University Hospital, Oslo, Norway
[c] Department of Gastrointestinal Surgery, Oslo University Hospital, Oslo, Norway
[d] Department of Oncology, Oslo University Hospital, Oslo, Norway

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ABSTRACT

PI3K/AKT signaling leads to reduced apoptosis, stimulates cell growth and increases proliferation. Under normal conditions, PI3K/AKT activation is tightly controlled and dependent on both extracellular growth signals and the availability of amino acids and glucose. Genetic aberrations leading to PI3K/AKT hyper-activation are observed at considerable frequency in all major nodes in most tumors. In colorectal cancer the most commonly observed pathway changes are *IGF2* overexpression, *PIK3CA* mutations and *PTEN* mutations and deletions. Combined, these alterations are found in about 40% of large bowel tumors. In addition, but not mutually exclusive to these, *KRAS* mutations are observed at a similar frequency. There are however additional, less frequent and more poorly understood events that may also push the PI3K/AKT pathway into overdrive and thus promote malignant growth. Here we discuss aberrations of components at the genetic, epigenetic, transcriptional, post-transcriptional, translational and post-translational level where perturbations may drive excessive PI3K/AKT signaling. Integrating multiple molecular levels will advance our understanding of this cancer critical circuit and more importantly, improve our ability to pharmacologically target the pathway in view of clonal development, tumor heterogeneity and drug resistance mechanisms. In this review, we revisit the PI3K/AKT pathway cancer susceptibility syndromes, summarize the known aberrations at the different regulatory levels and the prognostic and predictive values of these alterations in colorectal cancer.

Contents

☆ Scope of review: Comprehensive overview of the molecular mechanisms causing dysregulation of the PI3K/AKT signaling pathway and their functional and clinical impact in colorectal cancer.

* Corresponding author at: Department of Cancer Prevention, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway. Tel.: +47 227 81 728; fax: +47 227 81 745.
E-mail address: rlothe@rr-research.no (R.A. Lothe).

[1] Shared first authors.

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1. Context

The role of the PI3K/AKT signaling in cancer pathogenesis has been thoroughly investigated in recent years, and several drugs targeting the pathway are under development. The growth factor receptors, *RAS*, *PIK3CA*, *PTEN*, and *AKT*, are all altered at some level in most solid tumors. Consequently, this signaling cascade and the downstream effectors are considered attractive pharmacological targets [1,2]. However, development of secondary resistance, unanticipated feedback effects and pathway cross talk have challenged the efforts to design

Table 1
PI3K/AKT aberrations in colorectal cancer.

Function	Gene	Chromosome	Cytoband	Aberrancy in CRC	Frequency altered (%)	Predisposition
Ligands	AREG	4	q13.3	mRNA upregulation	1	
	EFNA5	5	q21	mRNA downregulation	–	
	EREG	4	q21.21	mRNA upregulation	–	
	IGF1	12	q23.2	Mutation	<1	Beckwith–Wiedemann
	IGF2	11	p15.5	Mutation	<1	Syndrome, Silver–Russel
				Copy number gain	18	syndrome
				Loss of imprinting	~50	
				mRNA upregulation	13–18	
Receptors	EGFR	7	p11.2	Mutation	4	
				Copy number gain	5–61	
				Protein increase	2–8	
	ERBB2	17	q12	Mutation	5	
				Copy number gain	2–15	
				Protein increase	10	
	ERBB3	12	q13.2	Mutation	6	
				Protein increase	10	
	ERBB4	2	q33.3–q34	Mutation	5	
	IGF1R	15	q26.3	Mutation	5	
				Copy number gain	<5	
				Protein increase	60–75	
	KIT	4	q12	Mutation	3	
				Copy number gain	2–9	
	MET	7	q31	Mutation	<2	
				Copy number gain	15	
				Protein increase	10	
Upstream components	AKT1	14	q32.33	Mutation	1	Proteus syndrome
				Protein increase	–	
	AKT2	19	q13.2	Mutation	1	
				Copy number gain	<2	
				Protein increase	–	
	AKT3	1	q44	Mutation	<2	
	KRAS	12	p12.1	Mutation	35–40	Noonan syndrome 3, Costello syndrome
	NRAS	1	p13.2	Mutation	9	Noonan syndrome 6
	IRS1	2	q36.3	Mutation	4	
				Protein increase	–	
	IRS2	13	q34	Mutation	2	
				Copy number gain	>40	
				mRNA upregulation	8	
	IRS4	X	q22.3	Mutation	4	
	PDK1	2	q31.1	Mutation	1	
	PHLPP1	18	q21.33	Mutation	0–2	
				Copy number loss	70	
	PHLPP2	16	q22.2	Mutation	1–3	
	PIK3CA	3	q26.32	Mutation	10–15	
	PIK3CB	3	q22.3	Mutation	2	
	PIK3CD	1	p36.2	Mutation	2	
	PIK3CG	7	q22	Mutation	4	
	PIK3R1	5	q13.1	Mutation	2–8	
	PIK3R2	19	p13.11	Mutation	<1	
	PTEN	10	q23.31	Mutation	2–10	PTEN Hamartoma Tumor
				Copy number loss	4–35	Syndrome (PHTS)
				Protein reduction	35–75	
				Protein mislocalized	80	
	PTENP1 (PTEN pseudogene)	9	p13.3	Copy number loss	8	
	SPRY2	13	q31.1	mRNA upregulation	10	
Downstream components	CSNK2A1	20	p13	mRNA upregulation	20	
				Protein increase	>20	
	DEPTOR	8	q24.12	mRNA upregulation	2	
	GSK3B	3	q13.3	Mutation	3	
	MLST8	16	p13.3	Mutation	1	
	MTOR	1	p36.22	Mutation	8	
	NEDD4	15	q21.3	mRNA upregulation	3	
	RICTOR	5	p13.1	Mutation	4	
	RPTOR	17	q25.3	Mutation	5	
	STK11	19	p13.3	Mutation	5	Peutz–Jeghers syndrome
	TSC1	9	q34.13	Mutation	4	Tuberous sclerosis complex
	TSC2	16	p13.3	Mutation	4	Tuberous sclerosis complex

therapeutically effective compounds [3]. Colorectal cancer is the third most common type of cancer worldwide with 1.4 million new patients and 700,000 deaths annually [4]. Considering this and that about 40% of the malignant tumors carry known activating PI3K/AKT alterations, the clinical potential of targeting the pathway becomes clear [5] (See Tables 1 and 2). In addition to new drugs, there is a need for molecular markers that could better stratify patients into clinically relevant subgroups.

Currently, there are several clinico-pathological biomarkers in use for prognostication of colorectal cancer, disease stage at diagnosis and the ability to perform tumor resection without residual disease being the two most important. Many patients are cured by surgery alone; about half of the patients with metastases to the regional lymph nodes (stage III), and more than 80% of those with disease confined to the bowel wall (stages I–II). However, to reduce risk of relapse, patients with stage III disease are recommended 6 months of adjuvant chemotherapy after surgery whereas the benefit from adjuvant treatment for stage II patients is controversial [6]. Nevertheless, risk stratification according to clinico-pathological factors alone is not very accurate, and some patients are under-treated and some are over-treated with (radio)chemotherapy using current guidelines. Better prognostication is therefore warranted, and the use of molecular biomarkers seems attractive. Although a wealth of molecular knowledge about colorectal cancer development exists (Fig. 1) [7,8], few biomarkers have been implemented in clinical practice [9–11]. Several prognostic gene expression signatures have been developed and for the most promising ones clinical trials are ongoing (reviewed in [12]).

The use of targeted therapy and the corresponding molecular predictive biomarkers is a topic of increasing interest in the management of colorectal cancer. Currently, tailored treatment is mostly offered patients with distant metastases. A comprehensive understanding of the cellular signaling pathways, combined with knowledge of effect mechanisms of approved drugs as well as for drugs in pipeline, is foreseen to pave the way for inclusion of patients without distant metastasis in clinical trials and assumed to improve effect on survival outcomes.

1.1. Molecular phenotypes of colorectal cancer

Colorectal cancers progress in a stepwise manner through dysregulation of various pathways (Fig. 1). About 85% of the malignant tumors present a chromosome instability (CIN) phenotype, whereas a second group (~15%) comprises mismatch-repair deficient tumors resulting in a microsatellite instability (MSI) phenotype [13–16]. Two additional classifiers are described, the hyper-mutator and CpG-island methylator phenotypes (CIMP) [5,17]. Both are to a large degree overlapping with MSI. Moreover, in analogy to the DNA-level described "-instability" phenotypes, we recently presented evidence for a transcriptome instability (TIN) phenotype in colorectal cancer [18]. Though through different means, somatic evolution leads to the accumulation of molecular

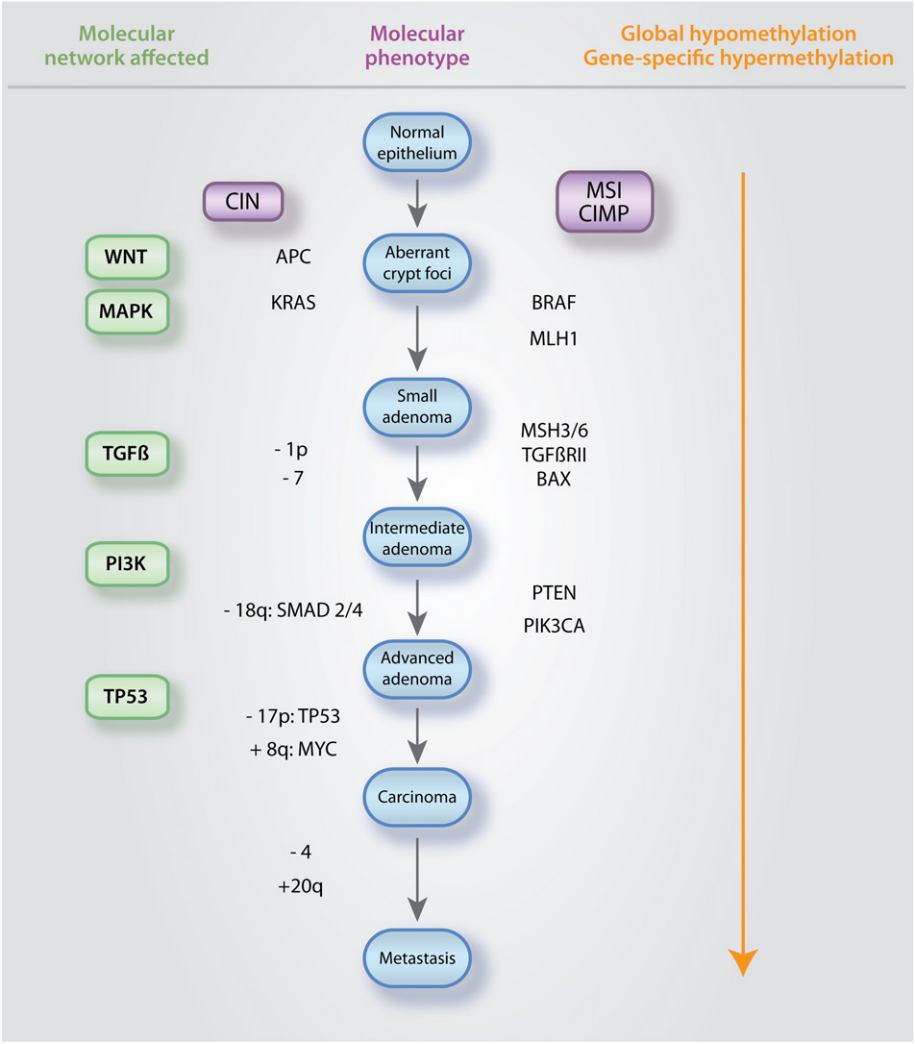

Fig. 1. The colorectal adenoma–carcinoma sequence with molecular networks and common aberrations. The key signaling pathways are indicated in green while instability phenotypes are colored purple. CIN: chromosomal instability; MSI: microsatellite instability and CIMP: CpG-island methylator phenotype.

changes that disrupt key regulatory networks. Progressively, the integrities of the WNT, MAPK, TGF-β, PI3K/AKT and TP53 signaling pathways are lost and tumors develop [7,19].

1.2. The PI3K/AKT signaling pathway

A fundamental premise for proper cell function is the correct transduction of inter- and intracellular signals. Mammalian cells harbor a plethora of sensors that transduce information from the extracellular environment into molecular signals, which are further relayed through the cell in signaling cascades and alter the state of various effector proteins that regulate transcriptional programs and metabolic pathways. The modular architecture allows multiple sensory inputs to be integrated into a specific cellular response making cells able to meet their own and the host organism's specific temporal needs. Genomic alterations cause signaling network deregulation that may result in severe syndromes and diseases, including cancer.

PI3K/AKT pathway activation (Fig. 2) is controlled by four principal types of sensors; the receptor tyrosine kinases (RTKs), which sample for growth factors, the cytokine- and G-protein coupled receptors, that are activated by a wide array of different ligands, and the integrins, which sense cell–cell and cell–matrix adhesion [20–23]. Upon appropriate binding, these sensors, in conjunction with their cofactors, activate downstream kinases in the phosphatidylinositide 3-kinase (PI3Ks) family. At the cell membrane, the main phosphoinositide (PI) is PI 4,5-bisphosphate (PIP_2). Class I PI3Ks activated either by RTKs or RAS phosphorylate PIP_2 yielding PI 3,4,5-triphosphate (PIP_3). Specific signals in the extra-cellular environment induce a change in the intracellular membrane composition and subsequently proteins with PIP_3 binding domains are recruited. Among these are the serine/threonine kinases PDK1 and AKT. PDK1 phosphorylates AKT on threonine-308 [24]. A second phosphorylation catalyzed by the mTOR complex 2 (mTORC 2) on serine-473 fully activates AKT [25]. This multi-subunit complex consisting of among others MTOR, RICTOR and PROTOR integrates the availability of amino acids together with TSC1/2 and thus restricts AKT activation in case of nutritional limitations [26,27]. PTEN and PHLPP1/2 are phosphatases that counteract the action of PIK3CA by dephosphorylating PIP_3 and AKT, respectively, and thereby balancing pathway activity [28,29]. Fully phosphorylated AKT activates a multitude of downstream targets. Among these, arguably the most prominent are the mTOR complex 1 (mTORC1), BAD, CASP9, various FOXO proteins, GSK3β, MDM2 and TSC1. However, more than 100 AKT target proteins have been described in the literature [30]. Through its numerous substrates, AKT exerts signals leading to cell growth and differentiation, angiogenesis and prevents apoptosis.

Even though a large number of gene products are implied in the PI3K/AKT signaling pathway only those that have been associated with colorectal carcinogenesis will be discussed here (Fig. 3). Interestingly, much of the initial knowledge on the alterations in the PI3K/AKT pathway was acquired through investigation on patients and families with hereditary diseases that predispose to cancer and will be revisited in the following section.

1.3. Lessons learned from PI3K/AKT pathway associated cancer susceptibility syndromes

Precise control of PI3K/AKT activation pathway is essential for maintaining tissue homeostasis and dysregulation of its activities contributes to a wide range of diseases such as diabetes and heart conditions, multiple hamartoma syndromes and various malignancies.

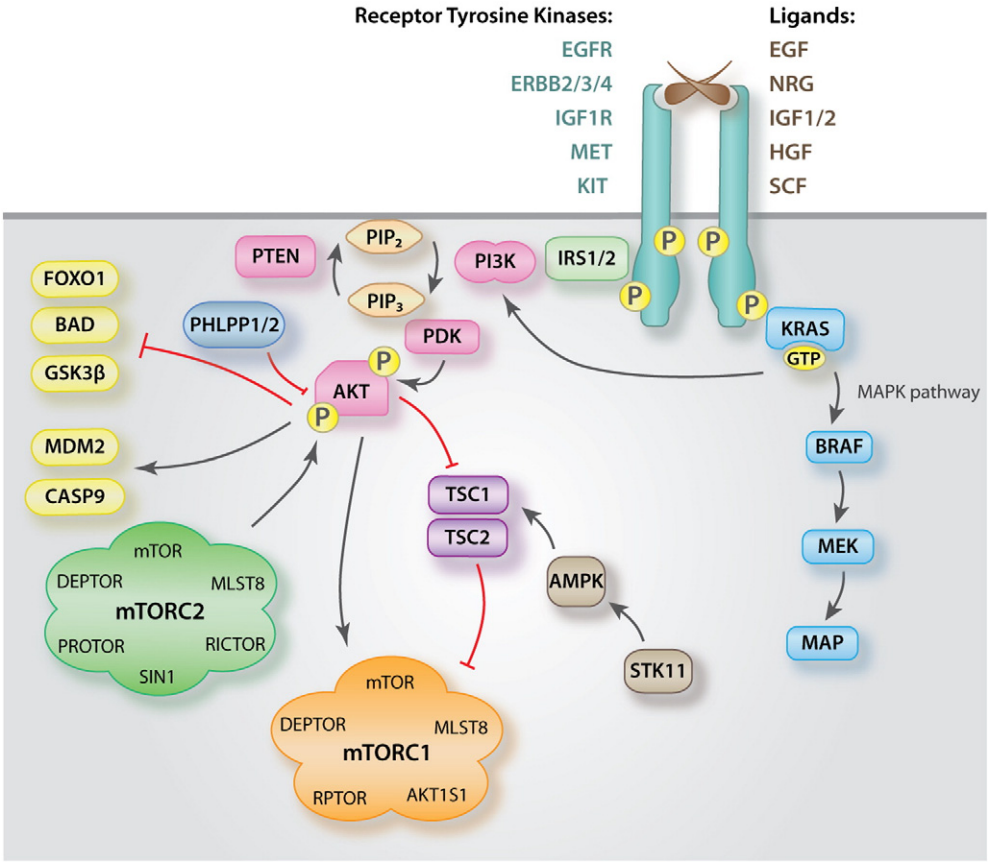

Fig. 2. The core components of the PI3K/AKT pathway. Activating interaction are indicated with black arrows while inhibitory ones are shown as red T-lines.

PTEN hamartoma tumor syndrome (PHTS)[2] is the collective term for a spectrum of rare disorders with highly variable clinical manifestations caused by *PTEN* germline mutations [31]. Characteristic symptoms include cerebral overgrowth, neurodevelopmental abnormalities, multiple hamartomas and other benign lesions as well as elevated risk of certain cancer forms. Intriguingly, mutations of *PTEN* give rise to a diversity of phenotypes and there is even significant intra-familial variability among affected relatives, underscoring the complexity of pathway interaction. Individuals with PHTS have an increased risk of developing breast cancer, follicular thyroid cancer, renal cancer and endometrial tumors [32], which also were some of the first malignancies to be associated with somatically acquired *PTEN* alterations [33]. Recent studies indicate that PHTS is also associated with elevated risk of developing melanomas and colorectal cancer [33,34]. PHTS include among others Cowden syndrome[3] (CS) [35,36], Bannayan–Riley–Ruvalcaba syndrome[4] (BRRS) [37,38], *PTEN*-related Proteus syndrome[5] (PS) and Proteus-like syndrome [32,39]. Based on early studies, the prevalence of *PTEN* gene alterations reported in individuals diagnosed with CS was estimated to be nearly 80%, and another 10% were found to harbor promoter mutations, resulting in decreased PTEN protein levels and as a consequence increased levels of phosphorylated AKT [40]. However, recent studies based on larger patient cohorts have established that the actual prevalence is 25–35% [41,42]. In addition to *PTEN*, *PIK3CA* and *AKT1* were recently identified as CS susceptibility genes and 9% and 2% of unrelated CS individuals with wild type *PTEN* carried *PIK3CA* and *AKT1* mutations, respectively (CS 5,[6] and CS 6[7]) [43]. Notably, no germline mutations were found in *PIK3R1* or *PIK3R2*. *PTEN* mutations are found in approximately 60% of patients having BRRS, while ~10% alternatively have larger deletions, including deletion of the *PTEN* promoter [40]. So far, a clear association between BRRS and increased risk of developing cancer has not been demonstrated. However, it is speculated that *PTEN*-mutation positive CS and BRRS are different presentations of the same syndrome, thus BRRS patients are recommended to be included in cancer surveillance strategies set up for CS patients [31,39].

The association of PS with PTHS remains controversial but there are case reports presenting individuals with *PTEN* mutations, hence the terms *PTEN*-related Proteus syndrome and Proteus-like syndrome [39]. Interestingly, the genetic alteration underlying the majority of PS cases was unraveled as late as 2011 by the use of exome sequencing, and turned out to be located in AKT1. Lindhurst and colleagues found that PS is associated with mosaicism for a somatic activating mutation in this gene [44]. This is not an inherited condition, but a mutation acquired during early stages of embryonic development. The patients demonstrate increased incidence of rare, benign tumors, such as ovarian cystadenomas, lipomas and meningiomas [45], and also a subset of testicular tumors and central nervous system tumors [32].

Tuberous Sclerosis Complex[8] (TSC) is yet another syndrome with a wide spectrum of clinical manifestations, but characterized by benign hamartomas in multiple organs. It arises from germline mutations in either *TSC1* or *TSC2* [46,47]. Aberrant *TSC2* accounts for most of the TSC cases, whereas *TSC1* mutations are the underlying cause in 10–30% of the cases. Despite the lack of a consistent genotype–phenotype correlation, patients with *TSC2* mutations usually present with a more severe disease than do patients with altered *TSC1* [48]. Cancers of the central nervous system, kidney, heart, lung and skin have been reported for this syndrome.

Fifty to 70% of all Peutz–Jeghers syndrome[9] (PJS) cases are caused by germline mutations in the tumor suppressor gene *STK11*, also known as *LKB1* [49–51]. STK11 is a serine/threonine kinase that via phosphorylation of AMPK activates TSC2, resulting in negative regulation of mTOR signaling. Hence mutations in *STK11*, either in the germline or somatically, indirectly result in hyperactivation of mTOR [52]. The PJS patients experience disease onset in early childhood and typically harbor a few, moderate to large sized hamartomatous polyps in the bowel or stomach. When the individuals reach their thirties, they have a risk of developing any cancer of 85–90%, with gastrointestinal and genitourinary cancers being the most common [53]. Consistent with Knudson's two-hit hypothesis of tumor suppressor genes, the tumors associated with PJS also have somatic *STK11* mutations or experience loss of heterozygosity (LOH) of chromosome 19p, where the *STK11* gene locus is located [54].

1.4. PTEN- and RASopathies

PTEN-opathies, a joint term that takes into account the wide phenotypic differences seen when *PTEN* or other components in the PI3K/AKT pathway are dysfunctional, was proposed last year [33]. Ultimately, disturbed homeostasis of the pathway leads to overactivation of AKT and dysregulation of downstream proteins and thus increased cell growth and decreased apoptosis. This explains to a large part the phenotypes observed, ranging from severe benign overgrowth to development of malignancies. Improved understanding of the underlying biochemical mechanisms dysregulating the PI3K/AKT pathway and the ultimate consequences of the alterations, are likely to contribute to the development of targeted therapies, not only for the inherited conditions included in the PTEN-opathies, but also for the sporadic malignancies harboring similar aberrations.

Analogous to the PTEN-opathies there is another group of syndromes, termed the RAS-opathies, which are caused by germline mutations in *KRAS*, or other components of the MAPK pathway [55]. Due to the scope of this article, only disorders caused by *KRAS* mutations will be mentioned here. *KRAS* is considered to be one of the most frequently activated oncogenes across a variety of cancer diseases, including colorectal cancer [56–58]. In recent years, heterozygous germline mutations in *KRAS* have also been associated with some cases of developmental disorders, including the autosomal dominant Noonan syndrome 3,[10] cardio-facio-cutaneous syndrome 2,[11] and Costello syndrome[12] [59–62]. However, detailed biochemical and structural analyses of the *KRAS* germline mutations have shown that they in general confer milder gain-of-function effects than do the somatically acquired cancer-associated mutations [63]. Furthermore, mutations in genes encoding associated components in the MAPK signaling pathway (*PTPN11, SOS1, NRAS, HRAS, NF1, RAF1, BRAF, SHOC2, MEK1* and *CBL*), are known to cause variants of the same disorders. These manifestations share features such as facial abnormalities, heart defects, impaired growth and development, and, in some instances, a predisposition to certain malignancies [64].

2. A glance into the somatic genome and epigenome of colorectal cancer

Most healthy cells carry two copies of each autosomal gene. Erroneous recombination and improper segregation upset this balance. Selection towards relaxation of cell growth constrains leads to the loss of tumor suppressor genes and gain of proto-oncogenes. In colorectal cancers, copy number changes are characteristic of chromosome instable

[2] OMIM #601728.
[3] OMIM #158350.
[4] OMIM #153480.
[5] OMIM #176920.
[6] OMIM #615108.
[7] OMIM #615109.
[8] OMIM #191100 and #613254.
[9] OMIM #175200.
[10] OMIM #609942.
[11] OMIM #615278.
[12] OMIM #218040.

tumors in contrast to those with microsatellite instability [65]. Losses at chromosome 17p and 18 and gains of 8q, 13q, and 20 are common events in the development of large bowel malignancies [66]. Copying billions of base pairs inevitably introduces small errors, but our genomes are normally remarkably stable. Genetic instability provides variation on which somatic evolution can work and is a hallmark of cancerous lesions [67].

Colorectal cancers were recently suggested to form two distinct groups according to their mutational load. The hyper-mutated tumors carry more than ten times as many alterations than do the non-hypermutated ones [5,68]. However, compared to other carcinomas, even the non-hypermutated colorectal tumors exhibit a relatively high mutational load with a median of about 60 non-synonymous exon mutations per sample [5,69]. Coding mutations may lead to partly or complete loss of protein function or altered regulation, and the recent application of high-throughput technology has given insight into the mutation frequencies of less studied players involved in cancer. However, whether the low-frequency alterations detected in PI3K/AKT signaling are important or are mere "mutational noise" remains unclear. In addition, until now, virtually all high-throughput mutational studies have been looking at exonic regions. However, it is likely that changes in the promoter or other regulatory sequences will be found to have functional consequences as more studies are investigating this genomic dark matter [70,71]. Increasingly evident is the importance of alterations of epigenetic regulators in cancer development and on clinical strategies [72]. In contrast, hot-spot mutations in the genes constituting the canonical PI3K/AKT pathway are well studied in all the major cancer types and a vast number of papers containing mutational data for colorectal cancer specimens have been and are continuously being published, emphasizing the interest in this particular signaling network.

2.1. Genetic alterations in the PI3K/AKT pathway

Residing at the cell surface, receptor tyrosine kinases (RTKs) are the first receivers of extracellular signals from the surroundings. There are numerous RTKs in the human genome, of which the family of epidermal growth factor receptors is the most frequently altered in carcinomas. Several of the genes encoding the various RTKs are established proto-oncogenes, and upon activation they indirectly influence cell growth, proliferation and survival. The epidermal growth factor receptor family consists of four members, namely *EGFR* (*ERBB1*), *ERBB2*, *ERBB3*, and *ERBB4*. A number of cancer types are associated with alterations of these receptors [73], and they are frequently reported to be aberrantly regulated in colorectal cancer development and progression. This is occasionally due to mutations or copy number alterations. However, most commonly such overactivation seems to be caused by overexpression of the receptors due to increased synthesis or reduced degradation.

EGFR is considered a proto-oncogene, but somatic *EGFR* mutations are found in less than 5% of colorectal cancer specimens [74–78]. Two recent reports even suggested that the recurrent S492R mutation is acquired through exposure to EGFR monoclonal antibodies rather than being a primary event in carcinogenesis [79,80]. An increase in *EGFR* gene copy number, mainly due to polysomy of chromosome 7, has also been reported. The frequencies range from 5% to 61% [76,81,82], which partly can be explained by the different techniques applied (FISH, CISH, PCR) in the various studies, as well as the thresholds used for defining *EGFR* copy number gain and amplification. Compared to *EGFR*, the mutation rates of the other members of this family, *ERBB2*, *ERBB3*, and *MET* and their corresponding signaling pathways, were until recently relatively little studied.

Two to 4% of CRC patients display *ERBB2* (17q12) amplification, and this is correlated with increased protein expression [5,75,83–87]. Activating mutations in the *ERBB2* kinase-domain are found in about 3% of colorectal cancers and co-occur with KRAS aberrations, suggesting that these mutations may also have independent roles in the carcinogenic process [5,75,88]. Mutations in either *ERBB2*, *ERBB3* or *ERBB4* are found in 3–13% of tumors [88–90]. When accounting for factors such as gene length and local mutation rate, *ERBB3* was in fact among the genes called as significantly mutated with 14/223 colorectal cancers carrying non-synonymous alterations [57].

The proto-oncogene *MET* encodes a growth factor receptor protein that becomes activated by hepatocyte growth factor (HGF) [91]. Signaling events initiated by this receptor include activation of the PI3K/AKT and the MAPK pathway, but both amplification of and mutations in the gene are rare in primary colorectal cancers [92–94].

Two other RTKs implicated in promoting oncogenic transformation are the IGF1 receptor (*IGF1R*) and *KIT* proto-oncogene. They are found mutated in 5.5% and 3.5% of colorectal tumors, respectively [5,89,93,95]. It has been speculated that mutated *IGF1R* interfere with anti-EGFR therapy. *IGF1R* gene amplification seems to be a rare event in colorectal carcinomas [81], whereas approximately 2–9% of tumors experience copy number gain of the KIT gene locus 4q12 [5,93].

KRAS is a signal transducer located upstream in the MAPK and PI3K/AKT networks, directly activating both BRAF and PI3K, respectively. The mutation frequency in colorectal cancer is between 30 and 40% [5,56,93,96]. These alterations are first and foremost activating point mutations, of which 95% reside in codons 12 and 13, hence designated "hot spots". Consequently, these are the locations most frequently studied. The mutations lock the protein in a permanent active state, resulting in constant signaling to downstream effectors. Since the official recognition of these mutations as predictors of response to EGFR-directed therapy in 2008 [97], the request for *KRAS* mutation status has increased even further. However, the number of gene mutations reported in the literature is probably somewhat underestimated. Base substitutions are occasionally found also in codons 61, 63, and 146, with frequencies ranging from 1% to 4% for each spot [98–101]. Thus, because of the potential of these mutations to predict drug resistance, it is argued that the mutation status of these codons also should be obtained [99,102]. Mutations in *KRAS* codons 19, 22, 117, and 164 are reported for single colorectal tumor specimens [103]. However, the functional consequence of these alterations is yet unknown. In addition to point mutations, *KRAS* gene amplification has been reported in a 1–2% of colorectal cancer samples [103–105]. Patients harboring this alteration do not seem to be responsive to anti-EGFR therapy and copy number status could therefore further improve the selection of patients suitable for such therapy.

NRAS shares a high degree of sequence identity with *KRAS*, but the mutation frequency of these two genes in colorectal cancer is not at all comparable. One to 9% of colorectal tumors display *NRAS* alterations at the DNA level [5,98,101,106]. Also in contrast to *KRAS*, the *NRAS* codon 61 is preferentially altered over codons 12 and 13, and early studies suggest that these alterations too can predict lack of response to EGFR-directed therapy [98,102,107].

Downstream of the RAS proteins in the MAPK signaling cascade is the RAS effector protein BRAF. BRAF is strictly not part of the PI3K/AKT signaling network, but in the context of colorectal cancer it is often mentioned in the same breath as the other pathway components. The status of the encoding gene is likely important both for prognostic and predictive purposes, and thus will be mentioned briefly. *BRAF* is found mutated in approximately 10% of colorectal cancers, a feature strongly associated with MSI tumors [108]. Interestingly the clinical importance both as a prognostic and predictive marker seem to be attributed to the CIN group of tumors [109].

Class IA PI3K is a heterodimeric enzymatic complex composed of one of three catalytic subunits (p110α, p110β, p110δ) and one regulatory subunit (p85). *PIK3CA* encodes the p110α catalytic subunit and is, next to *KRAS*, the most frequently mutated gene in the PIK3K/AKT pathway. It was initially reported that *PIK3CA* is mutated in more than 30% of colorectal cancers [110]. However, later studies indicate that the fraction of colorectal tumors harboring such alterations is more modest, approximately 10–15% [5,68,93,96,111,112]. The most common alterations are amino acid substitutions in the helical domain, such as

E542/E545/Q546, and in the kinase domain, M1043/H1047. These mutations cause an increase in the PI3K lipid kinase activity in vitro and in vivo [113,114]. Mutations in *KRAS* and *PIK3CA* are not mutually exclusive [5,96], suggesting a selective advantage of activating both the MAPK and PI3K/AKT signaling networks independently. Further, *PIK3CA* (3q26.3) has been suggested to be amplified in colorectal cancers, however, other studies have failed to confirm this observation [5,110]. *PIK3CB* and *PIK3CD*, encoding the two other isoforms of the p110 subunit (p110β and p110δ, respectively) are found mutated in about 2–3% of CRCs [5,93]. *PIK3CG*, encoding the class IB 110γ catalytic subunit, was recently reported to be mutated in approximately 4% of colon cancers [5,93,95,115]. *PIK3R1*, encoding the p85α regulatory subunit, is mutated in 2–8% of colorectal tumors [5,116,117]. Missense and nonsense alterations lead to loss-of-function of p85α and thereby release its inhibitory effect on the PI3K complex [113,116,117]. Interestingly, *PIK3R1* and *PIK3CA* were together with PIK3CB and *PIK3CG* classified as "high confidence driver genes" in a recent comprehensive study by Tamborero et al. across 12 tumor types, including colorectal cancer [115].

PTEN is one of the few phosphatases in the PI3K/AKT signaling pathway. By dephosphorylating PIP_3 to PIP_2 and counteracting the action of PI3K [118], it has an invaluable role in balancing the activity of the circuit. As discussed throughout the review, several mechanisms are involved in the loss of PTEN function in CRC. The reported range of *PTEN* mutation varies from 2 to 10% among different studies [5,68,75,93,96,119,120]. They are associated with MSI since the most frequently observed CRC alterations are found in two polyA sequences located in exons 7 and 8. This is closely followed by alterations in codon 130, exon 5 and codon 233, exon 7 [93], located within the phosphatase core motif and the membrane-binding C2 domain, respectively. Thus the variability in mutation frequency between different studies can be attributed to whether or not the samples analyzed were stratified for MSI and whether the mutation status for the whole coding region or just of exons 7 and 8 was obtained. LOH of chromosome region 10q23.3, where *PTEN* is located, is reported in 4–35% of CRCs [95,121–124]. More specific deletions of whole or parts of the *PTEN* gene using multiplex ligation-dependent probe amplification were detected in 8% [96].

AKT is located downstream of PI3K and PTEN in the signaling cascade and is encoded by the *AKT1*, *AKT2* and *AKT3* genes. Even though activating *AKT1* E17K mutations were reported in 6% of a small colorectal cancer series, later studies estimate that the number is close to 1% [5,49,89,93,125].

It is well established that loss of 18q is a frequent event in colorectal cancer; approximately 70% of all tumors harbor this deficiency [5,19]. Located in this region are genes such as *DCC*, *SMAD2* and *SMAD4*, the latter two being important factors in the TGFβ-signaling pathway. More recently, the PHLPP family of serine/threonine phosphatase was discovered. PHLPP (PH domain leucine-rich-repeats protein phosphatase) selectively dephosphorylates and inactivates AKT [28], thus undertaking a similar tumor suppressor role as PTEN at a level further down in the signaling cascade [126]. One of the two genes encoding PHLPP, *PHLPP1*, is located very close to *SMAD2/4* on 18q and is therefore also found deleted in many CRCs. Consequently, the assumption that 18q loss is favored due to the presence of *SMAD* genes is challenged by the identification of yet another important tumor suppressor gene that is part of the PI3K/AKT pathway rather than the TFGβ pathway. The PHLPP-encoding genes, *PHLPP1* and *PHLPP2*, the insulin receptor substrate genes *IRS1*, *IRS2*, and *IRS4*, as well as the mTORC genes, *DEPTOR*, *PRR4* (*PROTOR*), *RICTOR*, *RPTOR*, *MLST8*, *SIN1*, and *AKT1S1* are each mutated in 1–4% of all colorectal cancers. *MTOR* itself is mutated in a slightly higher fraction, around 8%. Mutations of *STK11* occurs in about 5% of CRC [5,93].

2.2. Epigenetic alterations in the PI3K/AKT pathway

Epigenetics refers to heritable changes not encoded in the nucleotide sequence. Two well-studied epigenetic mechanisms are histone modification and DNA methylation, both of which are important in regulation of gene transcription. Epigenetic dysregulation is commonly observed during cancer development. Colorectal tumors that exhibit aberrant DNA methylation are classified as CIMP positive, and are to a large degree overlapping with those determined to be microsatellite instable. In fact, in a significant proportion of tumors, hypermethylation of the mismatch repair gene *MLH1* is causing microsatellite instability [127].

Despite later years' vast increase in genes reported to be first and foremost hypermethylated in CRC, there are few PI3K/AKT signaling pathway members present on the list of epigenetically modified genes. The majority of the central genes in the pathway are oncogenes, thus it would not be unreasonable to assume that activation of these by hypomethylation of their gene promoter could be a recurrent event. In fact, hypomethylation of *KRAS* (and *HRAS*) in colorectal cancer was one of the first epigenetic aberrations reported [128], although in low frequencies. Also loss of imprinting (LOI), i.e. hypomethylation of the normally silenced maternal allele, of the *IGF2* locus was reported in the early hours of epigenetics [129–131]. Even with these findings, promoter hypomethylation does not seem to be a common mechanism of oncogene activation in the PI3K/AKT pathway. Neither is hypermethylation of the tumor suppressor genes in the pathway a frequent event. Although methylation of *PTEN* has been reported [123], the primers failed to discriminate between *PTEN* and its pseudogene *PTENP1*, which is methylated in high frequencies. Thus we and others have established that hypermethylation of *PTEN* is a rare event in CRC [132,133]. Lastly, two small studies reported low expression of PIK3CG due to promoter hypermethylation and methylation of *STK11* in 8% of the colorectal tumors included, respectively [134,135].

3. Regulation of the PI3K transcriptome and proteome in colorectal cancer

To affect phenotype, the copy number and epigenetic perturbations discussed above have to alter mRNA and ultimately protein expression. This notion is in fact used to filter those events driving malignant growth from those resulting from the genomic instability often seen in solid tumors [136,137]. However, changes in promoter methylation and DNA copy numbers are only two out of several mechanisms affecting to what extent a gene is turned into a functional protein. RNA abundance is a function of both gene transcription and RNA degradation and both the stability and translation is regulated by different non-coding RNAs such as microRNAs [138].

While high-throughput methods for studying RNA expression have been readily available for years, technology for genome-wide measurement of protein abundance has been lagging behind. This is markedly reflected in our relative knowledge of aberrations at these molecular levels. Recent advances in mass-spectrometry-based approaches are however beginning to change this [139–141]. As RNA, proteins are subject to multiple mechanisms of regulation. Chemical modifications such as phosphorylation, acetylation, methylation, acylation or proteolytic cleavage alter the physical structure of the polypeptide and hence its stability, sub-cellular localization, binding properties and/or catalytic potential. Thus, disruptions at both the RNA level and post-translational mechanisms may contribute to carcinogenesis through disabling tumor suppressors or increasing the activity of oncoproteins. In the following such aberrancies which may function as alternate mechanisms for PI3K/AKT pathway deregulation are discussed (Fig. 3).

At the apex of the signaling pathway are the growth factors. In colorectal cancer, elevated IGF2 mRNA expression is reported in about 13–18% of tumors. In about half of the cases, mRNA increase is attributable to copy number gain [5,129]. This excessive IGF2 expression presents an autocrine signaling loop that drives improper PI3K/AKT pathway activation through its cognate receptor IGF1R [142,143]. The functional importance of this alteration is further underscored by its mutual

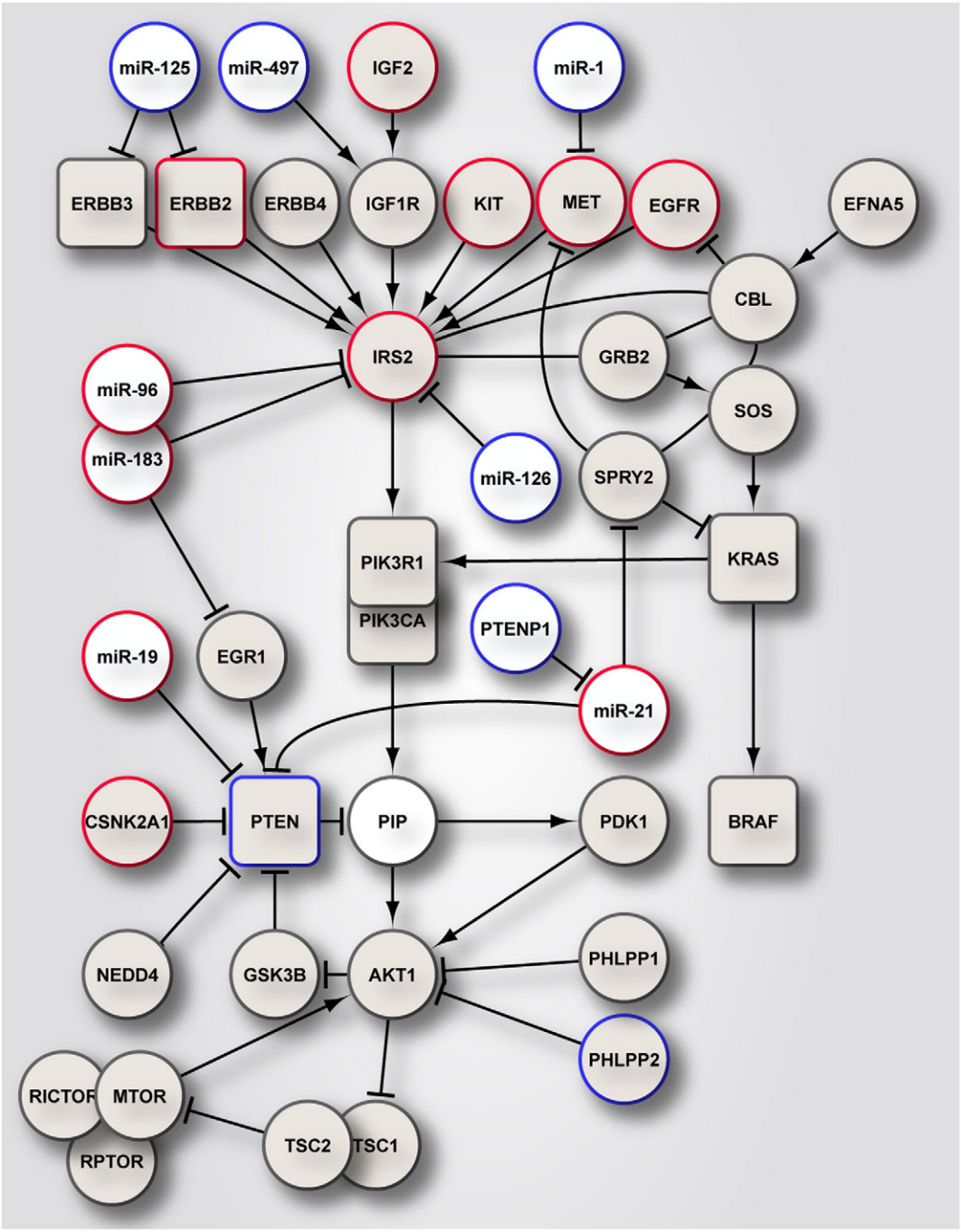

Fig. 3. The PI3K/AKT pathway with components and alterations. Blue color represents copy number loss and/or downregulation, whereas red color symbolizes amplification and/or overexpression. Rounded squares are genes frequently mutated in CRC [57]. Arrows and T-shaped lines indicate positive and negative interactions, respectively.

exclusivity with other pathway alterations such as *PIK3CA* mutations and *PTEN* loss [5].

By immunohistochemistry, cancers have been shown to have higher IGF1R levels than surrounding normal mucosa [144,145] and elevated protein expression is observed in 60–75% of patients [81,146]. Significant effort has been put into developing IGF1R inhibitors, and antibodies targeting the receptor have reached phase II clinical trials in colorectal cancer [147]. Considering the large proportion of tumors depending on IGF2/IGF1R mediated PI3K/AKT signaling and the success in targeting other receptor tyrosine kinases such as ERBB2 and EGFR in other cancers, this approach shows great promise.

EGFR is one of the best studied growth factor receptors in colorectal cancer, where protein upregulation is found in 2–8% of the tumors [86]. Growth factor receptors are known to be regulated among others through subcellular relocalization and degradation [148,149]. One such regulatory mechanism is CBL-mediated EGFR ubiquitination, leading to receptor internalization and signal attenuation [150–152]. The putative tumor suppressor EFNA5 (ephrin A5) has been demonstrated to exert an inhibitory effect on EGFR by inducing CBL-dependent degradation in colon cancer cells [153]. EFNA5 is an ephrin receptor ligand downregulated in colorectal primary tumors and cancer cell lines both at the mRNA and protein level. Moreover, ectopic expression of EFNA5

in colon cancer cells results in reduced proliferation, migration and resistance to chemotherapy [153]. These observations indicate that deregulation of either CBL or EFNA5 or other receptor cofactors could lead to hyperactivation of the PI3K/AKT and MAPK signaling pathways in colorectal cancer. Alterations of ephrins and their receptors are reviewed in Pasquale et al. [154].

The MET, ERBB2 and ERBB3 growth factor receptor proteins are strongly overexpressed in about 10% of colorectal tumors and may present alternative routes to activation of the MAPK and PI3K/AKT pathways [155]. MET is also upregulated at the mRNA level and its deregulation seems to be associated with inferior prognosis [156,157]. Further underscoring MET relevance, *MET* amplification was recently shown to present a resistance mechanism in relapsing tumors treated with anti-EGFR antibodies [92]. This effect was to a larger extent associated with MAPK than PI3K/AKT signaling and amplification was reported in only 2/196 untreated patients [92].

Insulin receptor substrates (IRS) are signaling adaptor proteins that serve as downstream messengers from activated cell surface receptors to various signaling pathways, including the PI3K pathway [158]. IRS1 is required for normal trophic actions of IGF1 in the intestinal epithelium as well as for antiapoptotic, but not mitogenic, effects of IGF1 in the intestinal crypts [159]. Increasing amounts of evidence indicate a role of IRS1 and IRS2 in intestinal carcinogenesis, and IRS1 protein expression has also been found to be higher in primary colorectal tumors and metastases compared to normal colonic epithelium [160–162]. Similar to IGF2, elevated IRS2 expression has been shown to be mutually exclusive with other PI3K/AKT alterations and is reported in about 8% of colorectal cancers [5]. Importantly, IRS2 overexpression is sufficient to activate PI3K/AKT signaling in cell line models [163].

Post-translational modifications of PTEN have been extensively studied and the protein is known to undergo phosphorylation, acetylation, ubiquitination, sumoylation and oxidation (reviewed in [164]). Loss of PTEN protein has been reported in 35–75% of patient tumors, which is much higher than what could be explained by mutations and copy number loss alone [165–168]. Appreciating its non-redundant tumor-suppressive roles and the fact that subtle variations in PTEN abundance affect the cancer susceptibility of mice, it becomes apparent that understanding its regulation is of high importance [169]. C-terminal phosphorylation of PTEN by CSNK2A1 and GSK3β leads to decreased stability [170,171]. CSNK2A1 protein expression has been reported as an independent prognostic marker and to be over-expressed in 22% of colorectal cancers [172,173]. Further, PTEN ubiquitination is important in regulation of both subcellular localization and stability [174,175]. NEDD4 is overexpressed in colorectal cancers and knockdown in cell lines leads to reduced growth also independently of PTEN [176]. Recently, NEDD4 was also shown to target both KRAS and PTEN for degradation in colon cancer cell lines [177].

In the PI3K complex, both the regulatory subunits PIK3R1 and PIK3CA, have been found elevated at the protein level in late stage disease [178,179]. Disruption of the PI3K/AKT pathway through AKT protein overexpression has been proposed as an early event in colorectal cancer progression [180], and elevated levels of AKT1 and AKT2 are reported in tumor lesions compared to matched normal colonic tissue [178]. The proteins are tightly controlled by several post-translational modifications, including phosphorylation, acetylation, ubiquitination and sumoylation and emerging evidence suggests that dysregulation of these modifications may result in overactivation of AKT in colorectal cancer. A recent study suggests that AKT1 may promote metastasis of colon cancer cells by inducing epithelial–mesenchymal transition (EMT) [181], whereas AKT2 has been suggested to play a role in metastasis and act synergistically with PTEN loss to promote malignant growth [182,183]. Simultaneous disruption of both AKT1 and AKT2 has been shown to result in inhibition of the growth and metastatic potential of colon cancer cells [184].

The serine/threonine protein phosphatase PHLPP1 acts as a tumor suppressor by negatively regulating AKT1 through dephosphorylation [28]. Loss of PHLPP1 expression occurs at high frequency in colorectal cancer, partly due to increased ubiquitination and degradation [185]. The deubiquitinase USP46 stabilizes PHLPP1 in colon cancer cells by reducing the rate of PHLPP1 ubiquitination, which causes inhibition of AKT1 and decreased cell proliferation and tumorigenesis of colon cancer cells [186]. Moreover, reduced USP46 protein level was in the same study found to be associated with low PHLPP1 expression in colorectal cancer patient specimens [186]. These observations collectively suggest that USP46 may act as a tumor suppressor in CRC, but further investigation is needed to confirm this notion.

Taken together, exploring RNA, protein level and potential post-translational regulators of PI3K/AKT components in colorectal cancer and preferentially integrated with genomic and epigenomic data, might extend our understanding of how this pathway contributes in disease development. However, discerning whether the observed deregulation is contributing to carcinogenesis, rather than being an effect of it, requires careful consideration. Furthermore, while DNA copy-number and mutations are categorical variables where tumor percentage is less of a concern, cellular composition of the sample can greatly affect the expression measured in bulk tumor samples. We will return to one such example when discussing microRNAs below.

3.1. Non-coding microRNA regulation of PI3K/AKT pathway components

MicroRNAs (miRNAs) are ~22 nucleotide non-coding RNAs that by binding specific mRNAs repress the expression of target genes. This binding reduces the stability or translational efficacy of the target transcript and in turn the abundance of the downstream protein product and may thus contribute to carcinogenesis through the deregulation of cancer genes. The annealing of miRNA to mRNA is flexible and accommodates non-Watson–Crick base pairs leading to promiscuity in the interaction and a single miRNA species could bind and regulate multiple different gene transcripts. In fact, more than a third of our protein coding genes are predicted to be under miRNA regulation [187,188]. However the task of identifying the real biological targets of each miRNA is challenging both due to the flexibility in the interaction and that the

Table 2
miRNAs consistently reported deregulated in CRC and with targets in the PI3K/AKT-pathway. Additional miRNAs expressed from the same cluster are indicated in parenthesis.

RNA (cluster)	Cytobands	CRC aberrancy	PI3K/AKT target genes
Mir-19 (17/92)	13q31.3, Xq26.2	Overexpressed	*PTEN*
Mir-21	17q23.2	Overexpressed	*PTEN*, *PTENP1*, *SPRY2*
Mir-96 (182–183)	7q32.2	Overexpressed	*IRS1*
Mir-183 (96–183)	7q32.2	Overexpressed	*EGR1*, *IRS1*
Mir-1	18q11.2, 20q13.33	Downregulated	*MET*
Mir-125	19q13.41, 11q24.1, 21q21.1	Downregulated	*PIK3CB*, *ERBB2*, *ERBB3*
Mir-126	9q34.3	Downregulated	*PIK3R2*, *IRS1*
Mir-143 (−145)	5q32	Downregulated	*KRAS*
Mir-145 (−143)	5q32	Downregulated	*AKT1*, *IRS1*
Mir-497 (−195)	17p13.1	Downregulated	*IGF1R*

effect will be dependent on the stoichiometry of the miRNA and its target transcripts [189].

Our genomes encode more than 1800 miRNA hairpins [190] and while their pivotal importance in eukaryotic biology is highlighted by their deep evolutionary roots and conservation [191], strong evidence of causal roles in cancer pathogenesis has been harder to establish even after a decade of profiling studies. More than ten years later, the best example may still be the deletion of mir-15/16 and subsequent loss of BCL inhibition in chronic lymphatic leukemia [192,193].

There have been dozens of studies looking at miRNA expression in colorectal cancer (reviews: [194,195] and [196]). Typically these report a handful of up- and downregulated miRNAs, but here we focus on those miRNAs that have shown consistent pattern across multiple studies and have confirmed targets in the PI3K/AKT pathway. It should however be kept in mind that each miRNA may target tens of different transcripts. Therefore, trying to understand their functional relevance in the context of only one or two substrates albeit useful may be an oversimplification of their cellular role.

Mir-1, which is downregulated in colorectal cancer, directly targets the MET growth factor receptor in the HT29 colon cancer cell line leading to decreased phospho-AKT levels [197]. MET and mir-1 is coordinately regulated through a feedback loop [198] and the expression of mir-1 is negatively regulated by insulin signaling [199]. Mir-125 is encoded thrice in our genomes; mir-125a once and mir-125b twice. The mature forms of the two variants differ by a single base and are believed to target similar transcripts. ERBB2 and ERBB3 receptor tyrosine kinases are both targeted by mir-125a and 125b in breast cancer cell lines [200] and their expressions are negatively associated in colorectal cancer cells [201]. In addition, mir-125b has been shown to target IGF2 in skeletal muscle [202]. Considering the prominence of IGF2 deregulation in colorectal cancer, this association may be interesting for large bowel neoplasms as well.

The mir-497/mir-195 cluster is epigenetically downregulated in breast cancer and suppresses growth in model systems [203]. In colorectal cancer cells, mir-497 regulates IGF1R and ectopic expression of mir-497 leads to reduced IGF1R protein, phospho-AKT and cell survival [204]. The encoding locus is deleted in about 1.5% of colorectal cancers [5].

Both mir-126 and mir-145 target the signaling adapter IRS1 in colon cancer cells and their overexpression leads to growth arrest [205,206]. IRS1 has also been shown to be targeted by two other colorectal cancer upregulated miRNAs, mir-96 and mir-183, respectively [207]. In addition to IRS1, mir-126 also binds PIK3R2 transcripts, encoding the regulatory subunit of the PIK3CA complex [208]. Importantly, ectopic mir-126 expression in colon cancer cell lines was reported to lead to a substantial reduction in phosphorylated-AKT levels.

Mir-21 is an established oncomir implied in numerous human malignancies. Overexpression has been shown to lead to increased tumor cell proliferation and migration through downregulation of PTEN protein levels, and their expression levels have been reported to be negatively correlated in primary colorectal tumors [209]. The *PTEN* pseudogene, *PTENP1*, which is transcribed, but not translated, retains miRNA binding and could thus act as a mir-21 sponge [210]. Therefore, either a decrease in PTENP1 levels or upregulation of mir-21 could phenocopy mutational inactivation of PTEN. Copy-number loss of *PTENP1* was reported in a fraction of colorectal cancer samples investigated [210]. While a recent study found mir-21 tumor expression to be exclusively localized to the stromal fibroblasts and not the cancerous cells [211], colorectal cancer cell lines express large amounts of mir-21 as measured by RT-PCR and small RNA sequencing (unpublished data). Relevant in this context, mir-21 has also been shown to target SPRY2, a negative regulator of KRAS activation and SPRY2 upregulation has been associated with poor prognosis [218–220].

The mir-17/92 cluster on 13q31.3 encodes six miRNAs; 17, 18a, 19a, 20a, 19b-1 and 92a-1 and is amplified in several hematological and solid cancers (reviewed in [212]). In colorectal cancer, overexpression is associated with 13q amplification and MYC upregulation [213]. Of the six encoded miRNAs, mir-19a and mir-19b have been consistently shown to repress PTEN and induce PI3K/AKT signaling [214,215]. Though mir-19 has been reported as the chief oncogenic 17/92 component in B-cell lymphoma [214], the cluster regulates a large number of additional transcripts, including TGFβ and WNT pathway components [216,217].

While mir-19 and mir-21 directly target the PTEN transcript, mir-183 inhibits PTEN indirectly through the EGR1 transcription factor [221]. The encoding mir-183-96-182 cluster is upregulated in colorectal cancer and all three miRNAs have been reported to have oncogenic properties [196,207]. As mentioned above, mir-183 and mir-96 in addition target IRS1 and could thus act in concert to modulate PI3K/AKT signaling. Moreover, GSK3β downstream of AKT is, through WNT signaling, an inhibitor of the mir-183-96-182 cluster and could thus form a feedback loop between these circuits [222,223].

Mir-143 and mir-145 are reported to be downregulated in many cancers including those of the colon and rectum, but emerging evidence suggests that this difference is due to different proportions of mesenchymal cells in cancers and control samples [224]. Expression profiling is usually done on mixtures of different cell populations and Chivukula and colleagues showed that mesenchymal, but not epithelial cells expressed mir-145/143. Earlier functional studies have shown that mir-143 targets KRAS and knockdown, and inhibition of this miRNA stimulates colon cancer cell growth [225]. Moreover, KRAS activation represses mir-143/145 expression and this feed-forward loop has been shown to be necessary for KRAS mediated cell line transformation [226,227]. The functional importance of mir-145/143 loss in colorectal cancers needs reevaluation, taking the cell-type specific expression into account [228].

Mir-520 and 525a target PIK3CA through 3′UTR binding [229]. In this study, in a small series of 17 tumors, 2 were found to carry mutations in the PIK3CA-3′UTR/mir-520/525 binding site and this was associated with an increase in PIK3CA/p110 protein expression. While this needs to be verified in a larger cohort, it illustrates how miRNA regulation may be disrupted through binding site inactivation. For *KRAS*, a SNP in the let-7 3′UTR binding site has indeed been associated with prognosis [230]. The importance of this mechanism for carcinogenesis will be exposed as more sequencing experiments include untranslated regions (UTR) and not just the exons.

Although a large number of in-vitro studies have shown the potential of miRNA deregulation driving PI3K/AKT activation, there is still a need to integrate it with the other molecular levels to conclude whether they represents physiologically important alternate mechanisms and drivers in colorectal cancers or are only markers of the transcriptional deregulation characterizing cancerous cells. To get an impression on to what extent miRNAs could contribute to PI3K/AKT deregulation in colorectal cancer we used TCGA data and compared the miRNA expression in samples with known PI3K/AKT alteration (*PI3KCA*, *PIK3R1* or *PTEN* mutation, *PTEN* copy number loss and/or IRS2, IGF2 overexpression) to those without (Fig. 4). Besides mir-483, which is encoded within IGF2, the difference was modest, but in light of the literature discussed above, mir-21 showed an interesting expression pattern across the two groups. Admittedly, this approach is crude, but as more data becomes available, similar analysis may reveal which miRNAs that are playing significant causal roles in colorectal carcinogenesis.

MicroRNA deregulation is firmly established in a wide variety of cancers and countless functional studies have demonstrated how many of these frequently observed deregulated miRNAs target both critical tumor suppressors and potent oncogenes (reviewed in [231]). Despite this, there are surprisingly few examples of this class of non-coding RNAs playing pivotal role in cancer pathogenesis. A possible explanation to this apparent discrepancy, may be that miRNA dysregulation instead of being linked to a few target genes, are contributing to malignancy through a general loss in signaling robustness [232]. There are several indications that could substantiate this notion. First, the primary role

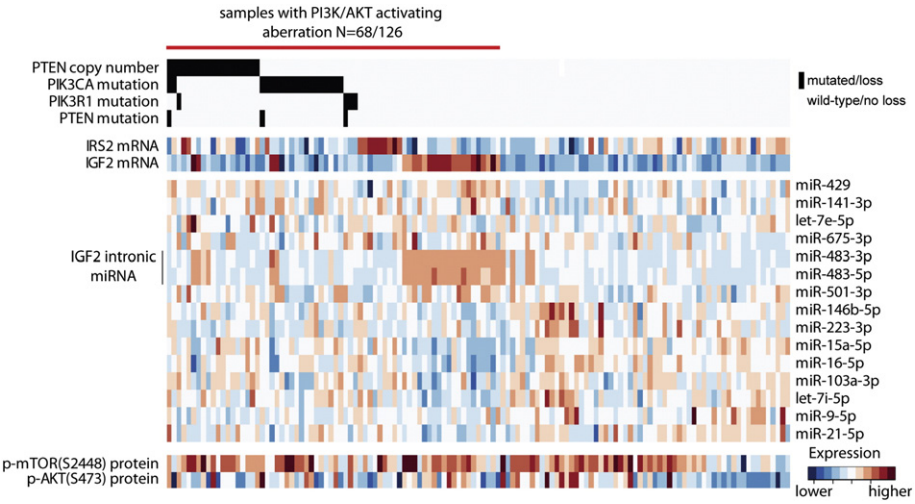

Fig. 4. MicroRNA expression and PI3K/AKT alterations in colorectal cancer. Heatmap showing row centered expression values for the 15 miRNAs with largest difference between samples with *PTEN* copy number loss, *PIK3CA*/*PIK3R1*/*PTEN* mutation or IRS2/IGF2 overexpression and those without. Primary tumor data on 126 colorectal cancer samples with available GISTIC DNA copy number, DNA/RNA/miRNA sequencing and reverse phase protein array data (RPPA) generated by the TCGA Research Network was included [5].

of miRNAs seems to be canalization and noise filtering [233,234]. In fact, in flies and mice targeted deletion of single or entire families of miRNAs give in many cases no observable phenotypic effect [235,236]. Second, miRNAs have in general a very modest (less than two-fold) effect on the expression of its target genes and by far the most important factor determining protein abundance is mRNA expression and protein stability [141,237]. In cancer, driving events typically lead to consecutive signaling through mutational hyperactivation (e.g. *KRAS*/*PIK3CA*) or large increases in growth factor or receptors (e.g. TCGA reports up to as much as 100-fold increase in growth factor levels in IGF2 driven tumors; [5]). Third, miRNAs are globally downregulated in cancer [238]. It has been shown that miRNAs have more oncogenic than tumor suppressive targets and a general loss of miRNAs could thus lead to excessive pro-proliferative and insufficient anti-apoptotic signaling. In fact, the non-redundant miRNA processing enzyme DICER1 functions as a haploinsufficient tumor suppressor [239]. In colorectal cancer, two other miRNA processing enzymes, TARPB2 and EXPO5 have been reported lost in MSI tumors with a concomitant reduction of miRNA expression [240,241]. Taken together this suggest that the loss and deregulation of miRNAs contribute to the disruption of key signaling networks, but more work is needed to determine whether they present driving events in colorectal cancer pathogenesis.

4. Clinical implications of PI3K/AKT alterations in CRC

In general, few biomarkers are currently valid for prognostic or predictive purposes in the setting of colorectal cancer [9–11]. The most commonly used predictive marker is *KRAS* mutation status; individuals with *KRAS* mutations in their tumor do not respond to anti-EGFR therapy [97]. Numerous studies have been set up in order to uncover similar relations for additional activating mutations in the PI3K/AKT pathway (see "Predictive biomarkers" section below) that seem to circumvent the effect of drugs targeting membrane proteins. As this has been proven to be a network of very complex nature, foreseeing mechanisms for primary and acquired resistance is challenging.

4.1. Prognostic biomarkers

The prognosis of colorectal cancer patients depends on several clinico-pathological factors such as disease stage, whether resection without residual disease can be carried out, tumor/bowel perforation, emergency operation, tumor differentiation, extramural venous invasion, few examined lymph nodes, lymph node ratio (stage III), distance to circumferential resection margin, age, co-morbidity and some others. Some of these factors are implemented in clinical guidelines as indicators for (neo)adjuvant treatment. However, prognostication based on clinico-pathological factors is not as precise as desirable, and much effort is being spent on developing improved molecular diagnostic tools, i.e. biomarkers with high sensitivity and specificity that will stratify the patients into high and low risk groups for relapse and hence guide treatment choices. Although many candidate genes have been launched, they have typically not been sufficiently validated for clinical use. The prognostic value of the main components of the PI3K/AKT signaling pathway has also been comprehensively studied. However, the outcome of these studies have been ambiguous, partly due to the use of different end-points, various technology platforms, and/or tested in relatively small patient series. The approach from biomarker discovery through validation studies towards clinical utility is demanding, and not all studies follow key reporting guidelines such as e.g. the Reporting of tumor MARKer Studies (REMARK) for presentation of accurate, complete and transparent prognostic data sets [242,243].

Here, we will shortly present some of the studies investigating the prognostic value of PI3K/AKT pathway components for CRC. As described earlier, EGFR is altered by various means in CRC (i.e. mutation, amplification, protein overexpression), but each type of alteration occur at low frequencies. The lack of standardized detection methods and scoring systems has made it difficult to establish the prognostic significance of EGFR. Further, numerous studies have evaluated the prognostic effect of *KRAS* mutation status; some of these have suggested the alterations to confer a negative prognostic impact [244–247], but other studies have shown contradictory results [248–253]. Thus, the potential of specific *KRAS* alterations as prognostic biomarkers in CRC is still not settled. Interestingly, an increasing number of studies are reporting on a prognostic value of *KRAS* mutations for smaller subgroups of patients, possibly explaining the inconsistency in the literature. In 2001, Andreyev and co-workers reported that only *KRAS* codon 12 mutations (G12V specifically) were significantly associated with poorer outcome for stage III patients, but not for those with stage II disease. Furthermore, codon 13 mutations did not have an impact on survival [254]. These findings were supported by two additional studies in 2009 and 2012 [255,256]. In contrast, Wangefjord and colleagues found that KRAS codon 13 mutations were associated with significantly reduced cancer specific survival in women, but not in men, although only in unadjusted analyses [257]. We recently confirmed these results on overall survival for women with stage III disease in a large population representative series of CRC (Merok et al., submitted

manuscript). Also for advanced CRC the question of prognostic value of *KRAS* mutations is controversial; hence no unambiguous conclusion has been reached on this subject.

Although BRAF is acting concurrently with the PI3K/AKT pathway, it should be noted that emerging evidence supports a role for *BRAF* mutations as a biomarker for prediction of poor prognosis for patients with MSS tumors [82,258], whereas for *BRAF*-mutated MSI tumors, the mismatch repair deficiency seems to take precedence over *BRAF* mutations when assessing outcome [109]. This result is supported by our own data in the study mentioned above (Merok et al., submitted manuscript).

Whether *PIK3CA* tumor mutations confer poor prognosis for the patients remains uncertain as they account for a rather small subgroup and often co-occur with *KRAS* or *BRAF* mutations. There are several studies arguing for and against a prognostic value. However, since the size of the patient cohorts differ and various end points are measured, the conclusion is likely to be the same as for *KRAS* mutations, that it will come down to better stratification and smaller subgroups. A recent study reported that *PIK3CA* mutations were associated with adverse outcome for patients with *BRAF* wild type tumors [259]. Exon 20 mutations have been suggested to be a negative prognostic factor in patients with stage III colon cancer [260], and to display a negative effect on progression-free and overall survival in patients with *KRAS* wild type chemorefractory metastatic CRC treated with cetuximab [98]. The latter observation was recently confirmed in a meta-analysis by Huang et al. [261]. Moreover, another study found that patients with concomitant exon 9 and exon 20 mutations had worse prognosis than did the patients with *PIK3CA* wild type or with *PIK3CA* mutation in either of the exons [262]. Notably, this applies to less than 5% of the patients [263]. In contrast, some recent reports have suggested that *PIK3CA* mutations are associated with longer survival and reduced rate of recurrence for patients that use aspirin regularly after diagnosis [264,265]. Although very interesting, conclusive data await documentation from larger patient series.

PTEN mutations or loss of PTEN expression often co-occur with alterations of other genes in the PI3K/AKT pathway, indicating that changes in this gene per se will moderately influence prognosis. Due to relatively low mutation rate and lack of standardized methods for detection and scoring of protein expression in addition to relatively small patient series, the value of PTEN as a prognostic marker remains unsettled. The clinical impact of PTEN alterations on CRC patient outcome was summarized in a recent review [266]. Also protein expression and activation of the downstream components phospho-AKT, S6RP and phospho-4E-BP1 have been found to be prognostic factors for disease-free survival of stage II CRC patients [267].

Some studies have also investigated the collective prognostic effect of several of the genes in the PI3K/AKT pathway. In one report CRC patients with altered *KRAS*, *BRAF*, and/or *PIK3CA* ($n = 316$) had reduced 3-year survival compared to patients with wild type tumors ($n = 244$) [268]. This was also confirmed in multivariate analysis adjusting for stage, MSI, location, age and gender. Eklof and colleagues collected information on *KRAS*, *BRAF*, *PIK3CA*, and *PTEN*, which was combined to a quadruple index used to assess the prognostic value of these markers in two patient cohorts [269]. In line with Barault et al., they found that patients with quadruple index-positive tumors had an impaired prognosis in univariate analysis in one of the cohorts ($n = 197$), but not in the second series ($n = 414$). However, when analyzing the genes separately, only *BRAF* and *KRAS* had prognostic value in each of the series, respectively, whereas neither *PIK3CA* nor *PTEN* added any significant prognostic information. Neumann et al. investigated *AKT* and EGFR expression in addition to mutation in the four abovementioned genes, and found that approximately 75% of the tumors had alterations in one or more of these components, and that this was associated with advanced disease [270]. Finally, in contrast to the studies arguing for a prognostic value of one or more of the genes in the PI3K/AKT pathway, Mouradov and colleagues reported that stage II and III CRC patient disease-free survival ($n = 822$) is independently predicted by CIN and MSI, rather than by mutations in *KRAS*, *BRAF*, *PIK3CA*, loss of 18q, and other individual genomic alterations [271]. In conclusion, the development of robust prognostic biomarker panels for stage II and III patients is ongoing, and whether such panels will include components of the PI3K/AKT pathway remains to be seen.

4.2. Predictive biomarkers

A paradigm shift in the era of targeted therapy of CRC was introduced when it became confirmed that CRC patients with distant metastasis and *KRAS* mutated primary tumors do not benefit from EGFR-directed monoclonal antibodies (cetuximab and panitumumab) [97,165,272]. Testing for these mutations is now mandatory before considering EGFR-targeted therapy. In the wake of this, the search for additional predictive markers has been highly prioritized as the summarized response rates for *KRAS* wild type patients are reported to be 26–41% and 11–17% for cetuximab and panitumumab, respectively [101]. In a recent study where the mutation status of additional RAS codons were assessed, it was reported that as much as 17% of patients with *KRAS* exon 2 wild type tumors possessed mutations elsewhere in *KRAS* or *NRAS* [107]. These mutations also conferred resistance to anti-EGFR therapy and, moreover, the refined wild type population displayed increased benefits from treatment than did the original wild type population. Besides this, it has so far been difficult to obtain consistent results for other markers in the EGFR signaling cascade. EGFR copy number alteration and overexpression, *BRAF* and *PIK3CA* mutations, as well as PTEN expression levels are putative predictive markers that are being thoroughly investigated [165,273,274]. It has also been reported that the expression levels of two EGFR ligands, epiregulin (EREG) and amphiregulin (AREG), might predict clinical benefit of cetuximab for patients with metastatic colorectal cancer. The ligands activate the PI3K/AKT pathway directly by binding to the extracellular domain of the EGF receptor, thus the level of sensitivity towards targeting therapy seems to be proportional to the expression levels of the ligands [76,275–278]. Even though the results attained so far look promising, there are currently no standardized protocols for measuring EREG/AREG mRNA and protein levels, thus further studies are required.

In terms of prediction of therapy response, the genes in the PI3K/AKT signaling pathway have been used one by one. Instead, it will probably be beneficial to collect the mutation profile of the whole pathway upfront, as this seems to improve the outcome [107]. Such an extended profile will first of all avoid treating patients that will not benefit, but rather, experience harmful effects of the therapy, and secondly, allow patient stratification into clinical trials with appropriate therapeutic strategies. This is indeed feasible with the advances in technology seen in recent years. Moreover, in parallel with treatment, it will be necessary to monitor the status of the pathway genes to detect acquired changes leading to resistance. Thus, a number of genomically driven clinical trials are selecting patients with advanced CRC based on *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* status. For an update on research trends, therapeutic strategies, and pathway inhibitors in clinical development see [279] for cancer in general and [280] for CRC specifically. However, the increase in agents developed for targeting the PI3K/AKT pathway, underscores its importance in cancer. And as many of the compounds in combination with standard chemotherapy and/or other specific drugs show promising results in clinical trials, there is rising optimism that we will see improved patient outcomes within reasonable course of time.

5. Concluding remarks

In colorectal cancers the most frequent PI3K/AKT pathway alterations are disruptions of *KRAS*, *PIK3CA* and *PTEN* and overexpression of *IGF2*. In addition to these, we have here discussed approximately 40 other network members which are less frequently altered, but could nevertheless contribute to hyperactivation of the pathway in a subset

of colorectal cancers. However, in a wider sense, databases include close to 350 PI3K/AKT pathway genes [281], raising the question as to what extent alterations in these additional components may contribute to pathway deregulation. The recent advent of large multi-molecular-level datasets is increasing our ability to obtain that answer, both by integrating information across molecular levels and by pooling across cancers of different tissues [282,283]. Increasing amounts of data will in the coming years provide the statistical power needed to identify additional real, but rare, driving events in a noisy background. The necessary cohort sizes are however also rapidly rising with the ever increasing resolution. As an example, gene expression is being superseded by exon-level analysis and single gene screening has already been replaced by exon sequencing. For mutations, the arguably best understood of the molecular levels, it has been estimated that about 2000 colorectal cancers/normal pairs has to be sequenced in order to identify drivers present in 1% of tumors [57]. The number required for detangling low-frequency drivers at the transcriptomic and proteomic levels is likely much higher.

For groups involved in clinical problems, TCGA and other similar consortia provide invaluable data for creating and testing hypothesis. Translational research does however require access to long-time high-quality patient follow-up and biobanks for technical validation and technology transfer. In addition, close integration of researchers and clinicians is critical when aiming to transform this deluge of data into improved patient healthcare.

Considering the enormous effort put into developing drugs specifically modulating the PI3K/AKT network, understanding the alternative (and peripheral) routes will be of utmost importance both to establish how resistance evolves, and also to understand how to target tumors in those patients without the most common driving events. Much has been learnt from other cancer types and diseases, but it should be emphasized that the signal circuitry is wired differently among tissue types, and caution must be taken. The most illustrative example may be how the $BRAF^{V600E}$ inhibitors (e.g. vemurafenib), which is highly effective in melanoma, fails to shut down MAPK signaling in colon cancer probably due to increased EGFR-mediated signaling, also including induction of the PI3K/AKT pathway [284,285]. Hence, combining $BRAF^{V600E}$ inhibitors with EGFR antibodies to counteract the overactivation of the EGFR caused by inhibition of BRAF has been suggested, and this concept is currently tested in phase I/II trials ([286], http://clinicaltrials.gov/show/NCT01719380). Another recent example is how colon cancer cells with *KRAS* mutations are able to escape KRAS inhibition through resorting to YAP1 activation as recently demonstrated [287].

The fundamental clinical challenge to all oncologic interventions is that selective pressure results in evolution of resistance. Compared to most other tumor types, colorectal cancer is characterized by a large degree of genomic instability and thus heterogeneity. From a Darwinian viewpoint, this makes the outlook of curative treatment dimmer [288]. However, by the same reasoning, the surgically imposed bottleneck gives a window of opportunity to eradicate reminiscent cells when the variation which selection can act upon is limited. Currently, targeted treatment is given mainly to patients with late-stage disease, but from an evolutionary standpoint, success is much more likely prior to or right after primary surgical resection where proper (neo-)/adjuvant therapy might lead to complete recovery. As an additional important benefit, the patients are in better condition and are thus more able to cope with the disabling side-effects commonly seen with targeted therapy. Finally, a shorter interval of an optimized aggressive neoadjuvant treatment protocol in early stages may increase the number of patients being cured.

Here we have provided a summary of the various alterations reported for the PI3K/AKT pathway in colorectal cancer. Considering the complexity and interconnectedness of cellular signaling networks, detangling the role of each component in normal physiology and malignancy will present formidable challenges to the research community. However, it will in the end be essential in order to provide better tools for stratification of patients into smaller subgroups to which specific targeted therapy may be administered, and will give invaluable clues as to how components in the network can be targeted by different strategies.

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