Abstract. Novel activating mutations in sporadic colorectal cancer (CRC) have recently been identified on major kinase encoding genes such as BRAF and PI3KCA. The presence of these activating point mutations, including the well characterized KRAS oncogene mutations, represent up to 75% of cases in CRC. These genes, that have been implicated in the adenoma-carcinoma transition, cause deregulation and constitutive activation of the MAP AKT/kinase pathways, rendering growth advantages to colon tumor cells. This review focuses on the key genetic alterations underlying the cumulative effect of multiple mutations within the colon cancer cell. Moreover, the currently available and alternative treatment approaches that may target these different genetic alterations are discussed, such as the novel BRAF inhibitor. Identification of novel mutations as well as differential gene expression analyzed by microarray reveal potential targets for combined therapeutic protocols which will result in personalized treatments in the near future.

Most sporadic colorectal cancers (CRCs) are thought to develop from benign adenomas. Identification of the genetic abnormalities that accumulate in a step-wise manner has led to the well-known model of the adenoma-carcinoma sequence initially characterized by Fearon and Vogelstein (1). The proposed model of colorectal carcinogenesis correlates specific genetic events with evolving tissue morphology. Progression from normal epithelium through adenoma to colorectal carcinoma is characterized by accumulated abnormalities of particular genes that ultimately will invade into the surrounding tissue and metastasize. Mutations in mismatch-repair genes cause microsatellite instability and the successive mutation of target cancer genes, which can occur at any point in the adenoma-carcinoma sequence. Each mutation alters the behavioral responses of the cells giving some growth advantage. The earliest genetic change is mutation and/or loss of the adenomatous polyposis coli (APC) tumor suppressor gene. KRAS mutations are found in 50% of CRCs and are thought to be relatively early events that correlate histologically with early to late adenoma. On the contrary, p53 mutations occur in 70% of sporadic CRCs and most probably occur before metastasis. Disruption of the TGF-βII/SMAD4 pathway and mutations in cyclin-dependent kinase inhibitors (CDKN2A) have all been identified as key factors in the development and progression of CRC (2).

Recent developments in collaborative studies and, most importantly, the completion of the human genome sequencing project have allowed for an accelerated effort to identify novel activating mutations. A combinatorial therapeutic approach targeting these different mutations may result in a better treatment alternative. In sporadic CRC, the KRAS-BRAF-MAPK kinase pathway is frequently mutated where KRAS mutations are inversely associated to BRAF mutations, with the latter being the most frequent in human cancer (3). On the contrary, the newly characterized PI3KCA mutations in CRC have been found to occur more frequently in association with KRAS or BRAF mutations than in isolation, suggesting a possible synergistic effect of signaling pathways controlled by these genes in CRC development/progression (4). RAS proteins are located on the inner surface of the plasma membrane and are attached to the membrane by a farnesyl residue. RAS proteins transmit extracellular signals.
that promote the growth, proliferation, differentiation and survival of cells. The signaling cascade starts from the plasma membrane where the growth factor (e.g., epidermal growth factor) binds to its enzyme-linked receptor causing receptor dimerization. The major downstream target of RAS-GTP is mitogen-activated protein kinases (MAPKs), but it is also known to activate other targets, e.g., phosphatidylinositol 3-kinase (PI3K), and phospholipase C epsilon (PLCe) (5, 6).

Activation of MAPK occurs through specific phosphorylation of both a threonine and a tyrosine separated by a single amino acid. The first component of the MAPK cascade is called RAF, which is activated on the plasma membrane by RAS-GTP. RAF phosphorylates mitogen-activated kinase kinase 1/2 (MEK1/2 kinase), which activates the extracellular regulated kinase 1/2 (ERK1/2 kinase or p44/42 MAPK) by phosphorylation. ERK1/2 phosphorylate a variety of downstream targets, which results in changes in several key growth factors; the catalytic activities of enzymes and proto-oncogenes that transduce signals promote growth and differentiation through this cascade (Figure 1).

Activated Kinase Pathways in Colorectal Cancer

There is growing evidence that activation of the RAF and PI3K pathways are involved in the pathogenesis, progression and oncogenic behavior of human CRC. In about 30% of all cancers these pathways are deregulated due to several proto-oncogene alterations, most notably the KRAS proto-oncogene. RAS mutations are an early event in the development of CRC (Dukes’ stage B and C) and result in a permanently active GTP-bound form of RAS. The high RAS activity is accompanied by raised ERK activity (7). During the process of oncogenic transformation, CRC cells escape from normal growth and differentiation control and acquire the ability to invade surrounding tissues and organs. The role of the resulting increased signaling through the RAF and PI3K pathways in CRC is closer than ever to being elucidated by the continuous research of the past few years.

The most important findings include the recently identified mutations on the PIK3CA gene encoding the p110α catalytic subunit that has been found to be mutated in over 25% of CRCs. The majority of the mutations cluster near to positions within the PI3K helical or catalytic domains and were described to confer increased kinase activity. PIK3CA abnormalities seem to occur at relatively late stages of neoplasia, near the time that tumors begin to invade and metastasize (8). Mutations of BRAF, a serine-threonine kinase of the RAF family, have also been associated with increased kinase activity. BRAF mutations have been found in 15% of CRC and many of those with such mutations are at early Dukes’ stage (A and B) (Figure 1).

Mutant PI3KCA in CRC

Physiological function. There are 3 different classes of PI3Ks; class I (subclass A and B), II and III. Class IA PI3Ks are composed of a 110 kDa catalytic subunit and a 85 kDa regulatory subunit and are activated by the binding of RAS to the p110α catalytic subunit or by receptor tyrosine kinases (RTKs) (9). The p110 catalytic subunit contains the RAS binding domain (RBD), to which RAS-GTP binds. The class I PI3Ks are the second best-characterized RAS effectors (5). Active PI3Ks phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2) at the 3'-OH position of the inositol ring, converting it to phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 creates secondary messengers that serve to recruit pleckstrin-homology (PH) domain-containing proteins, such as the AKT serine/threonine kinase, and the 3-phosphoinositide-dependent protein kinase-1 (PKD1). Once at the membrane, AKT is activated by PDK1 through phosphorylation of critical residues (Thr308 and Ser473) (10). AKT, in turn, phosphorylates numerous proteins, including the cell cycle regulator glycogen synthase kinase 3 (GSK3). GSK3 regulates C-MYC and Cyclin D1 levels by phosphorylation and subsequent proteasomal degradation (9). The PI3K-AKT pathway plays an important role in cell survival. AKT utilizes phosphorylation, not only to inactivate pro-apoptotic proteins such as BAD (Bcl-2 inactivation) (11) and caspase-9 (12), but also to inactivate members of the FOXO subfamily of forkhead-related transcription (FKHR). These transcription factors induce expression of pro-apoptotic proteins such as the FAS Ligand (FASL) (13) and BIM (14). There is evidence that AKT promotes cell survival by indirect activation of NFκB, a transcription factor that induces expression of various anti-apoptotic genes (15). AKT activity is negatively regulated by PTEN dephosphorylation (9, 16). PTEN, on the other hand, may suppress tumor cell growth by antagonizing protein tyrosine kinases and may regulate tumor cell invasion and metastasis through interactions at focal adhesions (17) (Figure 1).

Role of the PI3K pathway in cancer. The PI3K-AKT pathway is believed to play a central role in tumorigenesis since it is found to be frequently activated and deregulated in the carcinogenic process of various human cancers. Although genetic changes such as activating mutations along the PI3K-AKT pathway have been repeatedly documented, reports on the actual mechanism of activation and deregulation of the pathway are limited. Moreover, the AKT1 and AKT2 genes are amplified in various cancers (18). Deregulation may alternatively occur as a result of mutations in RAS (constantly active) or in some other components of the pathway that can interact with the
catalytic subunit p110 of PI3K and lead to its activation (19). As oncogenic \( RAS \) interacts with and activates PI3K (5), the activation of the PI3K signaling pathway via \( KRAS \) or \( PI3KCA \) mutations was considered to be one of the most common mechanisms involved in colorectal carcinogenesis. The PI3K pathway is reportedly more frequently activated in colon cancer cells (20). More than 80% of the reported \( PI3KCA \) mutations cluster in small regions within the helical (exon 9) and kinase (exon 20) domains, with E545K (helical domain) and H1047R (kinase domain) constituting two mutational hot-spots. The significant frequency of these mutations resembles the mutations found in \( KRAS \) and \( BRAF \) in colon cancer and is consistent with the notion that these mutations are activating. Functional studies have shown that E545K and H1047R mutant \( PI3K \) displayed increased lipid kinase activity in vitro (8). Finally, recent biochemical analysis by Samuels et al. (8) in colon cancer cells showed that mutant \( PI3KCA \) selectively regulated the phosphorylation of AKT and the forkhead transcription factors FKHR and FKHR-Ll. What actually happens is that \( PI3KCA \) mutations, under specific circumstances, confer resistance to apoptosis and facilitate tumor invasion. Although \( PI3KCA \) mutations have little effect on growth under standard conditions, they do reduce cellular dependence on growth factors.

Similarly the tumor suppressor PTEN that antagonizes PI3K activity is also mutated (17). Heterozygous PTEN knockout mice show increased tumor development (21), and overexpression of the \( PI3KCA \) as well as \( AKT \) genes has been shown to be able to transform chicken embryo fibroblasts (22). AKT, that has been reported to be activated downstream of mutant \( PI3KCA \), causes resistance to apoptosis in CRC (23). The difficulty of causing apoptosis-induced cell death in cancer where the PI3K pathway is activated by somatic mutations is becoming evident (8).

**Treatment approaches.** Treatment with the broadly effective PI3K inhibitor LY294002 revoked \( PI3KCA \) signaling and caused selective growth inhibition of \( PI3KCA \) mutant colon cancer cells HCT116 (H1047R, exon-20) and DLD1 (E545E, exon-9) (8). LY294002 is a potent PI3K inhibitor that aborts the activation of \( p53 \) by several drugs commonly used in cancer chemotherapy and attenuates \( p53 \)-dependent, chemotherapy-induced apoptosis of cancer cells. The decrease on PI3K signaling and sensitivity to apoptosis is brought about through growth suppression and decreased expression of AKT (Ser473) in cancer cells (24) (Figure 1). In addition, an unexpected positive role for PI3K on \( p53 \) activation by anticancer agents has been demonstrated, which suggests that the efficacy of PI3K inhibitors in cancer therapy may be greatly affected by the tumor \( p53 \) status (25). These data indicate that PI3K signaling is still a necessary process for progression and cell proliferation in cancer cells. Of interest is the finding that PTEN deficiency renders increased sensitivity to the PI3K pathway inhibitors and LY294002 was shown to actually inhibit ovarian tumor development with an activated PI3K pathway (26). On the other hand, the activation of the PI3K signaling pathway via \( KRAS \) oncogenic interactions or \( PI3KCA \) mutations has been closely associated with colorectal carcinogenesis. Bearing in mind that p110\( \alpha \) is a protein encoded by the \( PI3KCA \) gene, a potential novel target for CRC therapy may be developed. Combined treatment approaches are the trend nowadays. The development of inhibitors that would specifically target more that one major mutant forms, either on the same gene or different genes, would aid the development of treatments more applicable to a large number of patients.

**Mutant BRAF in CRC**

**Physiological function.** The best characterized RAS effectors are the serine/threonine RAF kinases. There are 3 different RAF kinases; ARAF, BRAF and CRAF (RAF-1). The RAF proteins are structurally related and share 3 conserved regions (CR1, CR2 and CR3). The CR1 domain is located on the N-terminal and contains the RAS binding domain (RBD) as well as a cysteine-rich domain (CRD), which also functions to bind RAS. The CR3 regions located on the C-terminal contain the kinase domain. Inactive RAF resides in the cytoplasm, but is recruited to the cell membrane upon binding to RAS-GTP, where it is activated through a number of phosphorylation events (Figure 1). Two critical phosphorylation sites (residues Thr598 and Ser601) are located within the activation segment of the kinase domain. Their replacement by alanines, has been shown to inhibit RAS-induced activation of BRAF. In contrast, mutagenic substitution of these 2 residues by acidic amino acids renders BRAF constitutively active and this mutant has been shown to induce PC12 cell differentiation (27) and to transform NIH3T3 fibroblasts, although less efficiently than mutant RAS (3). As compared to other RAF isoforms, BRAF requires less phosphorylation for activation because of its higher basal kinase activity, which might explain why BRAF is mutated in human cancers (28). Both BRAF and CRAF share highly related catalytic domains and downstream substrate-inhibitors that effectively block the activity of both proteins and could hold great promise as broad spectrum treatments for cancer. Thus, CRAF is considered to be an important target for the development of anticancer drugs.

**Role of the BRAF pathway in cancer.** In 2002 (3), a genome-wide screen for oncogenes showed the \( BRAF \) gene to be mutated in a variety of different types of cancer, including
colon cancers. Most BRAF mutations cluster within the activation segment of the kinase domain, with a single T179A transversion accounting for approximately 90% of the mutations. This mutation leads to a glutamic acid substitution at codon 599 (V599E). (Note: in 2003, an error in the NCBI gene sequence of BRAF was identified. The correct numbering of the mutated codon should be 600. To avoid confusion with published data, the old numbering system is used in this review). It is believed that the insertion of an acidic residue at position 599 renders a negative charge to the amino acid which mimics activation loop phosphorylation of Thr598/Ser601 in wild-type BRAF (3). In addition, substitution of V599 by positively-charged residues was also found in tumors. Wan et al. (29) managed
to determine the crystal structure of the wild-type BRAF and BRAF V599E kinase domains. To obtain crystals of suitable quality, it was necessary to co-crystallize the BRAF kinase domains, wild-type and mutant, in the presence of the small-molecule CRAF inhibitor, BAY43-9006 (30). The inactive captured conformation of BRAF shows that BRAF residues G595-V599 of the activation loop engage in hydrophobic interactions with residues G463-V470 of the P loop. In this conformation, the catalytic residues (T598 and S601) are not aligned for ATP and substrate binding. Oncogenic BRAF mutations, either in the P loop or activation loop, destabilize their interaction and disrupt the inactive conformation. Therefore, this crystal structure may also explain why the P loop and the activation loop are preferred mutational targets. Over 40 different BRAF mutations have been identified, half of which (including V599E) have been functionally analyzed and found to have elevated kinase activity. However, there are mutants that have decreased kinase activity compared to wild-type BRAF. Interestingly, the majority of these mutants can still activate ERK with the same intensity, via activation of CRAF (29). The mechanism by which this occurs is not known, although it has been shown that BRAF and CRAF do form heterodimers (31).

**Treatment approaches.** BAY43-9006, originally described as a CRAF inhibitor, has now been shown to target other kinases including BRAF (both wild-type and V599E mutant BRAF) (Figure 1). BAY43-9006 targets BRAF signaling *in vivo* and induces a substantial growth delay in melanoma tumor xenografts. However, BAY43-9006 provided only limited benefit in melanoma patients when used as a monotherapy (32). The reason for this lack of activity against melanoma is unknown to date, but BAY43-9006 has been shown to be relatively ineffective against V599E BRAF in cells and so it may not be potent enough to block oncogenic BRAF signaling in patients when used as a monotherapy. A combination of BAY43-9006 with novel conventional agents may prove successful in treating melanoma through cell priming and sensitization. A promising approach is to combine BRAF signaling inhibitors with apoptosis-enhancing agents (33). Wan and colleagues have shown that BRAF mutations, including the highly prevalent V599E mutant, promote cancer simply by increasing the catalytic activity of BRAF. These mutants activate ERK by activating another RAF family member, CRAF. Even though a number of BRAF mutants do not actually achieve significantly higher levels of catalytic kinase activity above the basal level (non-Ras-stimulated) wild-type BRAF, they still induce ERK hyper-phosphorylation. The RAF inhibitor BAY43-9006 was also extensively studied with success in the KRAS-dependent human colorectal cancer cell line HCT116 and combined with CPT-11 in DLD-1 cells. BAY43-9006 was also employed in clinical trials for CRC patients with considerable success (34).

In a recent study, where farnesyltransferase inhibitors (FTIs) were employed as an anticancer agent, they did not manage to influence either cell proliferation or the induction of apoptosis of BRAF mutant cells as on KRAS mutant cells. These results support the hypothesis that BRAF mutations activate BRAF independent of RAS via the RAS-RAF-MEK-ERK signaling pathway in CRC tumorigenesis. Moreover, overexpression of BRAF confers resistance against apoptosis induced by growth factor withdrawal or PI3K inhibition. In contrast, knockdown of BRAF expression and the inhibition of downstream signaling in WM793 human melanoma cells cause growth arrest and promote apoptosis. The identification of BRAF gene mutations may deregulate the apoptotic pathway and promote a novel CRC treatment targeting apoptosis (35).

**Therapies Targeted to Mutant KRAS in CRC**

There are 3 different mammalian RAS genes that encode 3 highly-related proteins, KRAS, NRAS and HRAS. The 3 RAS proteins are identical in their first 85 amino acid residues, which contain the effector domain (residues 32-40), *via* which RAS proteins interact which their effectors located on the N-terminal part. The most C-terminal part of RAS contains a CAAX motif which undergoes a number of post-transcriptional modifications. These modifications are required for RAS to anchor to the cell membrane. One such modification involves the enzyme farnesyltransferase, which catalyses the attachment of a farnesyl group to the cysteine residue of the CAAX motif (36). It has been shown that the different RAS proteins vary in their ability to activate different effector molecules. KRAS and NRAS have been shown to activate RAF more efficiently than HRAS, while HRAS has been demonstrated to activate PI3K more efficiently than KRAS and NRAS (37). There is increasing evidence that oncogenic RAS activates distinct effector pathways to induce transformation to different cell types. KRAS mutations are most common, followed by NRAS, whereas HRAS mutations are relatively rare. Many tumor types, like CRC, appear to be associated with KRAS mutations (38). Most RAS mutations are point mutations affecting residues 12, 13 and 61. These mutations render RAS insensitive to GAP-stimulated GTP hydrolysis (39). As a result, mutant RAS is locked in an active GTP-bound state.

Several therapeutic approaches to cancer have been developed pointing to reducing the altered KRAS gene product or to eliminating its biological function. The gene therapy approach has been employed using ribozymes, which are able to break down specific RNA sequences and inhibit gene expression aiming to prevent p21RAS protein synthesis.
Feng et al. (40) introduced, by means of adenovirus, an anti-RAS ribozyme into a human bladder cell line resulting in the reversion of the neoplastic phenotype. Another variant of gene therapy consists of utilizing antisense oligonucleotides, which are complementary to normal sense mRNA. These oligonucleotides bind to cellular mRNAs, preventing translation by ribosomes. In another experimental model, Cooper et al. (41) used liposome-associated antisense oligonucleotides that functioned as inhibitors of HRAS translation and reported a decrease in the growth of a tumor cell line growing in nude mice. Moreover, immunotherapy has also been used in treatment through passive or active immunization protocols of tumor cell growth. Adenovirus-mediated transfer of the single-chain Fv fraction of an anti-RAS antibody to colon carcinoma cells induced tumor regression in nude mice (42).

Even though gene therapy is one of the most revolutionary therapeutic approaches of the last decade and immunotherapy has found successful applications, much attention has been focussed on the inhibition of p21RAS farnesylation, either by inhibition of farnesyl transferase (FT) or synthesis inhibition of farnesyl. RAS proteins lose their ability to associate with the cell membrane and become active (Figure 1). In vivo, the enzyme FTIs have shown anti-proliferative activity, apoptosis induction and the blockade of morphological alterations associated with RAS transformation (43). Although very efficient in reducing HRAS-induced tumor growth in mice, FTIs have shown no effect in clinical trial I subjects with CRC (44). This is because KRAS and NRAS, but not HRAS, can be modified by an alternative mechanism when farnesylation is blocked.

On the other hand, a new therapeutic approach is the targeting of apoptotic molecules, such as the TNF-related-apoptosis inducing ligand (TRAIL), that are affected by these oncogenes. TRAIL itself has attracted most research efforts to date, and agonistic antibodies to the TRAIL-R1 receptor have been developed (45). A recent study (46) demonstrated that mutant RAS (RAS V12 and HRAS V12) can sensitize resistant cells (CaCo2) to TRAIL by the up-regulation of TRAL-R1 (DR4) and -R2 (DR5) receptors, partially through a MEK-dependent pathway. The combination of inhibitors with an immunotherapy approach like TRAIL may help overcome cell resistance to induced apoptosis and cytotoxicity. In addition, recent findings identified another novel mechanism by which MYC sensitizes cells to apoptosis-induced death (47).

Mutated Gene Signatures on Specific Cell Types

A great variety of genes and transcription factors are regulated by the RAS and, as a consequence, by the RAF/MEK/REK and/or PI3K/AKT pathways, depending mainly on the cell context. When core molecules found early on these pathways become mutated, downstream genes and transcription factors become deregulated. It is the outcome of a large microarray study that mutated KRAS (V12) regulated genes involved in cytokine signaling, cell adhesion and colon development. In contrast, HRAS (V12) mainly regulated genes involved in controlling cell morphology, correlating to an epithelial-mesenchymal transition only observed in these cells (48). In an effort to characterize sequences of genes that are targets of specific signaling pathways in growth factor stimulating human cells, transcription factor binding sites upstream of PI3K and the MEK/ERK signaling pathway were identified. It is the outcome of another study that groups of human genes regulated by discrete intracellular signaling pathways share common cis-regulatory elements (49). Similarly, using microarray gene expression as a tool, the BRAF mutation signature was revealed in melanomas harboring either BRAF or NRAS mutations. There seem to be gene-specific signals in addition to common MAPK activation that result from the diverse effects of BRAF and NRAS on other signaling pathways, giving rise to different transcriptional changes (50). In a different cancer model of thyroid cancer, samples were genotyped for their common activating mutations of BRAF RET/PTC1 and RAS (K-, H-and N-). This study led to the conclusion that the mutational status is the primary determinant of gene expression variation within these tumor models (51). Such findings may lead to clinical and diagnostic advances in an effort to predict success for therapies designed to prevent the consequences of these mutations.

Conclusion

Tumorigenesis is a multistep process and it generally takes many years for a cancer to develop. In humans, it is believed that for a normal cell to become malignant a number of genetic changes are required. These changes occur in a stepwise manner and include both activation of proto-oncogenes and inactivation of tumor suppressor genes. Gain-of-function mutations (activating mutations) of the KRAS, BRAF and PI3KCA genes drive a cell towards cancer through the MAPK pathways which affects the development of CRC. Kinases of the ERK MAPK pathway are potentially useful as targets for the treatment of CRC. Currently, many research groups are working on the design of new inhibitors and the precise role of the large numbers of MAPK involved in CRC is being studied and targeted. In the clinical development of therapies designed to block the function of several important signaling cellular proteins, apart from targeting RAF kinase, alternative target agents aim at other parts of the pathway, such as MEK, the apoptosis signaling pathways including NF-kB, Bcl-2 and the TRAIL receptor. Finally, the identification of novel
mutations, as well as differential gene expression analyzed by microarrays, reveal potential targets for combined therapeutic protocols which will result in personalized treatments in the near future.

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