Review Article

Clinical Relevance of KRAS in Human Cancers

Sylwia Jančík,[1] Jiří Drábek,[1] Danuta Radzioch,[2] and Marián Hajdúch[1]

[1] *Laboratory of Experimental Medicine, Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University and University Hospital, 775 20 Olomouc, Czech Republic*
[2] *Department of Experimental Medicine and Department of Human Genetics, McGill University, Montreal, Canada H3G 1A4*

Correspondence should be addressed to Marián Hajdúch, hajduchm@gmail.com

The *KRAS* gene (Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) is an oncogene that encodes a small GTPase transductor protein called KRAS. KRAS is involved in the regulation of cell division as a result of its ability to relay external signals to the cell nucleus. Activating mutations in the *KRAS* gene impair the ability of the KRAS protein to switch between active and inactive states, leading to cell transformation and increased resistance to chemotherapy and biological therapies targeting epidermal growth factor receptors. This review highlights some of the features of the *KRAS* gene and the KRAS protein and summarizes current knowledge of the mechanism of *KRAS* gene regulation. It also underlines the importance of activating mutations in the *KRAS* gene in relation to carcinogenesis and their importance as diagnostic biomarkers, providing clues regarding human cancer patients' prognosis and indicating potential therapeutic approaches.

1. Introduction

In 1982 Chang and Der, two postdoctoral fellows working in Geoffrey Cooper's laboratory, discovered Kristen Rat Sarcoma Virus and Murine Sarcoma Virus; retroviral oncogenes related to rodent sarcoma virus genes. The human *KRAS* gene is a homolog of these two oncogenes [1]. A normal form of human c-Ras has been called KRAS or KRAS2 (Kristen Rat Sarcoma Viral oncogene homolog or alternatively Kristen Murine Sarcoma Virus2 homolog). In 1983, Der described an abnormal form of the p21 protein expressed by colon and lung carcinoma cell lines and showed that the gene encoding this protein is able to transform NIH3T3 cells [2]. This finding was later confirmed by Parada and Weinberg [3], who described the transformation of NIH3T3 cells by an activated KRAS oncogene. Aberrant p21 proteins were encoded by the altered *KRAS* gene and their expression in carcinoma tissue was causally linked to an abnormal state of activation [2]. Since then, it has been accepted that KRAS is one of front-line sensors that initiate the activation of an array of signalling molecules allowing the transmission of transducting signals from the cell surface to the nucleus, thus affecting cell differentiation, growth, chemotaxis, and apoptosis. A signal transduction cascade initiated by the activated form of KRAS is depicted in Figure 1. As a result of these effects, KRAS elicits changes in the cytoskeleton and consequently affects cell shape, adhesion and migration [4, 5].

In the following paragraphs, KRAS protein, gene, oncogenesis, and cancer therapy is reviewed.

2. KRAS Protein

2.1. KRAS Protein Structure, Function, and Localization. KRAS belongs to a group of small GTP-binding proteins, known as the RAS superfamily or RAS-like GTPases. More than 150 RAS-like genes have been identified in mammalian genomes [6]. The entire RAS superfamily is characterised by the presence of a catalytic G domain, but includes members with distinct evolutionary specializations with respect to different cellular process [7]. The RAS subfamily (RAS, RHO, RAB, ARF, RAC, and RAN) includes the most frequently studied proteins, such as Harvey-Ras (H-RAS), neuroblastoma-Ras (N-RAS), and two variants of Kristen-RAS (K-RAS)—one, known as KRAS4A, which is weakly expressed in human cells and the dominant form, known as KRAS4B, which is much more highly expressed.
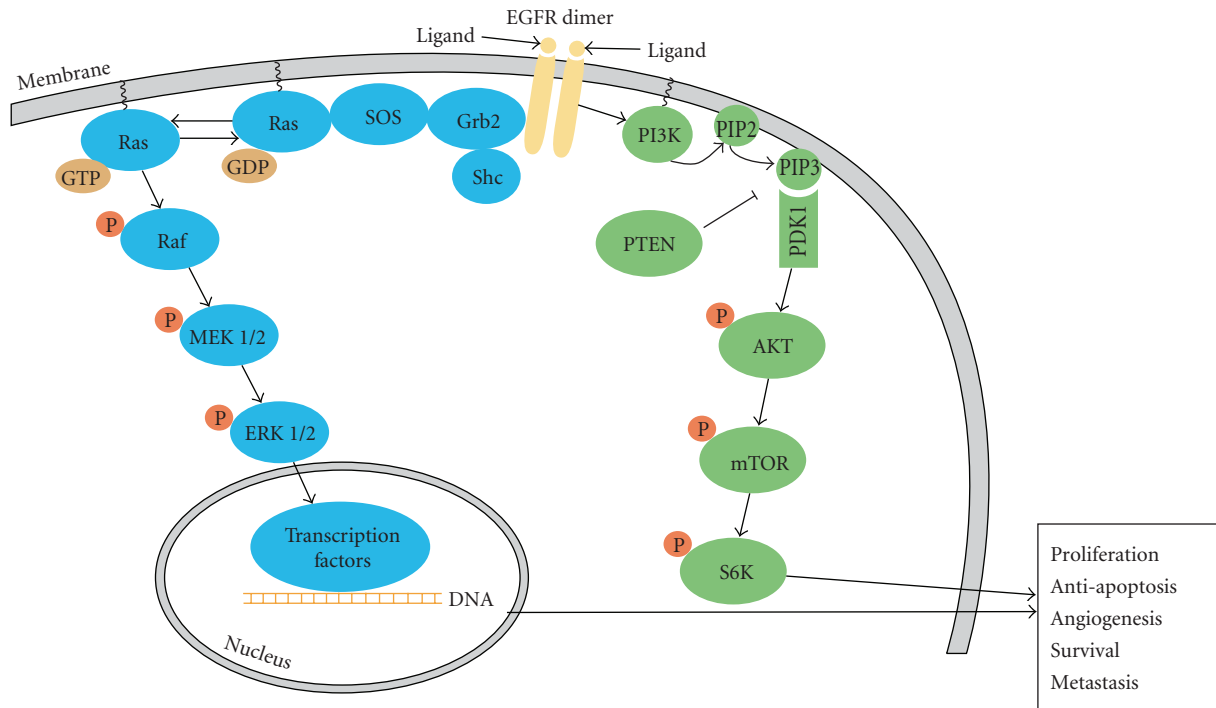
FIGURE 1: Signaling pathway of the KRAS protein. Following EGF binding to its receptor and activation of tyrosine kinases, the KRAS protein becomes activated by binding to GTP, transducing the activation signal to the nucleus by MAPKs and PI3K/AKT-mediated cascades. Specifically, the active state of the KRAS protein is facilitated by binding to the Grb2 protein, which interacts with the SH3 domains of the SOS protein, a member of the nucleotide exchange factor family. In the GTP state, KRAS is able to activate downstream proteins and to regulate cell transformation.

The *KRAS* gene product, KRAS protein, contains 188 amino acid residues with a molecular mass of 21.6 kD and participates in intracellular signal transduction [8]. As mentioned above, the KRAS protein remains inactive until it binds to GTP, as depicted in Figure 2. The switch from an inactive to an active form is regulated by intracellular signals. Once the GTP is bound to the KRAS protein, KRAS undergoes conformational changes that involve two regions of the protein, thus activating it. These two important regions are known as Switch 1 (aminoacids 30–38) and Switch 2 (aminoacids 59–67), which form an effector loop, controlling the specificity of the binding of this GTPase to its effector molecules. This conformational change in the KRAS protein affects its interactions with multiple downstream transducers—GTPase-activating proteins (GAPs)— which amplify the GTPase activity of the RAS protein 100,000-fold [9]. The change also affects interactions with guanine-exchanging/releasing factors (GEFs/GRFs) promoting the release of GTP. The KRAS protein also has intrinsic GTPase activity, stimulated by GAPs, which acts as a timer associated with direct interactions with the effectors [10]. Mutations found in an oncogenic form of the RAS p21 protein impair GTPase activity and make the KRAS protein unresponsive to GAP proteins. Mutated forms of p21 rapidly exchange GDP for GTP, which it prefers as a substrate, thus inducing the active state. Such aberrant forms of KRAS protein deregulate many effectors, thus affecting several important cellular pathways. Many GTP derivatives targeting
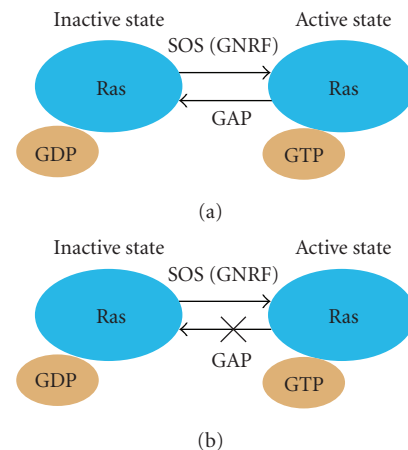

FIGURE 2: Activity states of the KRAS protein. Mutational change in exon 1 of KRAS leads to permanent "on" status.

RAS or RAF effectors have been developed to repair the defective GTPase activity that influences the aberrant RAS signalling [11]. However, little is known about the specificity and transport of compounds modified by GTPs through the plasma membrane.

KRAS contains four domains. The first domain includes 85 amino acids at the N-terminus and is identical in the three forms of RAS (KRAS, NRAS, and HRAS). The second
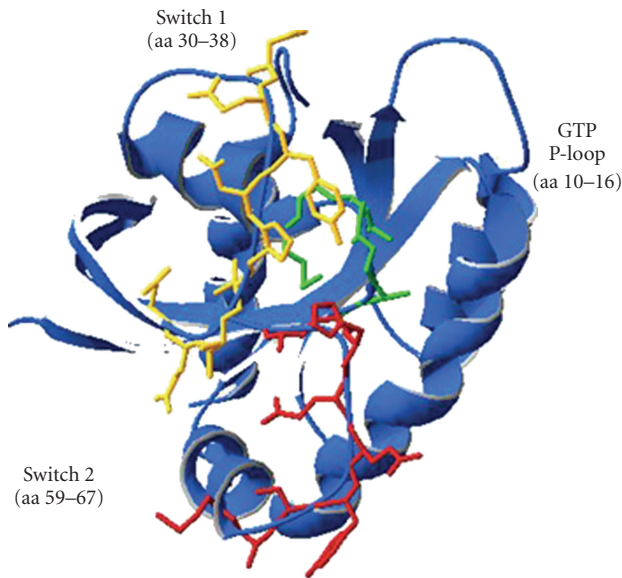
Figure 3: Model of the KRAS protein with important domains highlighted. Model of 3GFT molecule was rendered in Swiss-PdbViewer v4.0.1 (http://spdbv.vital-it.ch/). Switch 1, Switch 2, and GTP P-loop domains are highlighted by colour change.

domain contains 80 amino acids, with lower sequence identity (70–80%) among the three forms of RAS protein. These regions are important for the signalling function of the KRAS protein and jointly form the G-domain (amino acids 1–165, Figure 3). The G-domain of the KRAS protein includes the GTP-binding pocket, where P-loop-phosphate binding loops (aminoacids 10–16 and 56–59) interact with the b-phosphate and c-phosphate of GTP. Residues 116–119 and 152–165 interact with the guanine base. The region between amino acids 32 and 40 (the core effector region) is essential for the interactions between the putative downstream effectors and GAPs. RAS protein also contains a hypervariable region (HVR) at the C-terminus (amino acids 165–188/189; the third domain), which guides posttranslational modification and determines plasma membrane anchoring. This region plays an important role in the regulation of the biological activity of RAS protein [12].

Switch regions I and II play important roles in the binding of regulators and effectors. The phosphate binding pocket-P loop permits temporary binding of GTP to the RAS protein. This is also the region of GTPase activity, which negatively regulates the RAS protein via a RAS-GTP hydrolysis reaction and binding of guanosine diphosphate.

The KRAS protein acts like a plasma membrane-localized molecular switch, regulating multiple signal transduction pathways [13]. It is synthesized in the cytosol, where it is farnesylated by farnesyl transferase at the cysteine residue of the carboxy-terminal motif CAAX (where C represents cysteine, A is an aliphatic amino acid, and X is any amino acid). The carboxy-terminal motif forms the last domain of the KRAS protein. The AAX amino acid motif is cleaved by proteases, whereas the C-terminal carboxyl residue of the KRAS protein is methylated. Cleavage of the AXX peptide motif and methylation occur at the cytosolic surface of the endoplasmic

reticulum and are mediated by the RAS-converting enzyme Rce1 [14]. C-terminal farnesylation plays an important role in membrane localization. In the splice variant KRAS4A, the AXX motif undergoes additional palmitoylation by palmitoyl transferase, resulting in proper targeting of KRAS4A to the membrane. However, there is no detectable palmitoylation of the predominant splice variant KRAS4B, which probably reaches the plasma membrane via a microtubule-dependent mechanism, thus avoiding the Golgi apparatus [13, 15]. Posttranslational farnesylation and carboxymethylation are believed to be important for the oncogenicity of the RAS protein. Treatment with farnesyl transferase inhibitors has been shown to inhibit anchorage-independent growth of both KRAS-transformed mouse fibroblasts and human tumour cells containing KRAS and NRAS mutations. Signal transduction of the KRAS protein does not exclusively occur at the plasma membrane. Activation of downstream signalling pathways by KRAS can also be triggered by signals from subcellular compartments, such as the endoplasmatic reticulum and the Golgi apparatus [16, 17].

2.2. Why Is the KRAS Protein an Important Target to Study? While wild-type KRAS usually promotes cell cycle progression, it can also induce growth arrest, apoptosis, and replicative senescence when increased to abnormal levels. This can be triggered by cellular stress, ultraviolet or ionizing irradiation, heat shock, and some cytokines. In these circumstances, triggering of growth arrest can represent a defence mechanism against inappropriate activation of RAS.

It has been demonstrated that the wild-type KRAS gene is a tumour suppressor that is frequently lost during tumour progression in many types of cancer [18]. Once the KRAS gene mutates, it acquires oncogenic properties (Table 1) and seems to be causally involved in the development of various human cancers [19, 20]. Loss of the wild-type KRAS allele has been observed in both human and mouse tumours, indicating that absence of the normal allele may facilitate transformation by one copy of the oncogenic KRAS allele [21]. Oncogenic mutations in the KRAS gene prevent the hydrolysis of GTP, thus permanently activating the RAS molecules [22]. Expression of a mutated KRAS gene in fibroblasts has been shown to augment metalloproteinase 2 (MMP2) expression in the matrix and enhance the invasion of cancer cells [23]. Overexpression of this mutated form of KRAS also inhibits glycosylation of the integrin β1-chain, resulting in altered polarisation and increased adhesiveness of colon cancer cells. In addition, expression of this oncogenic form of KRAS protein has been shown to be associated with upregulated carcinoembryonic antigen (CEA) expression and disturbance of epithelial cell polarization [24].

3. The KRAS gene

3.1. Polymorphism/Structure and Localization of the KRAS Gene. There are two copies of the KRAS gene in the human genome, designated KRAS1 and KRAS2. The mRNA encoded by the main KRAS2 is 5.5 kb long, and differs from

TABLE 1: Consequences of oncogenic KRAS on cellular physiology and biology.

RAS effector pathways	Main function of the RAS signalling pathways	Oncogenic RAS perturb the intrinsic biochemical properties of cell pathways
MAPK	proliferation	increase
PI3K/AKT	survival	
NORE1/RASSF1	apoptosis	deregulation
RAL-GDS	membrane vesicle trafficking	
JAK/STAT3	growth arrest and differentiation	
TIAM1/RAC	cytoskeletal organization	
PLCε/PKC	calcium transport signalling	
AF6	cell-cell junctions	
PKCξ	transcription	
PI3K/PDK1	translation	

transcripts of the transforming Kristen murine viral gene by only six codons [8]. Analysis of human placental and embryonic cDNA libraries has revealed that 900 bp of the KRAS1 gene is homologous to the corresponding sequence of the Kristen Murine Sarcoma Virus2 homolog, with one intervening sequence, and 300 bp of the KRAS2 is fully homologous to the viral counterpart. The KRAS1 gene is a pseudogene derived from KRAS2 by alternative mRNA splicing. Therefore, KRAS1 should be officially named KRAS1P [8].

In 1983 McBride and colleagues found that the protooncogenes KRAS1 and KRAS2 are localized at human chromosomes 6 and 12, respectively [25]. Later, KRAS1 and KRAS2 were mapped by in situ hybridization to chromosome positions 6p11-12 and 12p11.1-12.1, respectively [26].

Sequencing showed that the KRAS2 gene has six exons. Of these, 2, 3, and 4 are invariant coding exons. Alternative splicing of exon 4 produces two mRNA forms, known as 4A and 4B. Exon 5 can be skipped during alternative splicing, giving rise to isoforms KRASA and KRASB. The 6th exon encodes the C-terminal region in KRASB and is not translated (the 3′untranslated region, 3′UTR) in KRASA. KRASB is the predominant splice variant of KRAS2, and is referred to, briefly, as KRAS [27].

There are indications that allelic losses of chromosome region 12p commonly occur in human cancers, and a frequently deleted region is near the KRAS gene at position 12p12-13 [28]. Further, recent studies on lung adenocarcinoma suggest there is an association between the incidence of allelic losses in the 12p12-13 region and KRAS gene mutation [29].

3.2. Diagnostics of KRAS Mutations in Human Cancers. Diagnostics of KRAS gene mutations in clinical setting is limited by two factors: first, in the time of testing, KRAS mutated tumour cells may be in minority, outbalanced by wild type tumour cells and wild type non-tumour cells present in the sample. Second, analytically preferable snap-frozen tumour samples are rarely available for KRAS mutation testing. Instead, formalin fixed paraffin-embedded (FFPE) tissue is used. There, integrity of DNA may be severely compromised by procedure of formalin fixation (especially by its long duration and low pH).

All the known principles of DNA polymorphism detection are applicable to KRAS mutation detection and demand a dedicated review outside of the scope of this paper. More than 60 methods described can be divided into sequencing methods [30–37], methods based on specific interaction with oligonucleotide, methods based on specific interaction with enzyme [38–40], and conformational methods [41–47]. While many specificity and/or sensitivity enhancement of methods were described as well [48–53], analytical validation, systematic comparison, and assessment of methods side by side is lacking. To authors best knowledge, only Communauté Européene (CE) marked KRAS mutation detection kits are supplied by DxS (mutations in codons 12 a 13 are tested using principle of ARMS-PCR [54] and Scorpion primers [55], Vienna-Lab (reverse dot blot assay format), TIB Molbiol (KRAS LightMix clamped hybridization probes for codon 12), and Invigene (qPCR with sequence suppressor agent StopPrimer for the unwanted excess component, applicable for first two nucleotide positions in codons 12 and 13).

3.3. Regulation of KRAS Gene Expression. KRAS expression is regulated both during the initiation of transcription by the binding of proteins to its promoter and during transcriptional elongation by microRNAs affecting KRAS mRNA stability. Both human and murine KRAS gene promoters contain a nuclease hypersensitive polypurine-polypyrimidine element (NHPPE). The G-rich strand of NHPPE located in the proximal promoter sequence is able to form an intramolecular parallel G-quadruplex, consisting of three G-tetrads and three loops, which recognizes and binds nuclear proteins that are involved in transcriptional repression of KRAS expression. Accordingly, it has been reported that sequestration of nuclear proteins that bind to NHPP by an oligonucleotide mimicking the KRAS G-quadruplex resulted in 40% inhibition of KRAS transcription, compared to controls [56].

The transcription of KRAS is regulated, in part, by an interaction between the promoter region and the 65 kDa ESXR1 protein and, in part, by microRNAs (miRNAs). ESXR1 is a human protein with an N-terminal homeodomain in the nucleus and a C-terminal proline-rich repeat region I in the cytoplasm. The N-terminal fragment of ESXR1 binds to the TAATGTTATTA consensus sequence in exon-1 of the KRAS gene, thus inhibiting its mRNA expression [57]. miRNAs contain a 21-22 nucleotide long noncoding sequence that is able to regulate gene expression [58]. In 2005 it was estimated that there are more than 500 miRNAs, which collectively regulate approximately 30% of all human genes, including the RAS gene family [59].

Regulation of gene expression by miRNAs probably occurs as a result of imperfect hybridization of the miRNA to the complementary sequences located in the 3′untranslated region (3′UTR) of target messenger RNA (mRNA) species. This interaction between miRNA and mRNA both decreases mRNA stability and represses protein synthesis by preventing access to ribosomes [60]. Interestingly, many altered miRNAs have been identified in human cancers [61–63], including some of the most thoroughly analyzed miRNAs—let-7 [64], lin-4 [65], and bentam [66]—all of which regulate cell proliferation and differentiation. Cancer-altered miRNAs can be divided into two main groups. Members of one group, the oncomirs, are upregulated in cancer and can act like oncogenes. The second group, the anti-oncomirs, probably act as tumour suppressors by targeting oncogenes, repressing the cell cycle and division of cancer cells [67]. For example, miRNA-let7 is an oncogene-antioncomir pair that negatively regulates RAS protein levels and decreases cell proliferation rates [68, 69]. KRAS, NRAS, and HRAS harbour multiple let-7 miRNA complementary sites (LCSs) in their 3′UTRs [70]. Zhang et al. [18] found that reducing the activity of let-7 in HeLa cells resulted in a 70% increase in RAS protein levels, while Takamizawa et al. [68] found that let-7 expression was 80% lower in 60% of lung cancer adenocarcinoma and squamous cell carcinoma lines than in normal lung tissue. Moreover, a correlation between low levels of let-7 miRNA and significantly higher RAS protein expression has been found in lung squamous cell carcinomas. These results suggest that let-7 is able to downregulate the expression of RAS in human carcinomas.

These molecular findings provide a strong rationale for developing novel therapeutic treatments aimed at decreasing KRAS protein expression in cancer cells. In many cases KRAS protein expression is dramatically increased due to mutations in the *KRAS* gene sequence, thus making cells refractory to current therapies, such as those involving use of epidermal growth factor receptor inhibitors [71].

4. KRAS in Oncogenesis

Activating *KRAS* gene point mutations have been detected in many types of human tumours [72]. Such oncogenic forms of the *KRAS* gene are prevalent in pancreatic carcinomas (>80%), colon carcinomas (40–50%), and lung carcinomas (30–50%), but are also present in biliary tract malignancies, endometrial cancer, cervical cancer, bladder cancer, liver cancer, myeloid leukemia [73, 74] and breast cancer [75].

Mutations in the *KRAS* gene have important effects on the process of carcinogenesis, which depend on the cells and tissues involved [76]. The mutations found most frequently in the *KRAS* gene of cancer cells are located at positions 12 and 13 in exon 1, and less frequently in codons 61, 63, 117, 119, and 146 [77, 78]. These mutations are located near to the GTP binding site. Allelic mutations result in amino acid changes, namely Gly to Asp, Ala, Arg, Ser, Val, or Cys in codon 12 and Gly to Asp in codon 13 [79]. Somatic missense mutations at positions 12, 13, 61, and 63 enable perturbation of the intrinsic GTPase activity of the KRAS
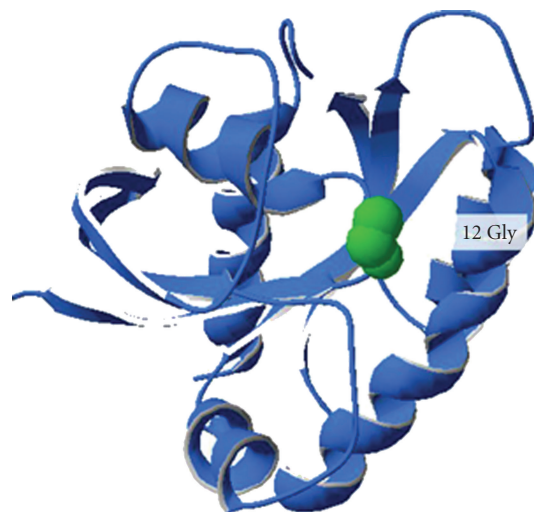

Figure 4: Position of codon 12 in KRAS molecule. Wild type aminoacid at codon 12 is shown in green.

protein, resulting in reductions in GTP hydrolysis capacity. Mutations in codons 12 (Figure 4) or 13 are known to lead to conformational changes in the KRAS protein.

Mutation in codon 12 of the *KRAS* gene causes the encoded KRAS protein to "freeze" in its active state for a much longer duration than its nonmutated counterpart [11]. Mutations resulting in the substitution of amino acids 116, 117, 119, and 146 reduce the nucleotide affinity of the KRAS protein, thereby affecting the rate of GDP/GTP exchange. The oncogenic forms of the RAS protein have a profound effect on the downstream effector pathways, resulting in much higher proliferation rates of cancer cells expressing such forms.

The transforming ability of the KRAS oncogene may result from overexpression of the mutant *KRAS* allele or from deletion of the wild-type allele [80]. Overexpression of KRAS can also be induced by the loss of p16INK4 (CDKN2A), p19INK4 (CDKN2D), or p53 [81]. However, studies by Zhang et al. (2001) have shown that the wild-type *KRAS* allele can suppress the oncogenic function of the mutated allele [18].

In addition, the radiosensitivity of tumour cells is altered by oncogenic RAS expression, probably as a result of the effect of the KRAS mutation on several intercommunicating pathways [82].

4.1. Pancreatic Cancer. The prevalence of mutations in the *KRAS* gene at the time of diagnosis is highest in pancreatic cancers (>80% of cases), notably pancreatic adenocarcinomas predominantly harbour KRAS forms with a guanine to thymine transversion in codon 12 [83]. Wei and colleagues examined samples collected from 30 patients with pancreatic cancer and found that 24 of them showed mutations at codon 12 and only one at codon 13 [84]. However, concurrent KRAS mutations frequently occur in patients with pancreatic cancer [85]. A positive association has been found in patients with pancreatic cancer between tobacco exposure

and mutations in the *KRAS* gene [86]. Similar associations have also been reported for coffee drinking [87], and milk, butter, and alcohol consumption [88, 89]. However, no direct evidence of a causal relationship between these dietary components and mutations in the *KRAS* gene has been presented.

4.2. Colon Cancer. The second highest incidence (about 50% of cases) of mutations in the *KRAS* gene is found in colon cancers [90, 91]. The progression of colon carcinomas can be divided into at least three stages. The first stage is characterized by the development of a small, benign tubular type of adenoma or polyp with sporadically detectable *KRAS* mutation(s) [92]. The second stage is more aggressive and is usually associated with patches of definitive carcinoma cells, which may grow into invasive cancers characterising the third stage. Mutations of the *KRAS* gene have been identified in tissues from both adenoma and carcinoma cases, but at much lower frequencies in colon adenoma tissues than in carcinoma tissues [93, 94]. The incidence of mutation in the *KRAS* gene has been found to be low and to occur mainly in the small adenomas of patients with familial adenomatous polyposis, who have a predisposition to colon cancer [94] in the *KRAS* gene associated with colon cancer appear most often in codons 12 (28%) and 13 (8%) of exon 1 and less frequently in codon 61 [95]. In colorectal cancer, the main substitution (Gly to Asp) has been found to occur in codon 12. Mutation from GGT (Gly) to GTT (Val) in codon 12 has been observed more frequently in primary metastatic carcinoma, suggesting that this mutation may confer a more aggressive phenotype in colorectal carcinoma [96]. A mutation in codon 13, resulting in the substitution of Gly with Asp, observed in colon cancer has been shown to be associated with reduced survival rates [97]. This kind of *KRAS* gene mutation has also been shown to occur more frequently in unstable, than in stable, colon tumours [97, 98].

4.3. Lung Cancer. Losses of *KRAS* wild type alleles in both mouse and human lung adenocarcinomas and squamous carcinomas have been found in many studies, notably in 67% to 100% of chemically induced murine lung adeno- carcinoma cases harbouring a mutant KRAS. In humans, *KRAS* mutations appear in 10–30% of lung carcinoma cases, demonstrating strong associations with a history of smoking [99] and poor prognosis [100, 101]. Among both current and former smokers, *KRAS* gene mutations have been identified in 30% of lung adenocarcinoma cases. Further, although some researchers have found sporadic KRAS mutations in non-smokers with early onset of cancer, smoking history is an important factor and is correlated with increased occurrence of mutations in the *KRAS* gene in lung cancer cases [102]. Mutations in the *KRAS* gene in codons 12 and 13 were detected in 21% of NSCLC (non-small cell lung cancer) tumour samples examined in the TRIBUTE III trial [103]. NSCLC patients have a tendency to accumulate activating mutations in either the EGFR or *KRAS* genes. However, a clinical study has shown that mutations of these two genes

are, in general, mutually exclusive. EGFR mutations are more often found in patients who have no history of smoking.

4.4. Breast Cancer. Although higher *KRAS* mutational fre- quency is primarily found in cancers of the pancreas, colon and lung, possible links between KRAS hyper- activity and human breast cancer have been explored recently. Hollestelle at al. found mutations in 12.5% of cases [104] but the Sanger COSMIC database version 28 (http://www.sanger.ac.uk/genetics/CGP/cosmic/) records only a 5% incidence [105]. The lower frequency of KRAS mutations in breast cancer cell lines suggests that the gene mutation may be less important in breast cancer carcino- genesis than in other forms of cancer, although mutations at a "hotspot" in the *KRAS* gene have been found in a small subset of breast cancers.

5. KRAS and Cancer Therapy

5.1. Tyrosine Kinase Inhibitors of the EGFR1 Gene. Many clinical trials have shown that a poorer response to chemotherapy, a shorter time-to-progression, and worse overall survival are consistently associated with specific mutations in oncogenes. *KRAS* is one of the most frequently mutated oncogenes in many cancers, and it is also one of the most important predictors of resistance to targeted therapy using EGFR1 tyrosine kinase inhibitors (EGFR-TKIs) [106].

Two of the most important EGFR-specific TKIs are gefitinib (Iressa, ZD1839) and erlotinib (Tarceva). The first indications of the predictive strength of the association between the *KRAS* gene and therapeutic responses to the EGFR-TKI gefitinib were originally observed in NSCLC patients with tumours bearing the wild-type form of the *KRAS* gene and constitutively activated EGFR1 gene, due to activating mutations in exons 18 to 21 or high copy number/amplification of the EGFR1 gene. Clinically, better responses to tyrosine kinase inhibitor treatment were observed in patients with adenocarcinomas and well- differentiated tumours, female patients, non-smokers, and people of Asiatic origin [107–109]. Clinical research data show that gefitinib monotherapy is well tolerated and active against a wide range of tumour types, including colon, head, neck, breast, prostate, and lung cancers, especially NSCLCs [110].

EGFR-TKIs are usually used as the second line therapy in patients after failure of chemotherapy. However, gefitinib did not pass the registration procedure in the European Union because insufficient clinical benefit was demonstrated, probably because European clinical trials did not include suf- ficient "good responders" (Asians, women, adenocarcinoma patients and non-smokers). Clinical data also suggest that the drug represents a new therapeutic option for NSCLC patients with brain metastases [111].

After the successful TRIBUTE and TALENT clinical trials, erlotinib (Tarceva) was also approved by the US FDA in 2002 as a second or third line treatment for NSCLC after failure of standard chemotherapy. However, molecular analysis revealed that patients who have activating mutations

in the *KRAS* gene (exon 1: codons 12, 13, or 61) with or without increases in EGFR copy numbers did not derive benefit from this therapy and had about a 96% chance of disease progression [71].

Similarly, Eberhard et al. first observed the relationship between *KRAS* mutations and the outcome of erlotinib therapy in a randomized phase III clinical trial in which the drug was used, in combination with first line "gold standard" chemotherapy (carboplatin and paclitaxel), to treat advanced NSCLC patients [103]. Patients with the *KRAS* mutation exhibited a shorter time to progression (three months) and a shorter overall survival (four months) when treated with a combination of erlotinib and first line chemotherapy, such as treatment with cisplatin, compared to the group with wild-type *KRAS*, for whom the time-to-progression was 12 months. Most NSCLC patients in the erlotinib treatment study had expressed wild-type *KRAS*, and their KRAS status had greater prognostic than predictive value as a biomarker [112]. However, in colorectal cancer, mutations in the *KRAS* gene are important predictive (as well as prognostic) biomarkers, since the effectiveness of treatment with cetuximab and panitumumab is impaired in tumours with the activating mutation. Information regarding the status of the *KRAS* gene allows the selection of appropriate therapies for patients who do not display activating mutations and the selection of alternative therapies for patients with mutations. Although results pertaining to the role of KRAS in the prognosis of clinical outcome or prediction of therapeutic responses to EGFR1 tyrosine kinase inhibitors are interesting, they need to be validated in larger and prospective trials, using standardized and sensitive mutation detection techniques. If the associations are confirmed, knowledge of the mutation status of KRAS in NSCLC tumours could help physicians decide which patients should receive gefitinib and/or erlotinib.

Interestingly, *KRAS* gene mutations also seem to provide strong predictive indication of therapeutic responses to other classes of tyrosine kinase inhibitors, as recently demonstrated for the imatinib mesylate (Glivec). Imatinib is the standard drug for patients with chronic myeloid leukaemia (CML) and patients with gastrointestinal stromal tumours (GISTs), expressing the bcr-abl fusion protein and tyrosine kinase receptor c-kit, respectively. Further, drug resistance to imatinib is usually attributed to mutation of the imatinib-binding sites of these proteins [113], although amplification of the bcr-abl fusion gene or overexpression of multidrug resistance proteins may be involved in some cases [114]. However, a recent study by Agarwal et al. demonstrated that CML patients resistant to imatinib frequently expressed mutated KRAS proteins [115].

5.2. Monoclonal Antibodies as EGFR1 Inhibitors.

Colorectal cancer is another frequent neoplasia that is associated with activation of the EGFR1 pathway, so it is not surprising that novel and successful therapeutic strategies for this condition involve EGFR1 protein kinase inhibition. In contrast to NSCLC, two monoclonal antibodies against EGFR1, rather than small molecular inhibitors of EGFR1, are generally used for treating colorectal cancer: cetuximab (Erbitux®) and panitumumab (Vectibix®). In accordance with the effect of small molecular EGFR1 inhibitors in NSCLC, KRAS alterations play a critical role in the response of colorectal cancer patients to such therapeutic monoclonal antibodies. Indeed, KRAS mutation status is the most important predictor of resistance to cetuximab or panitumumab; both the median progression-free survival of cetuximab-treated patients and overall survival was recently found to be superior in a *KRAS* wild-type group than in a *KRAS* mutant group (31 versus 10 weeks, and 16 versus 7 months, respectively) [116]. On September 27, 2006, the US FDA approved the completely humanized monoclonal anti-EGFR1 IgG2 antibody—panitumumab (Vectibix) for clinical use in the third line treatment of patients with metastatic colorectal carcinoma who had progressed after standard chemotherapy. KRAS Panitumumab therapy was tested in a randomized study involving 463 patients [117], and the results showed that the wild-type *KRAS* gene is essential for its therapeutic activity. Progression-free survival of patients with wild-type versus mutant *KRAS* gene tumours was 12 versus seven weeks, while response rates obtained in another study were 17% versus 0% [118]. These findings strongly indicate that *KRAS* gene status should be routinely tested as a critically important diagnostic biomarker to determine which patients will derive therapeutic benefit from EGFR1 inhibition. Indeed, analysis of the *KRAS* gene status in colorectal cancer cases has become *conditio sinequa non* for deciding whether or not to apply cetuximab or panitumumab therapy in routine clinical practice and FDA has updated Vectibix and Erbitux labels in 2009 to include this information.

5.3. Chemotherapy and Radiation.

Surprisingly, the effects of *KRAS* gene mutations on tumour sensitivity to cytotoxic chemotherapies and radiation have only been explored in a few studies. However, expression of a 12 Val mutated form of KRAS has been shown to increase the resistance of cancer cells to radiation therapy [119]. Similarly, the presence of oncogenic KRAS has been found to significantly increase the sensitivity of cells to a novel class of anticancer agents, cucurbitacins, in a p53- or p21-dependent manner [120]. In contrast, an ovarian cancer cell line TOV-21G bearing a mutant allele of *KRAS* is reportedly significantly more sensitive to cisplatin and radiation, but not to paclitaxel or campthotecin, than the corresponding *KRAS* wild type line [121].

However, results of clinical studies by Rosell and colleagues (1995) showed that patients with a mutation in the *KRAS* gene had poorer clinical responses to paclitaxel monotherapy than wild type controls, suggesting that *KRAS* gene status is a predictive marker of paclitaxel resistance [122]. In a phase III retrospective study on NSCLC patients (TRIBUTE), randomly treated with carboplatin and paclitaxel with erlotinib or placebo, patients with KRAS mutant tumours showed poorer clinical outcomes when treated with erlotinib plus chemotherapy compared to chemotherapy alone [103]. An updated clinical trial (CRYSTAL) involving 540 metastatic colorectal cancer patients demonstrated that

that cetuximab in combination with FOLFIRI (folic acid, fluorouracil, and irinotecan) in first line therapy is highly effective against KRAS wild type, but not mutant, tumours. However, further analysis of the data showed that neither the response nor the progression-free survival of patients treated with chemotherapy alone were significantly affected by *KRAS* gene status, although the overall survival of patients with KRAS mutant tumours was significantly shorter than that of patients with KRAS wild type tumours [123]. Recently we have also shown that EGFR may represent a predictive molecular marker for poor response to preoperative chemoradiotherapy in locally advanced gastric carcinoma [124]. Responses to chemoradiotherapy were found in 60% of EGFR-negative patients, but only 13% of EGFR-positive patients ($P = .044$), and pathologic complete responses were observed in 29% of patients with EGFR-negative staining, but none (of eight) EGFR-positive patients ($P = .16$).

The above findings regarding the role of the *KRAS* gene in tumour responses to cytotoxic therapies appear to conflict somewhat. The predictive and prognostic significance of oncogenic KRAS seems to have been mixed in many studies, and the contributions of variations in the gene to clinical outcome appear to differ according to tumour types and therapeutic interventions. Clearly, further studies are urgently needed to confirm and clarify the findings in large prospective biomarker-oriented clinical trials.

5.4. Angiogenesis Inhibitors. Other clinical trials have also demonstrated that activating mutations in the *KRAS* gene can contribute to tumour progression by affecting the expression of vascular endothelial growth factor (VEGF), which plays a critical role in tumour angiogenesis. Inhibition of KRAS expression by selected KRAS antisense oligonucleotides has been shown to be associated with significantly reduced secretion of VEGF-A165 into the medium of colorectal cancer cell cultures [125]. In addition, in a cohort of patients with pancreatic tumours, 25/33 (76%) with *KRAS* mutations showed higher VEGF expression, and their median survival was shorter, than those with tumours expressing the wild-type allele [126]. Similar findings have also been reported from a study of NSCLCs, in which higher VEGF expression was observed in 50% of tumours bearing a *KRAS* gene mutation [127]. Although these studies suggest that *KRAS* gene status could play an important role in responses to anti-VEGF targeted antiangiogenic therapy, a recent study by Hurwitz and Saini [128] showed that groups of patients bearing either *KRAS* mutant or wild-type tumours derive therapeutic benefit from first line application of the anti-VEGF monoclonal antibody bevacizumab (Avastin). Furthermore, although both groups of patients (i.e., those with wild-type KRAS and mutated *KRAS* genes) benefited from adding bevacizumab to chemotherapy, both progression-free survival and survival was better for wild-type KRAS patients, both with chemotherapy alone and with chemotherapy plus bevacizumab. Bevacizumab did not increase the percentage of patients with mutated KRAS who responded to treatment.

5.5. Future Therapies. An optimal therapeutic drug should be able to specifically target the mutated *KRAS* gene or its product, have minimal systemic toxicity and be orally active. Unfortunately, drugs like this remain to be developed and less efficient strategies need to be used in clinical trials. However, in addition to the cancer therapies mentioned above, several therapeutic agents and strategies can directly suppress the activating mutant form of the *KRAS* gene, and thus improve the efficiency of chemotherapy and biological therapy.

One possible approach for inhibiting KRAS expression is to use antisense oligonucleotides or viral constructs delivering antisense sequences in order to inactivate the mutant oncogene RNA message [125]. In addition, synthesis of mutated KRAS protein has been repressed by applying a small interfering adenovirus-mediated RNA (siRNA), and the specifically designed siRNA was shown to have prolonged anti-proliferative effects against various tumour cancer cell lines expressing mutated KRAS proteins [129]. Another, similar strategy to target mutant KRAS mRNA is based on designing an mRNA ribozyme that specifically interacts with a mutated form of the KRAS mRNA and encodes catalytic RNA molecules that bind to the mRNA substrate by base-pair complementation, leading to translation arrest and/or degradation of the specific mRNA. The KRAS-specific ribozyme strategy has also been shown to suppress successfully the proliferation of KRAS-mutated tumour cells [130].

Recently an interesting novel strategy employing farnesyltransferase inhibitors (FTIs) was shown to inhibit the biochemical transactivation initiated by the mutated *KRAS* gene. Farnesyl transferase is an enzyme that primarily regulates zinc metabolism by the addition of a farnesyl group to the cysteine residue of a protein. At least 30 proteins (including KRAS) require posttranslational farnesylation to reach their membrane positions and function properly in cell signalling. Farnesyl inhibitors represent a novel class of biologically active anticancer drugs that inhibit cell growth. After the discovery that RAS proteins have to be farnesylated to become functionally active, several farnesyl inhibitors were developed. However, Phase II and Phase III clinical trials conducted to date have found that KRAS FTIs might not be sufficient to inhibit the mutated-overactive forms of KRAS protein. The reason for this is probably incomplete inhibition of farnesylation, because garnesylation of KRAS protein by geranyltransferase I leads to suppression of the effects of farnesyltransferase inhibitors [131, 132].

It should be noted that although KRAS inhibitory strategies have shown promise in preclinical trials and have been partially successful in clinical trials, there are insufficient data on their efficacy in combination with anti-EGFR1 strategies to recommend their routine use as yet.

6. Conclusions

The evidence from various studies summarized in this review demonstrates that the KRAS protein is an important signal transducer involved in the regulation of various

cellular responses during cell proliferation, differentiation, and survival. A pivotal function of KRAS protein in the regulation of the MAPK and PI3K/AKT pathways is its effect on the proliferation rate of both normal and cancer cells. Activating mutations of the KRAS protein, which frequently occur in cancer cells, indicate poorer prognosis and increased resistance to some cancer therapies.

Overall, this review summarizes novel approaches allowing the management of cancers with or without *KRAS* mutations, and highlights the importance of early identification of somatic mutations in the *KRAS* gene in cancer biopsies. The latter assists oncologists to select the best available therapies for their cancer patients.

Acknowledgments

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