Targeting the Ras/Raf/MEK/ERK pathway in hepatocellular carcinoma (Review)

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Abstract. Although the biological basis of hepatocellular carcinoma (HCC) remains unclear, effective treatments and improvement of the survival rate remain worthwhile research goals. Abnormal protein signaling pathways contributing to uncontrolled cell proliferation, differentiation, survival and apoptosis are biomarkers of the carcinogenic process. Certain mutated components or overexpression of the rat sarcoma virus (Ras)/rapidly accelerated fibrosarcoma (Raf)/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway are increasingly being studied in HCC carcinogenesis. The present review addresses the effect of the Ras/Raf/MEK/ERK signaling pathway on the pathogenesis of HCC, and provides an update on the preclinical and clinical development of various inhibitors targeting this core signaling pathway, which include various Ras inhibitors, Raf inhibitors and MEK inhibitors for HCC.

Contents

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers, with an incidence rate ranking sixth highest and a mortality rate ranking third highest, accounting for 7% of all cancers in the world (1). In particular, there is a high incidence in China, with ~4/10,000 cases per year. Current treatment options for HCC, including surgical approaches, locoregional ablative techniques and interventional ablation treatments, could increase the 5-year-survival rate to 75% (vs. 30% prior to these treatments), however, <20% of HCC patients qualify for these treatments (2). Although the promising 5-year-survival rate of HCC cases has been increased due to advances in surgical techniques, nutritional support and perioperative management, long-term survival after surgical resection remains low due to the high rate of recurrence and metastasis (3,4). Novel evidence-based therapies for HCC are urgently required. Recently, biological studies have pointed to aberrant rapidly accelerated fibrosarcoma (Raf)/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway activation as being central for cancer growth, survival and motility, as well as for targeted therapy resistance mechanisms (5-7). For example, sorafenib is a Raf-1 kinase inhibitor and is the only approved drug therapy for HCC. In patients with advanced or metastatic HCC and compensated cirrhosis, sorafenib offers disease control in ~40% of treated patients, with a time to progression of 5.5 months and a median survival time of 10.7 months, ~3 months longer than that of placebo-treated patients (8). Hence, there is an eagerness to dissect the molecular mechanisms of invasion and metastasis for novel insights and interventions against the recurrence of HCC.

Undoubtedly, the rat sarcoma virus (Ras)/Raf/MEK/ERK signaling pathway contributes a core effect in regulating cell proliferation, differentiation and survival in the signaling networks (9). On this account, it has been studied and discussed to determine the pathogenesis of several types of human cancers, including HCC (10). Not merely the Ras/Raf/MEK/ERK signaling pathway, but also the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway has been studied to determine whether it is connected with the pathogenesis of HCC (11). It is notable that the interaction between the Ras/Raf/MEK/ERK and PI3K/Akt signaling pathways may lead to the regulation of cell growth and development, more than either alone.

In the present review, the function of the Ras/Raf/MEK/ERK signaling pathway in HCC is elaborated on and its therapeutic potential as a target for the intervention and treatment of HCC is expounded.

2. Ras/Raf/MEK/ERK pathway

The mitogen-activated protein kinase (MAPK) cascade consists of serine/threonine kinases, which convert extracellular molecules such as growth factors, hormones, tumor-promoting substances and differentiation factors, into intracellular signals for regulating cell proliferation, differentiation and survival (12,13). There are four core protein kinases, Ras, Raf, MEK and ERK, in the Ras/Raf/MEK/ERK signaling pathway. Ras, Raf and MEK are members of multi-gene families; of those, Ras has three members (Ki-Ras, N-Ras and Ha-Ras), Raf has three members (A-Raf, B-Raf and Raf-1) and MEK has five gene family members (MEK1, MEK2, MEK3, MEK4 and MEK5). At the cell surface, the activation of the Ras/Raf/MEK/ERK signaling pathway is initiated by ligand binding to receptor tyrosine kinases (RTK), then, in the nucleus, the phosphorylation of four core protein kinases, Ras, Raf, MEK and ERK, in turn regulating gene transcription (14). The specific activation pathways are as follows.

There are a number of extracellular signals, including growth factors, hormones, tumor-promoting substances and differentiation factors, which activate the Ras/Raf/MEK/ERK signaling pathway. When extracellular signals bond with an appropriate RTK (an Src homology 2 domain-containing protein), the C-terminus of the growth factor receptor [for example, FGFR, Flt-3, platelet-derived growth factor receptor (PDGFR), insulin-like growth factor receptor-1 (IGFR-1) and macrophage colony stimulating receptor among others] that has been activated is linked with the RTK. The tyrosine kinase domain of the excessive phosphorylation of RTK acting as a carrier protein, such as sex muscle abnormal protein-5 or organization control-1, recruits guanine nucleotide exchange factors [for example, mammalian son-of-sevenless (SOS)] to the cytomembrane where they stimulate Ras-GDP conversion to Ras-GTP, resulting in Ras protein activation (14,15). Ras phosphorylation then recruits Raf to the membrane where it becomes activated, likely via an Src-family tyrosine (Y) kinase. Raf is responsible for the serine/threonine phosphorylation of MEK1. The MEK family has five genes, namely MEK1, MEK2, MEK3, MEK4 and MEK5. The five genes are all double specificity kinases, which means that they can phosphorylate serine/threonine residues along with tyrosine residues. Of those, Ras and Raf activate downstream target proteins of MEK1 and MEK2 through phosphorylating the activation domain of two serine residues. MEK1 phosphorylates ERK1/2 at specific T and Y residues. The ERK family has four members, namely JNK1/2/3, ERK1/2, ERK5 and p38 MAPK. ERK1/2 is the only downstream protein target of MEK1/2 phosphorylation. When activating ERK1/2 serine/threonine kinases, they will generate a series of effects (for example, the phosphorylation and activation p90 ribosomal six kinase-1) (16,17). There are 460 ERK1/2 targets, including downstream and upstream substrates (18,19). Therefore, the regulation of the Ras/Raf/MEK/ERK signaling pathway plays an important role in cell proliferation, differentiation and survival by suppressing MEK and ERK activities.

There is a feedback pathway regulating the activity of B-Raf, Raf-1 and MEK1 through the activation of ERK. With regard to Raf-1, ERK phosphorylation can improve or lower the its activity, which depends on the site phosphorylated. With regard to B-Raf and MEK1, ERK phosphorylation can lower their activity. There is also a negative feedback pathway preventing the activation of Ras through the phosphorylation of SOS by ERK. Target protein phosphorylation, such as that of Ras, Raf, MEK and ERK, can enhance or inhibit the associated signaling pathway, even phosphorylating different sites of a target protein playing a different role in regulating the pathway (20,21). Therefore, regulating the Ras/Raf/MEK/ERK signaling pathway is a complex process, which plays an important role in cell proliferation, differentiation and survival (Fig. 1).

3. Ras/Raf/MEK/ERK pathway activation in HCC

A large amount of preclinical and clinical evidence has shown that the abnormal activation of the Ras/Raf/MEK/ERK signaling pathway frequently results in HCC. Ito *et al* showed that MAPK/ERK is activated and its associated gene expression is upregulated in 58% of HCC cases (22). Hoffmann *et al* demonstrated that the mRNA of Ras, MEK, ERK and MAPK was overexpressed in 33, 40, 50 and 50% of HCC patients, respectively (23). Similarly, H-ras has been found to be activated in ~93.8% of HCC cases (24). A study further showed that the expression Raf and its downstream genes, MEK and ERK, were upregulated in samples of hepatocirrhosis and HCC (25). Western blot analysis demonstrated the overexpression of Raf-1 in 91.2% of hepatocirrhosis and 100% of HCC patients. Furthermore, the Raf-1 expression level in HCC patients was significantly high compared with that of hepatocirrhosis patients (26). All research results showed that the activation of the Ras/Raf/MEK/ERK pathway may lead to HCC development functionally.

The cellular mechanisms behind the activation of the Ras/Raf/MEK/ERK signaling pathway are not yet completely clear in HCC. However, activation by RTKs is hypothesized to be the main mechanism. It has been verified that EGFR, IGFR, vascular endothelial growth factor receptor (VEGFR) and c-Met are overexpressed. Of those, EGFR accounts for 47.1% of cases in HCC, and the overexpression of EGFR is responsible for the invasiveness and recurrence of HCC (27). Wiedmann and Mössner showed that the EGFR inhibitors erlotinib and lapatinib inhibit not only RTKs, but also serine/threonine kinases along the Ras/Raf/MEK/ERK pathway, in two phase III placebo-controlled trials (28). Similarly, the overexpression of VEGFR has been found in HCC cell lines and in the serum and tissues of HCC patients (29,30). Furthermore, the overexpression of c-MET accounts for 20-48% of cases in HCC (31). Activation of c-MET plays a role in bringing the growth factor receptor-bound protein 2/SOS complex to the plasma membrane. As a result, GTP along with Ras activates a protein cascade that contributes to the downstream protein kinase phosphorylation of ERK by Raf and MEK (32).

Hepatitis virus infections also play an important role in the activation of the Ras/Raf/MEK/ERK pathway in HCC.

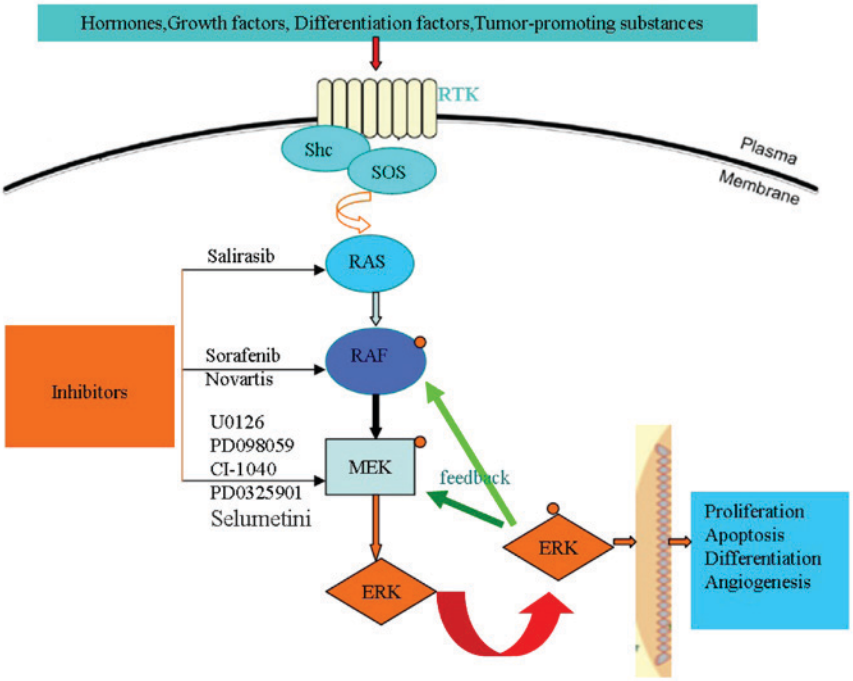

Figure 1. Activation of the Ras/Raf/MEK/ERK signaling pathway. ● indicates a key protein regulated by phosphorylation. RTK, receptor tyrosine kinase; SOS, mammalian son-of-sevenless; Shc, Src homology 2 domain-containing protein; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase.

Hepatitis B virus X protein has also been demonstrated to have an essential effect on the progression of HCC through activating the Ras/Raf/MEK/ERK cascade (33) and then contributing to the loss of function of the tumor suppressor p53 (34). Hepatitis C virus (HCV) core protein activates the kinase Raf-1 and MAPK/ERK pathway through interacting with 14-3-3 protein (35). Schmitz *et al* confirmed that the mechanism of HCC carcinogenesis may be via the activation of the Ras/Raf/MEK/ERK pathway by HCV infection (36). There are at least two functions of the HCV core protein, including the activation of the Ras/Raf/MEK/ERK pathway and an anti-apoptotic effect. HCV core protein can activate ERK phosphorylation alone without hepatocyte mitogen-mediated signaling. However, HCV core protein along with tissue plasminogen activator may contribute to the effect on MEK1 or further upstream of the protein kinase (37).

In recent years, genomic sequencing research has revealed the associated gene changes of the Ras/Raf/MEK/ERK signaling pathway in HCC. The B-Raf gene, one of the human isoforms of Raf, has been found to be mutated or deleted in HCC, accounting for ~23% of cases. As a result, this may lead to activating oncogenic Ras in HCC (38). In addition to the mutation or deletion of B-Raf, codon 12 of the K-Ras and N-Ras genes is also mutated or deleted in HCC, accounting for 4.69 and 41.37% of cases (24,38,39). Challen *et al* found that mutations of the K-ras and N-ras genes in codon 61 occurs in 5.3 and 15.8% of HCC cases (40). All the reported mutations of K-Ras, N-Ras and H-ras are somatic missense mutations (for example, changes to the amino acids of codons 12, 13 and 61) in HCC. Mutations in Ras family genes can phosphorylate the Ras/Raf/MEK/ERK signaling pathway, then deregulate signal transduction (41). However, Taketomi *et al* examined 61 patients through a dot-blot and elaborated that the mutations of Ras proto-oncogenes in codons 12, 13 and 61 had little effect in HCC (42). Therefore, further study is required to clarify the controversy.

4. Targeting Ras/Raf/MEK/ERK pathway in HCC

As the abnormal activation of the Ras/Raf/MEK/ERK signaling pathway plays a major role in HCC cell proliferation, differentiation, survival and apoptosis, a number of studies have been focused on the inhibitors of the core protein kinases Ras, Raf, MEK and ERK in the Ras/Raf/MEK/ERK signaling pathway (Fig. 1); a number of these studies are preclinical, while others are clinical studies (Table I).

5. Targeting Ras

Activating Ras mutations have been observed in ~30% of all cancers. However, according to studies of the pathogenesis of cancer at present, the specific function of Ras is not yet settled. Salirasib [also known as S-trans,trans-farnesylthiosalycilic acid (FTS)] is a synthetic low-weight molecule of a S-farnesyl cysteine analog that expresses a potent inhibitory effect on Ras. Its mechanism of action is likely to be associated primarily with the dislodgment of the mature protein from membrane domains that interact with Ras and with the subsequent accelerated degradation of the dislodged Ras proteins (43,44). These effects of FTS are manifested by a decrease in the amount of cellular Ras accompanied by interruption of the Ras-dependent Raf-1/ERK signaling cascade (45). FTS plays an antitumoral role in several non-hepatic cancer cell lines (46), and a phase I clinical trial of gemcitabine and FTS has demonstrated that FTS is well tolerated in patients with solid non-liver tumors (47). As its expression is well tolerated,

Table I. Inhibitors of the Raf/MEK/ERK signaling pathway for HCC in preclinical and clinical studies.

Drug	Targets	Phase	Outcome	Ref.
Salirasib	Ras	Preclinical	IC_{50} in μM range, therapeutic potential of HCC was further confirmed in a xenograft model	(48)
Sorafenib	Raf	Phase III	Significantly improved OS and TTP	(55)
Novartis	Raf	Preclinical	Inhibition the growth rates of HCC cells and xenograft tumors	(56)
U0126	MEK	Preclinical	Not favorable for clinical use due to poor solubility and low bioavailability	(62)
PD098059	MEK	Preclinical	Not favorable for clinical use due to poor solubility and low bioavailability	(59)
CI-1040	MEK	Phase I	28% SD for 5.5 months	(64)
PD0325901	MEK	Phase II	All patients experienced at least one adverse event	(76)
Selumetinib	MEK	Phase II	35% SD for 6 weeks; PFS, 1.4 months; TTP, 1.4 months; OS, 4.2 months	(73)

MEK, mitogen-activated protein kinase kinase; IC_{50}, half maximal inhibitory concentration; HCC, hepatoceullar carcinoma; OS, overall survival; TTP, time to progression; SD, stable disease; PFS, progression-free survival; ERK, extracellular-regulated protein kinase.

salirasib may become a drug of choice for the treatment of HCC by targeting Ras and mammalian target of rapamycin (mTOR) protein kinase (48). A study by da Silva Morais *et al* indicated that a high concentration of salirasib can inhibit liver cancer cell proliferation *in vivo* in rats following a partial liver resection (49). Its mechanism of inhibitory effects is mediated, at least in part, by the inhibition of ERK phosphorylation. Furthermore, the study by Nicolas *et al* demonstrated that salirasib injection can prevent the development of liver tumors in a subcutaneous xenograft model (50).

6. Targeting Raf

Certain special distinct classes of compounds have been developed as potential Raf kinase inhibitors. However, thus far, sorafenib (Nexavar) is the most successful anti-Raf inhibitor. Sorafenib, an orally available anti-Raf compound, is the only small molecular target kinase to receive Food and Drug Administration approval for the treatment of advanced HCC (51). Sorafenib suppresses the serine/threonine kinase subtypes of Raf, which are well known to regulate the Raf/MEK/ERK signaling pathway and inhibit tyrosine kinase receptors, including VEGFR2, PDGFR and IGFR. For this reason, sorafenib inhibits angiogenesis and tumor growth (52). Sorafenib has demonstrable preclinical and clinical activity against certain types of cancer (for example, HCC, and ovarian, breast and pancreatic cancer). However, in pharmacokinetics, pharmacodynamics and adverse events, sorafenib has exhibited individual differences in clinical research (53,54). An updated meta-analysis evaluated sorafenib administration and found that it could significantly increase OS time and TTP in patients with advanced HCC. Additional large-scale, well-designed randomized controlled trials are planned to further evaluate the efficacy of treating advanced HCC with sorafenib (55).

NVP-AAL881 (Novartis, Basel, Switzerland) is an oral Raf and VEGFR2 small molecule inhibitor that has been shown to inhibit cell proliferation and tumor growth in a subcutaneous xenograft model of HCC (56). NVP-AAL881 can abnormally activate ERK and STAT3, and inhibits the migration of HCC cells. NVP-AAL881 inhibits the growth of HCC cells in a dose-dependent manner, as observed through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. In HCC cells under low serum culture conditions, the inhibition effect of NVP-AAL881 is enhanced. Furthermore, with increasing concentrations of NVP-AAL881, the inhibition of ERK, MEK and STAT3 phosphorylation is enhanced in HCC cell lines (57). More recently, it has been shown that NVP-AAL881 administration markedly inhibits the growth of HCC xenograft tumors compared with controls (58).

7. Targeting MEK

As Ras inhibitors are challenging to identify and almost no biological functions of B-Raf inhibitors are known, more attention is being focused on the study of MEK and ERK inhibitors. Numerous MEK inhibitors have been developed; PD98059 was the first MEK1/2 inhibitor to be found, which combines with the inactive forms of MEK1/2 to prevent its phosphorylation, then inhibits the phosphorylation of ERK1/2 and blocks cell signal transduction (59). U0126 is the second MEK1/2 inhibitor to be identified, and its inhibitive effect is greater than that of PD98059. U0126 is widely used in *in vitro* experiments (60). In HepG2 cell cultures *in vitro*, the phosphorylation of p38 and ERK1/2 was inhibited by PD98059, and this effect was also demonstrated *in vivo* (61). The two types of MEK1/2 kinase inhibitors are non-ATP-competitive, acting by inhibiting the MEK excitation instead of directly inhibiting the activity of MEK. Due to the low solubility and bioavailability of PD98059 and U0126, they have not entered into clinical trials and are only applied in experiments *in vitro* (62).

CI-1040 is the first MEK1/2 inhibitor to enter into a clinical trial (63). Despite effective inhibition of the MEK pathway and demonstration of antitumor activity in HCC cell models, its development was halted after phase II trials due to poor clinical efficacy (64,65). However, the reduced adverse events associated with CI-1040 has led to more investigation of potent analogues. PD0325901 is the congener of CI-1040, which was

found through replacing the hydroxyl and fluorine of CI-1040. It was also to optimize diphenylamine of CI-1040 through pharmacological probe in the study of the Ras/Raf/MEK/ERK signaling pathway (66). To improve the poor solubility and rapid clearance of CI-1040, the diphenylamine core and the hydroxamate side chain were optimized with resulting improvements in solubility, potency in cell-based assays and oral bioavailability. Compared with CI-1040, this compound represents a 100-fold improvement in potency in cell-based assays (0.33 nM in colon 26 cells) and, perhaps more significantly, greater solubility and better stability in human liver microsomes and hepatocytes. Due to its increased solubility, compared with CI-1040, the pharmacological effect and biopharmaceutical properties are significantly improved (63). However, as all patients treated in the phase II trial of the drug experienced at least one adverse event, PD0325901 could not be developed further (67,68).

The oral, non-ATP competitive MEK1/2 inhibitor selumetinib (previously known as ARRY-142886 and AZD6244), is a benzimidazole derivative (69). The drug is the second MEK inhibitor to enter into clinical trials (70). A large quantity of effective results have been shown in preclinical studies only, using cell cultures and animal models (71). Selumetinib has been demonstrated to play a role in contributing to the inhibition of ERK1/2 phosphorylation in a number of cancer cell lines, with a half maximal inhibitory concentration as low as 8 nmol/l and the sustained inhibition of ERK activity achieved with a concentration of 10 mg/kg/day in a subcutaneous xenograft model of HCC (72). In a multi-center, single-arm phase II study of selumetinib in advanced or metastatic HCC patients, the study was stopped at the interim analysis due to a lack of radiographic reaction. The drug has not yet been shown to significantly decrease the time to progression. Selumetinib is well tolerated, but the treatment effect is not ideal in advanced HCC (73). Another phase II study showed that 14 out of 17 evaluable HCC patients succumbed; of the remaining 3 patients, 2 experienced progression and 1 remained alive without progression (74). The median progression-free survival time was 1.4 months. The median time to progression was the same. The median survival time was 4.2 months (75).

8. Conclusion

Studying the Ras/Raf/MEK/ERK signaling pathway has provided novel insights and novel target drugs for HCC treatment. On the one hand, although such drugs exhibit improved therapeutic effects compared with conventional chemotherapeutic drugs, they present potential problems and challenges for HCC therapy, such as adverse events and resistance. On the other hand, although the Ras/Raf/MEK/ERK signaling pathway plays an important role in the regulation of HCC cell proliferation, differentiation, survival and apoptosis, its exact functional relevance in the settings of this complex signaling network and HCC tumorigenesis are far from being fully understood. Furthermore, a key challenge for Ras/Raf/MEK/ERK pathway inhibition will likely be the level of cross-talk and negative feedback along parallel pathways (such as the PI3K/AKT/mTOR pathway). Preclinical data suggest that certain problems and challenges may be overcome by combining Ras/Raf/MEK/ERK pathway inhibitors with other pathway inhibitors, but this must be confirmed in clinical studies.

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