MINIREVIEWS

Hypoxia inducible factor in hepatocellular carcinoma: A therapeutic target

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Abstract

Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed and deadly cancers worldwide; its incidence has been rising in the United States due to the increase in hepatitis C associated cirrhosis and the growing epidemic of obesity. There have been no effective therapeutic options in the advanced disease setting beyond sorafenib, a multi-targeted tyrosine kinase inhibitor that showed significant survival benefit. Because of this, there is an urgent need to search for novel pathways in sorafenib experienced patients. This review will focus on the role of hypoxia and hypoxia-inducible factor alpha (HIF-1α) in cancer development, specifically in HCC. We will discuss the biology of HIF-1α, the pathways with which it interacts, and the function of HIF-1α in HCC. Furthermore, we will review studies highlighting the relevance of HIF-1α in the clinical setting, as well as the pre-clinical data supporting its further investigation. Finally, we will conclude with a discussion of the potential role of a HIF-1α mRNA antagonist for the treatment of HCC, and hypothesize the ways in which such an inhibitor may be best utilized in the management of advanced HCC. Hypoxia plays a significant role in the development of HCC. HIF-1α is a key transcription factor involved in the hypoxic response of cancer cells. It activates transcription of genes responsible for angiogenesis, glucose metabolism, proliferation, invasion and metastasis in HCC. Its involvement in multiple, essential tumor pathways makes it an attractive potential therapeutic target in HCC.

Key words: Hypoxia; Hypoxia-inducible factor alpha; Hepatocellular carcinoma; Vascular endothelial growth factor

Core tip: Beyond sorafenib, limited systemic treatment options exist for the treatment of advanced hepa-

tocellular carcinoma (HCC). Hypoxia and hypoxia-inducible factor alpha (HIF-1α) have emerged as important factors in the development of HCC. This review focuses on the scientific background and pre-clinical data of HIF-1α and will conclude in a discussion of the clinical relevance of this transcription factor and its potential therapeutic role, particularly in combination with other therapies, in HCC. A phase IB clinical trial to investigate a HIF-1α mRNA antagonist in HCC patients who have failed first line systemic treatment is currently underway.

INTRODUCTION

Incidence of hepatocellular carcinoma

Worldwide, hepatocellular carcinoma (HCC) remains the 5th and 7th most common cancer diagnosed in men and women, respectively; it is responsible for over 500000 deaths annually[1]. HCC typically occurs in the setting of chronic liver disease and cirrhosis, which are closely related to risk factors such as hepatitis B or C infection and alcohol abuse. In the United States, the incidence of HCC is rising, owing in large part to the increase in hepatitis C associated cirrhosis, and the latency that exists between timing of hepatitis C infection, cirrhosis, and ultimately development of HCC[2]. Although the recent successful treatment of hepatitis C has brought about excitement in the medical community, the potential for patients with preexisting, residual cirrhosis to develop HCC may increase due to prolonged lifespan after treatment of viral infection. Moreover, the rising incidence of obesity and its association with metabolic syndrome has paralleled the rise in HCC, with insulin resistance, increased tissue necrosis factor activity, and non-alcoholic steatosis all implicated as possible mechanisms of pathogenesis for HCC[3].

Current treatment options in HCC

For early stage disease, which represents only 15% of all patients with HCC, potentially curative thera-peutic options include liver resection, transplantation, and ablation. Nevertheless, recurrence of disease or development of metastases commonly occurs in patients post-resection, with 50% of patients recurring within two years; the three-year survival rates post recurrence have been 10%-40%, depending on the stage of disease[4,5]. The majority of patients with HCC often present with advanced disease not amenable to cure or locoregional therapy, and only systemic therapy can be offered.

Systemic therapy for HCC

Considered chemotherapy-refractory, HCC has seen very few successful advances within the realm of systemic treatment. With the success of the randomized, phase III SHARP trial by Llovet and colleagues in 2008, sorafenib, an oral multikinase inhibitor of the vascular endothelial growth factor receptor, platelet-derived growth factor receptor, and Raf serine/threonine kinases, has established itself as the standard systemic treatment for advanced, unresectable HCC, based on an improvement in overall survival (OS) of nearly three months compared to placebo[6]. Since then, unfortunately, no agent has come to demonstrate a similar, significant survival advantage in the first-line setting. Moreover, despite numerous studies of various treatment options, no agent has proven beneficial as a standard second-line therapy. Therefore, investigation of novel and effective therapy in the second-line setting is urgently warranted.

Hypoxia, HIF-1, and cancer

Hypoxia, a reduction in tissue oxygen tension due to inadequate oxygen supply, has been implicated in pathways promoting tumor growth. Although hypoxia itself is toxic to cancer cells, it also appears to induce a series of adaptive, "pro-survival" changes in the tumor, which include a shift from aerobic to anaerobic metabolism, an increase in erythropoietin to promote rise in hemoglobin, and an increase in growth factors leading to angiogenesis[7]. Furthermore, hypoxia has been associated with resistance to chemotherapy and radiotherapy, and is closely related to poor clinical outcomes. Hypoxia-inducible factor (HIF-1) is an important transcription factor involved in the hypoxic response of cells, and functions in tumor development and progression. One of its target genes, vascular endothelial growth factor (VEGF), is one of the major components of angiogenesis and tumor proliferation. Among the subunits of HIF-1, HIF-1α has been implicated in cancer progression. This review will therefore focus on the scientific background of HIF-1α, its biology, existing pre-clinical data, and its potential role in the treatment of advanced HCC.

HIF-1: BIOLOGY AND SIGNIFICANCE

Structure of HIF-1

HIF-1 is a heterodimeric transcription factor which consists of the oxygen-sensitive HIF-1α and the constitutively expressed HIF-1β [also known as aryl hydrocarbon receptor nuclear translocator (ARNT)]. Both subunits contain basic helix-loop-helix (bHLH) motifs and PER-ARNT-SIM (PAS) domains needed for dimerization with hypoxia response elements (HRE) in the promoter region of target genes[8] (Figure 1).

HIF-1α degradation
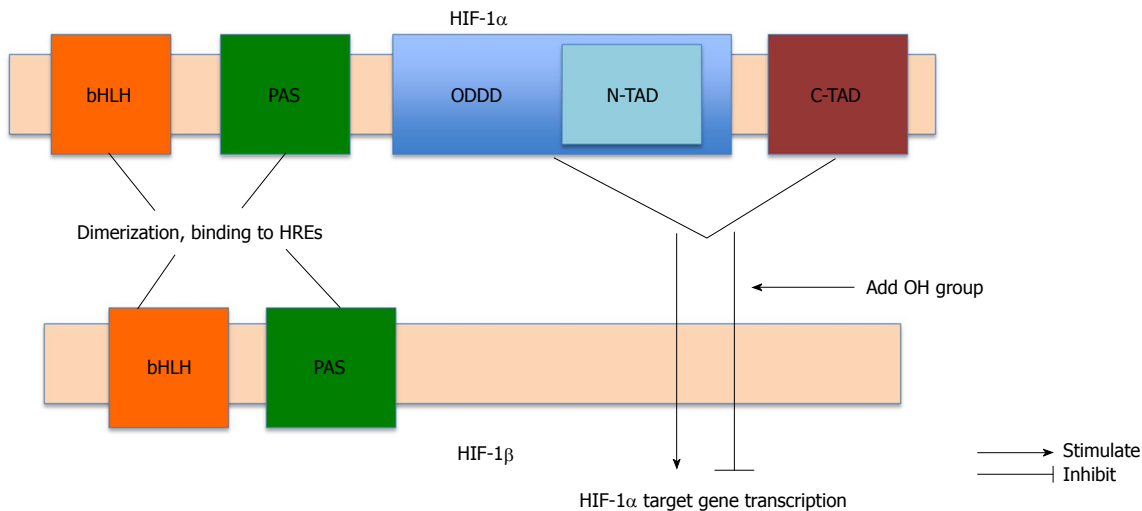
The expression of HIF-1α is determined by both its

Figure 1 Structure of hypoxia-inducible factor-1. Hypoxia-inducible factor (HIF)-1 is a heterodimer comprising HIF-1α and HIF-1β. Both subunits contain basic helix-loop helix (bHLH) motifs and PER-ARNT-SIM (PAS) domains needed for dimerization and binding to hypoxia response elements (HREs) in the promoter region of target regions. HIF-1α contains two transactivation domains: the NH₂-terminal transactivation domain (N-TAD), located on the oxygen-dependent degradation domain (ODDD), and the carboxy-terminal transactivation domain (C-TAD). Both transactivation domains are essential for promoting HIF-1α target gene transcription. Hydroxylation of N-TAD and C-TAD of HIF-1α leads to inhibition of HIF-1α target gene transcription.

synthesis, which is regulated in an oxygen independent manner, and its degradation, which is regulated in an oxygen dependent manner[9].

In the presence of oxygen or "normoxia," prolyl hydroxylases modify proline residues 402 and 564 in the NH₂-terminal transactivation domain of the oxygen-dependent degradation domain of HIF-1α, which allows for the interaction of this subunit with von Hippel Lindau (VHL) tumor suppressor protein[10-12]. VHL is then recognized by E3 ubiquitin-protein ligase which targets HIF-1α for ubiquitination and proteasomal degradation[13,14]. In addition to hydroxylation of proline residues, hydroxylation of the asparagine 803 residue in the carboxy-terminal transactivation domain (C-TAD) of HIF-1α (in the setting of normoxia) *via* factor inhibiting HIF-1 (FIH-1) blocks transcriptional coactivation of HIF-1α with p300 and CREB binding protein (CBP), and hence inhibits transcription of target genes[15,16] (Figure 2).

HIF-1α and hypoxia
Under hypoxic cellular conditions, hydroxylation decreases due to inactivation of proline hydroxylases, leading to the inability of VHL to bind to HIF-1α and diminishes the degradation of HIF-1α. Stabilized HIF-1α, in turn, accumulates and translocates from the cytoplasm into the nucleus, where it dimerizes with HIF-1β and interacts with cofactors, such as p300/CBP, to bind to DNA on HREs, ultimately activating target gene transcription and mRNA, and eventually protein synthesis (Figure 2).

HIF-1α synthesis
In addition to this oxygen dependent mechanism of regulation leading to degradation, HIF-1α synthesis is mediated by growth factor binding to tyrosine kinase receptors, causing an activation of the phosphatidylinositol 3-kinase (PI3K) and ERK mitogen-activated protein kinase (MAPK) pathways, which represent the primary pathways responsible for cell proliferation and survival[17]. PI3K activates Akt and mammalian target of rapamycin (mTOR). In the MAPK pathway, a series of kinase activation occurs from Ras ultimately to ERK. Both the PI3K and MAPK pathways converge in activating proteins that upregulate the translation of HIF-1α mRNA into protein (Figure 3).

HIF-1α: Function in cancer
Nearly 100 HIF-1 target genes have been identified[18,19]. Transcription of these target genes produce factors essential for tumorigenesis, such as angiogenesis, glucose metabolism, survival, invasion and metastasis[18,20]. Directly activated by HIF-1, VEGF is a potent growth factor stimulating proliferation of endothelial cells and promoting angiogenesis, particularly in areas of hypoxia[21]. Furthermore, hypoxia and HIF-1α cause an increased production in enzymes and glucose transporters involved primarily in oxygen-independent, anaerobic glycolysis[22,23]. Hypoxia and HIF-1α induce growth factors, such as insulin-like growth factor-2 and transforming growth factor-α, which bind to their receptors, inducing a signal transduction cascade leading to cell proliferation and survival, and in turn stimulating further production of HIF-1α[19]. To promote invasion and metastasis, HIF-1α induces a process called epithelial-mesenchymal transition by suppressing E-cadherin, which plays a role in maintaining epithelial integrity[24,25]. The reduction of E-cadherin therefore will leave more space for tumor cells to invade through the epithelial layer and eventually metastasize. In addition, HIF-1α upregulates expression of matrix metalloproteinases, which have been associated
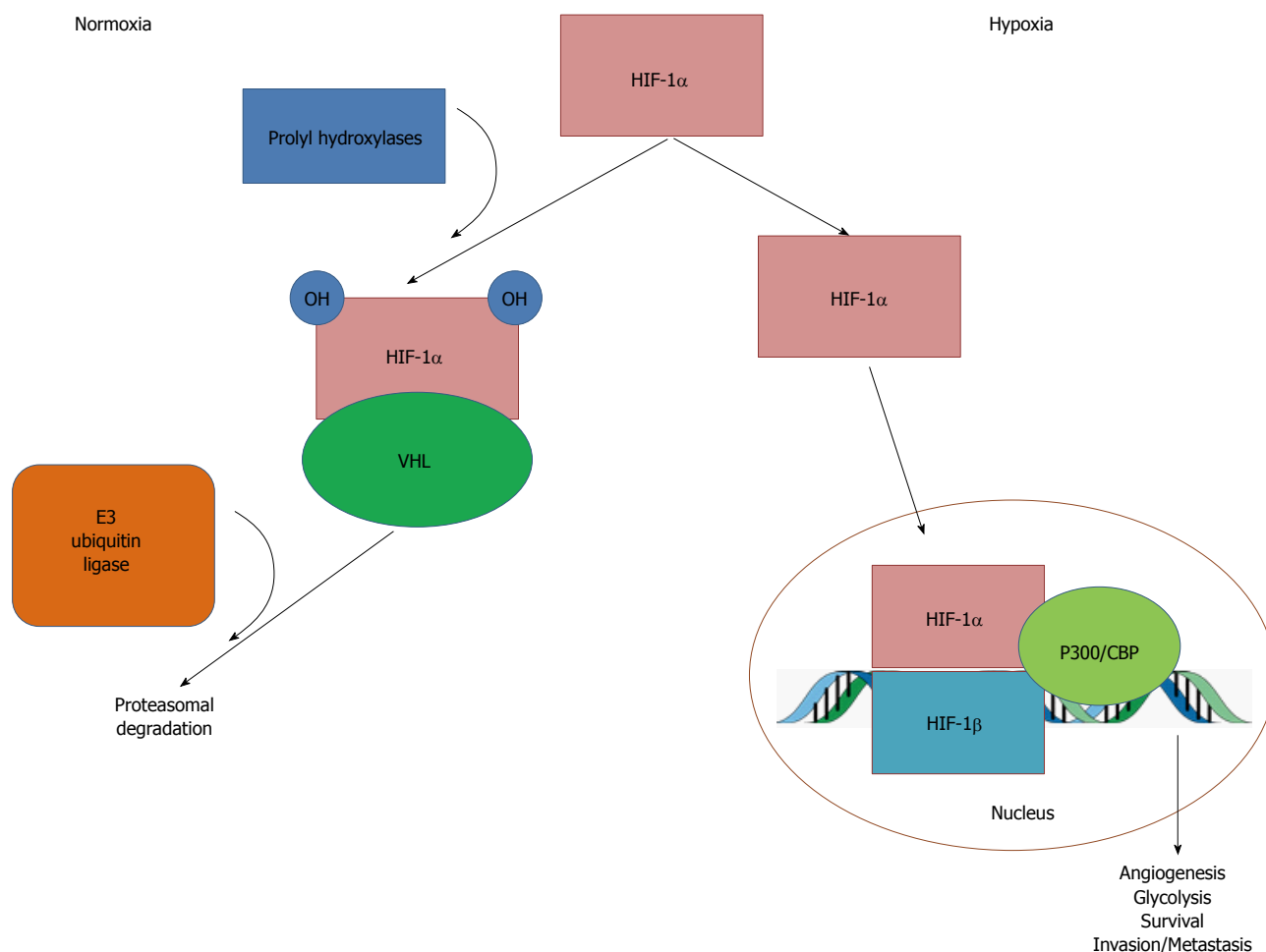
Figure 2 Oxygen-dependent mechanism of hypoxia-inducible factor-1α degradation. In the presence of oxygen (normoxia), hypoxia-inducible factor (HIF)-1α undergoes hydroxylation *via* prolyl hydroxylases. This causes HIF-1α to interact with von Hippel Lindau (VHL) tumor suppressor protein, which is in turn recognized by E3 ubiquitin ligase, which targets HIF-1α for ubiquitination and degradation. Under hypoxic conditions, reduced oxygen leads to inactivation of prolyl hydoxylases, which diminishes hydroxylation and, therefore, reduces degradation of HIF-1α. Stabilized HIF-1α accumulates and translocates into the nucleus, where it dimerizes with HIF-1β and interacts with cofactors, such as p300 and CREB binding protein, to bind to DNA on hypoxia response elements (HREs). This activates transcription of HIF-1α target genes, leading to angiogenesis, glycolysis, survival, and invasion and metastasis of cancer cells.

with the degradation of extracellular matrix (ECM) including basement membrane, removing another defense mechanism to allow tumor cells to successfully invade[26,27].

Opposite to tumorigenesis, HIF-1α also interacts with the tumor suppressor p53 gene, which in turn promotes transcription of pro-apoptotic genes. More specifically, p53 activates transcription of BAX which acts at the mitochondrial level to promote release of cytochrome C, activating a series of caspase signaling, and ultimately leading to apoptosis[28]. P53 has also been demonstrated to downregulate BCL2, an anti-apoptotic protein[29] (Figure 3). Interestingly, different downstream effects of the cell-death pathway have been reported with HIF-1α, depending on its interaction with different target genes in various types of cancer cells. In ovarian cancer cell lines, HIF-1α has been associated with better survival outcomes[30], which may be explained by the usual function of HIF-1α activating transcription of p53. However, overexpression of HIF-1α in the setting of an inactivating mutation of p53 in ovarian cancer cells has been shown to decrease apoptosis, and is associated with shorter survival in these patients[30]. In early stage esophageal cancer patients, overexpression of HIF-1α and BLC2 is associated with resistance to photodynamic therapy[31]. Furthermore, the mutation rate of p53 has been reported from 30% to as high as 67% in HCC, depending upon geographic region, with sub-Saharan Africa and East Asia with higher reported incidence[32,33]. HIF-1α expression in such tumors combined with a P53 mutation may potentially contribute to a worse prognosis in HCC.

RELEVANCE IN HCC

Hypoxia in HCC
Hypoxia plays a significant role in HCC development. Since HCC typically arises in the setting of cirrhosis induced by chronic liver injury, fibrinogenesis that results from liver injury and cirrhosis leads to reduction in vascularization, which contributes to hypoxia[34]. As
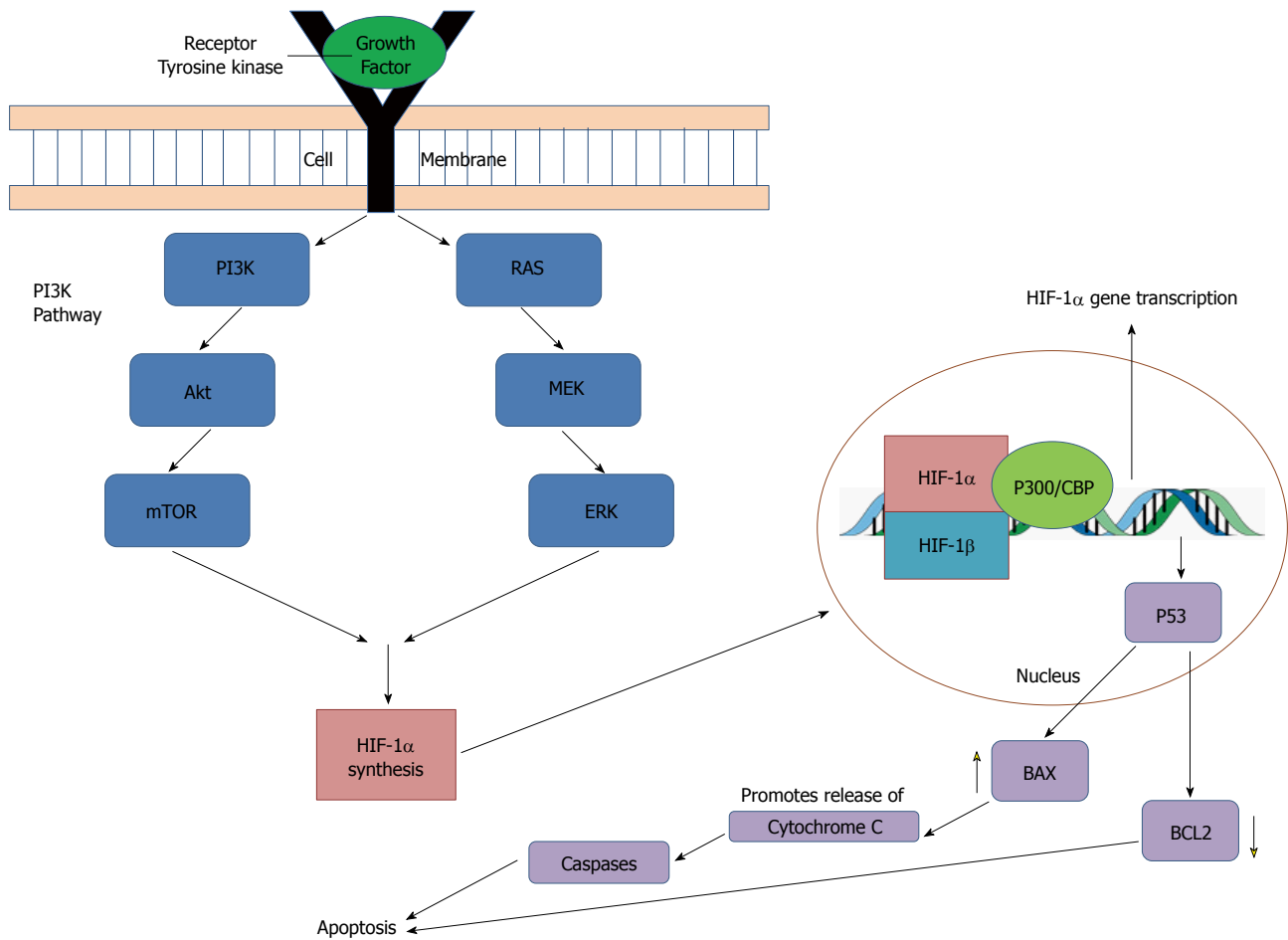
Figure 3 Hypoxia inducible factor 1α: protein synthesis and relation to apoptotis. Hypoxia-inducible factor (HIF)-1α synthesis is upregulated by growth factor binding to tyrosine kinase receptors, causing activation of two pathways essential for cell proliferation and survival: the phosphatidylinositol 3-kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway. Extracellular signal-related kinase (ERK) and mitogen/extracellular signal-related kinase (MEK) represent members of the MAPK family which are activated as part of a signaling cascade. HIF-1α also interacts with p53, a tumor suppressor gene, which leads to transcription of pro-apoptotic genes. p53 activates transcription of BAX which acts on mitochondria to promote release of cytochrome C, activating a series of caspase signaling, which ultimately promotes apoptosis. In addition, p53 also downregulates BCL2, an anti-apoptotic protein. Together, these actions serve to increase apoptosis. mTOR: Mammalian target of rapamycin.

a result, HCC may present as cavitary lesions in the liver due to rapid growth of tumor, leading to necrosis and hypoxia. Although hypoxia can suppress cell proliferation and survival in normal cells, HCC cell lines exhibit normal cell cycle despite hypoxia, due to HIF-1α upregulated growth factors, such as VEGF which promotes tumor proliferation, and hexokinases which help generate ATP to provide an energy source for HCC cells[35,36].

HIF-1α overexpression in HCC
Identified as a poor prognostic factor in patients with varying malignancies[37], high HIF-1α expression, as measured by immunohistochemical analysis using monoclonal antibodies, has been correlated with worse clinical outcomes in patients with HCC[38]. In a study by Yang *et al*[39], HIF-1α exhibited high expression in intratumoral tumor tissue, and was closely associated with capsular infiltration and portal vein invasion; more importantly, it was associated with shorter disease free survival (DFS) and overall survival (OS). Xiang *et al*[40]

evaluated HIF-1α expression in HCC patient tumor samples and correlated expression with response to treatment of abdominal lymph node metastases with external beam radiotherapy (EBRT). They found that high intratumoral HIF-1α expression was associated with worse OS rates, and lower response to EBRT. Due to its association with clinical outcomes, HIF-1α may therefore also serve as a potential biomarker for response to treatment in HCC.

HIF-1α: Pre-clinical data in HCC
Experiments in hepatoma cells by Xia *et al*[41] suggest that an interaction between HIF-1α and TNF-α leads to binding to a proliferation-specific transcription factor, Forkhead box M1 (FoxM1), enhancing proliferation of hepatoma cells and resistance to apoptosis. Furthermore, Xu *et al*[42] found that HIF-1α induced cell proliferation and cell cycle progression in hepatoma HepG2 cells by influencing expression of Cyclin A and Cyclin D. In the setting of hypoxia, HIF-1α has also been demonstrated to facilitate transcription of MDR

(multi-drug resistance) related genes in HepG2 cell lines, contributing to resistance to chemotherapeutic agents, such as 5-Fluorouracil (5-FU)[43]. The activity and relationship of HIF-1α in tumorigenesis have been examined in xenograft assays, where tumor cells are subcutaneously injected into immunodeficient mice to evaluate for gene involvement in tumor growth. Embryonic stem (ES) cells in HIF-1α knockout mice exhibit impaired vascularization in xenografts, and experience early in utero demise[44]. Although these studies involving cancer cell lines or xenografts are limited by the lack of genetic heterogeneity that is found in actual human tumors and the interactions that may occur between tumor and stromal environment, they serve as a step towards further investigations *in vivo*.

FUTURE DIRECTION: POTENTIAL THERAPEUTIC ROLE OF HIF-1α

Strategies targeting HIF-1 levels have a therapeutic potential in HCC, since they may interrupt multiple pathways implicated in angiogenesis, tumor metabolism, invasion and survival. Down-regulation of the HIF-1 complex *via* activation of hydroxylases, through inhibition of HIF-1α binding to coactivators, and through small molecule inhibitors has been studied. In a study by Knowles *et al*[45], ascorbate was found to suppress HIF-1α protein expression in human cancer cell lines, by activating hydroxylases and promoting HIF-1 degradation. Kung *et al*[46] reported reduced growth of tumor in xenograft assays *via* injection into nude mice of a fusion protein compromising GAL4 fused to the C-TAD of HIF-1α, which blocks binding of HIF-1α to its coactivators, p300/CREB. Furthermore, small molecule inhibitors, such as topotecan, a topoisomerase inhibitor, have been reported to negatively affect ribosome entry on HIF-1α mRNA, preventing translation of protein[47].

Another avenue of investigation has been the development of a HIF-1α mRNA antagonist. SPC2968 is a HIF-1α mRNA antagonist, which is a locked nucleic acid (LNA) antisense oligonucleotide, causing a down-modulation of HIF-1α mRNA and protein. LNA oligonucleotides represent a new class of nucleic acid analogs, in which conformational changes in the chemical structure lead to higher affinity for mRNA and higher potency in downregulation. This agent has undergone phase I testing in advanced malignancies to find the maximum tolerated dose and dose limiting toxicities. Though a reduction in tumor size was noted, there was no correlation with clinical efficacy. A further exploration of this HIF-1α mRNA antagonist (RO7070179) is underway in a Phase Ib proof-of-mechanism trial investigating this agent in patients with HCC after failure of at least one line of systemic therapy[48].

The therapeutic potential for HIF-1α directed therapy also lies in the possibility of combining treatment with other targeted therapies to enhance efficacy and prevent resistance. For example, a HIF-1α inhibitor may be combined with drugs that target the MAPK-RAF-ERK pathway, such as Sorafenib and Regorafenib. Liang *et al*[49] reported the ability to overcome intratumoral hypoxia-related Sorafenib resistance in HCC cells by treating them with EF24, which causes VHL-dependent HIF-1α degradation and NF-κB inactivation. Combination of HIF-1 inhibitors with mTOR inhibitors, which act upstream of HIF-1α, such as Everolimus, may also downregulate synthesis of HIF-1α and attenuate downstream signaling. Given the regulatory role of HIF-1α in apoptotic pathways, combination therapy with Stat3 inhibitors, which upregulate expression of p53 (downstream of HIF-1α, or with BCL2 inhibitors (also downstream of p53), would increase cancer cell apoptosis and augment the effect of HIF-1α inhibition (Figure 2).

Though HCC is considered relatively chemoresistant, possibly owing to a HIF-1 induced increase in multidrug resistance gene expression[50], a HIF-1α inhibitor may ultimately aid in absorption of systemic chemotherapy agents, such as doxorubicin, and enhance its effectiveness in HCC[51].

Finally, while early phase studies demonstrated no clinical efficacy with HIF-1α mRNA antagonists, the finding of tumor size reduction with therapy may be clinically significant for HCC patients who are not transplant candidates due to large tumor size. Given that there is no effective treatment which significantly reduces tumor size, a therapeutic intervention to downstage tumor may open the possibility of liver transplant in these patients for whom cure was previously not considered.

Understanding the biology and various pathways implicated in the pathogenesis of HCC is essential for the development of effective targeted interventions in advanced HCC. HIF-1α inhibition, particularly in combination with other therapies, is a promising area of research with the potential to help further the advances in systemic treatment of HCC.

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