Role of hypoxia-inducible factor in clear cell renal cell carcinoma and its application in targeted therapy

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Abstract: Hypoxia is a common feature of many solid tumors such as renal cell carcinoma (RCC). Hypoxia-inducible factors (HIF), namely HIF-1α, -2α, and -3α, have been found to play important roles in tumors. As a key mediator of hypoxic responses, HIF-1α activates hypoxia-responsive genes, which are closely correlated with processes related to tumorigenesis and cancer progression, including cell proliferation, metabolism, angiogenesis, invasion, metastasis, and therapy. As 70-80% of RCCs are clear cell renal cell carcinomas (CCRCCs). With regard to the etiology of CCRCC, the article presents molecular pathways such as VHL-HIF-EPO pathway. The downstream effects of this pathway in RCC include upregulation of vascular endothelial growth factor production and increase in related inflammatory reactions. Importantly, HIF is a key component in this pathway. Previous findings indicate that upregulation of HIF-1α is associated with poor prognosis in CCRCC patients. Thus, the article discusses HIF-1α as a therapeutic target for CCRCC. The HIF-1α overexpression observed in CCRCC may be regarded as a proposing signaling or an indicator for the potential of HIF-α targeted therapy in CCRCC.

Keywords: Hypoxia-inducible factor, clear cell renal cell carcinoma, targeted therapy

Introduction

Currently, renal cell carcinoma (RCC) accounts for 3% of all adult tumors and 95% of all renal malignancies [1]. The number of new emerging cases is reaching 210,000 per year [2]. RCC has multiple subtypes with high heterogeneity [3], the classification of RCC has modified in 2004 [4]. The International Society of Urological Pathology (ISUP) 2012 consensus conference suggested modifications to the existing WHO 2004 categories [5]. The new classification includes 15 subtypes, of which three predominant types in malignant RCC tumors are clear cell renal cell carcinomas (CCRCs, covering 70-80%), papillary renal cell carcinomas (10-15%) [6], and chromophobe renal cell carcinoma (5%). A small proportion of rare types, including papillary adenoma, oncocytoma, carcinoma of the collecting ducts of bellini, renal medullary carcinoma, MIT family translocation renal cell carcinoma, carcinoma associated with neuroblastoma, mucinous tubular and spindle cell carcinoma, tubulocystic renal cell carcinoma, acquired cystic disease associated

Gene mutations have accepted generally as the etiology of RCC. Among other causes, the von Hippel-Lindau (VHL) mutation and its protein product are an important component of the cell's response to hypoxia [7]. Mutations in this cancer suppressor gene, VHL, play a critical role in the development of the most prevalent CCRCs by causing a loss of a segment of chromosome 3 [2, 8]. In the case of CCRCC, loss of function in both VHL alleles could be caused by chromosomal mutations or altered methylation in the promoter. Some studies have indicated
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that 19% of CCRCC cases present with promoter aberrant methylation, 27-56% present with gene mutation, and 12.5% present with both promoter methylation and gene mutation; however, in around 25% of the cases, none of these has been observed [9-11].

Hypoxia and hypoxia-inducible factors (HIFs)

Hypoxia is a common feature in locally advanced solid tumors that has been found to be associated with decreased response to treatment and malignant progression, causing an increasing probability of recurrence, spread, and metastasis. HIFs are a group of transcription factors for most hypoxia-inducible genes responding to changes in available oxygen in the cellular environment, decreases in oxygen levels, or hypoxia [12]. HIF is a major regulator of the adaptation of tumor cells to hypoxic stress.

Structure and function of HIFs

HIF is widely expressed in various tissues in mammals, and the nuclear transcription complex containing HIF mediates the hypoxia response of mammalian cells. Semenza, and Wang while extracting the nucleus from hypoxic cells [13] discovered HIF first in 1992. As a heterodimer, HIF is formed of two subunits, i.e., 120-kD HIF-α and 91-94-kD HIF-β (Figure 1). Being the active subunit, the coding region of the HIF-α gene is located in q21-24 on chromosome 14 and regulated by hypoxic signaling. The HIF-β gene is coded in q21 chromosome 1 and is expressed constantly in the nucleus and cytoplasm of both normal and hypoxic cells, where it functions as a cellular skeletal protein.

Both the subunits belong to the family of basic-helix-loop-helix transcriptional factors (bHLH), which have the PER-ARNT-SIM (PAS) structure [14]. In the N-terminus of the subunits, there are bHLH and PAS structures, which are necessary for the formation of the heterodimer and for binding with DNA [15]. The consensus HIF-1 binding sequences (hypoxia response elements, HREs) have been published earlier, known as A/G TACGTGGG [16].

HIF is required for embryonic development in mice [17], and plays a key role in ischemic cardiovascular disease, stroke, and cancer [18-20]. The synthesized HIF protein, however, degraded rapidly by the intracellular, oxygen-dependent ubiquitin-proteasome under normoxic conditions, but is stably expressed under hypoxic conditions [12]. In mammals, active HIF-α subunits contains three subtypes, i.e., HIF-1α, -2α and -3α [21]. The activity of HIF is mainly regulated by oxygen through these subunits. HIF-1α is expressed throughout the whole body. Delineation of the mechanisms that regulate HIF-1 activity in these contexts has become a major challenge in contemporary molecular and cell biology [22]. In contrast, HIF-2α not only exists in some specific cell types including endothelia, hepatocytes, Type II alveolar cells, and macrophages [23], but can also be found in the nucleus of CCRCCs [24]. In the kidney, for example, HIF-1α expression can be detected predominantly in tubular cells. In contrast, renal interstitial fibroblasts, endothelial cells, and some glomerular cells show HIF-2α positivity [25]. Likewise, recent increasing evidence supports that different subunits play different roles. For example, HIF-1α is thought to be the main hypoxic inducible transcriptional factor in breast cancer and endothelial cancer, while HIF-2α, on the other hand, plays an important role in angiogenesis in hepatocellular carcinoma and RCC [26, 27]. It is accepted generally that HIF-1α and HIF-2α are involved in transcriptional regulation, and HIF-3α plays inhibits HIF-1α, HIF-2α, but has no ability of transcription activation. Interestingly, a recent study regarding the function of HIF-3α in zebra fish showed that HIF-3α has strong transactivation activity in this species. HIF-3α not only mediates hypoxia-induced growth and developmental retardation but also possesses hypoxia-independent activities. A recent report revealed that the transactivation activity is conserved in
human HIF-3α, which is upregulated similar to HIF-1α and HIF-2α in human cells. These findings suggest that HIF-3α is also an oxygen-dependent transcription factor and activates the transcriptional response to hypoxia [28].

Usually, the stable expression of HIF-β in the cytoplasm plays a structural role. Under cellular hypoxic conditions, HIF-β translocates from the cytoplasm to the nucleus, thus causing more HIF-β to be present in the nucleus, which consequently forms dimers with HIF-α; when the oxygen tension returns to normal, HIF-β again translocates to the cytoplasm, and the intramuscular high HIF-α level rapidly vanishes [29].

**Target genes of HIF**

HIF is a transcriptional factor for many target genes. It is involved in the processes of cancer progression, including angiogenesis, erythropoiesis, glucose metabolism, cell proliferation, and apoptosis [30]. For example, the level of HIF-1α is significantly higher in primary and metastatic CCRCC than in benign tissues [31].

Most tumor cells become hypoxic under conditions of uncontrolled growth and in the hypermetabolic state. In response to this hypoxic environment, tumor cells adopt one or both of the following strategies: increasing glycolysis or angiogenesis. During these activities, HIFs play a very important role in maintaining the homeostasis of the tissue and cells under the hypoxic conditions.

Erythropoietin (EPO) is one class of HIF-target genes. Vascular endothelial growth factor (VEGF) is one of the EPOs, which is directly involved in angiogenesis [32]. Endothelin-1 (ET-1), platelet derived growth factor (PDGF), angiopoietin 2 (ANGPT2) and others also play important roles in angiogenesis, but they participate in the process through more complex mechanisms, rather than through the direct way as VEGF does [33].

Another essential process in cancer biology that HIF is associated with is glucose metabolism. HIF-α regulates the expression of many enzymes in the glycolytic pathway, which allows tumors to survive in hypoxic environments. In the absence of oxygen, tumors use anaerobic glycolysis for metabolizing glucose [34]. Glucose transporter 1, 3 (GLUT1, 3) and glycolytic enzymes, which include hexokinase (HK), lactate dehydrogenase A (LDHA), aldolase A (ALDA), enolase 1 (ENO1), monocarboxylate transporter 4 (MCT4), pyruvate dehydrogenase kinase 1 (PDK1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphofructokinase L (PFKL), phosphoglycerate kinase 1 (PGK1), and MAX interactor 1 (MXI1), participate in glucose metabolism. HIF activates GLUT1 and 3, thus mediates cellular glucose uptake. Under hypoxia, pyruvate, a product of glycolysis, is converted to lactate by HK and LDHA [35].

**Regulatory role of HIF in CCRCC**

Several molecular pathways are involved in the oncogenesis of CCRCC. The VHL-HIF-EPO pathway is one of the well-studied pathways involved in the etiology of CCRCC. Under normoxic conditions, the VHL gene product, the ubiquitin ligase responsible for regulating HIF-α protein levels, efficiently targets HIF-α for rapid proteasome-dependent degradation. Usually, the subunit HIF-α has a shorter life, and HIF-α protein is rapidly degraded under normoxic conditions [36, 37], as it is degraded by an event largely mediated by a functional VHL [38, 39]. As shown in Figure 2, the functional protein product of VHL, pVHL, forms a pVHL-E3 ubiquitin ligase (pVHL-E3) complex with elongin B, elongin C, Cui2, and Rbx1 [40]. This pVHL-E3 complex can bind with HIF-1α under normal oxygen tension, which leads to its polyubiquitination...
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[41]. Its α subunit can be hydroxylated by three different prolylhydroxylases, which can be further ubiquitinated by the pVHL-E3 complex and degraded through the ubiquitin-proteasome proteolytic (UP) pathway. Therefore, HIF-α is hardly detected in normal cells.

Importantly, in the case of cancer hypoxia, the VHL gene is mutated; the formation of the VHL protein itself or that of the E3 ubiquitin ligase complex is either decreased or blocked. Degradation of HIF-α is decreased, leading to its accumulation in the cytoplasm [42]. HIF-α therefore translocates into the nucleus, and thus HIF-α can be observed in the cytoplasm as well as the nucleus of cancer cells [43]. In the nucleus, subunits HIF-α and HIF-β form dimers, which trans-activate the target genes of HIF, especially EPO. EPO mRNA forms EPOR with its receptors for participating in tumor progression, for example, by upregulating the production of VEGF, increasing inflammatory reactions, and decreasing intrinsic drug-induced apoptosis. In the end, these events eventually increase the likelihood of tumor angiogenesis and progression.

Under normoxic conditions, a functional VHL gene produces pVHL, which forms a pVHL-E3 ligase complex and mediates the polyubiquitination and proteasomal degradation of HIF. Under hypoxia, the degradation of HIF-α is decreased, leading to its accumulation in the cytoplasm. This HIF-α translocates into the nucleus, where it dimerizes with HIF-β. These dimers transactivate downstream target genes, especially EPO. EPO mRNA forms EPOR with its receptors for participating in tumor progression, for example, by upregulating the production of VEGF, increasing inflammatory reactions, and decreasing intrinsic drug-induced apoptosis. In the end, these events eventually increase the likelihood of tumor angiogenesis and progression.

**Therapy for CCRCC**

**Suppressing HIF expression**

Tumors are formed by over-differentiation and proliferation of cancer stem cells (CSCs). The shrinkage of tumor was used as an efficient parameter in the clinic to determine after radiotherapy and/or chemotherapy. In fact, after a certain period of remission, tumors recur, as only the fast-differentiating tumor cells are killed but CSCs are not and they show strong resistance to radio- and/or chemotherapy. Therefore, traditional treatment methods such as those involving the use of interferon and interleukin have only seen 15-20% efficiency in CCRCC [44]. Thus, the cancer could not be really cured, as CSCs are not killed; at the same time, the CSCs may cause resistance to radiation and chemotherapeutic medication. There are several mechanisms underlying the resistance of CSCs to radio- and chemotherapy. One of these mechanisms is the upregulation of ATP-binding cassette (ABC)-transporting protein, which includes ATP-binding cassette B1 (ABCB1), C1 (ABCC1), and G2 (ABCG2). All three genes are multidrug resistance genes. These ABC-transporting proteins act by transferring the chemotherapeutic medicine out of the cells by utilizing the ATP energy generated in the cells, thus resulting in lower concentration of the drugs, which eventually compromises the effectiveness of the chemotherapy medications and confers drug resistance to the cells [45]. Furthermore, the ABC-transporting protein has also been reported as one of the direct downstream target genes of HIF in breast cancer. This is consistent with the finding that the ATP-binding cassette transporting protein expression in hypoxia-induced CSCs is one of the mechanisms underlying the chemotherapy resistance [46]. In addition, immunohistochemical analyses conducted to determine if HIF is involved in drug resistance through an indirect process revealed that HIF expression is also increased in other types of tumors. For example, in oropharyngeal cancer, the overexpression of HIF was found to be closely related to radiotherapy resistance and poor prognosis; in addition, the degree of its expression had a predictive and prognostic significance in individuals undergoing curative radiation therapy [47].

Given the important role of HIF, an HIF suppressor would provide a potential therapeutic strategy. In a different study aimed at identifying micromolecular HIF suppressors, it was found that 3-(5’-hydroxymethyl-2’-furyl)-1-benzylindazole (YC-1) could inhibit the activity of HIF and the growth of the transplanted tumor [48]. After YC-1 was administered and reached to tumor tissue, the expression of HIF-α as well as angiogenesis was obviously decreased; furthermore, the expression of the downstream target genes
of HIF, such as VEGF, was also suppressed. HIF can also directly interact with heat shock protein 90 (HSP90), which binds to the HIF-α PAS domain. After separation of HIF-1α and HSP90, ubiquitin-proteasome-induced HIF-1α degradation is increased under normoxic conditions. HSP90 inhibitors such as geldanamycin, 17-allylamino-geldanamycin (17-AAG), and proteasomal can induce HIF-α degradation in a VHL-dependent way [49, 50]. In addition, 2-methoxyestradiol (2ME2) not only interrupts the aggregation of microtubules, but also decreases HIF levels and VEGF mRNA expression [51].

**Targeted therapy for RCC**

Targeted therapy involves the design of corresponding bio-micro medication at the cellular and molecular level according to certain specific signal transduction pathways or some oncogenic genes that have already assured from development of tumor. After administration into the body, these drugs for targeted therapy specifically interact with or interrupt relevant signal transduction pathways or oncogenic gene domains and kill tumor cells and/or attenuate vascular growth without disturbing the surrounding normal tissue and cells. Therefore, molecular targeted therapy is also called a bio-missile. Currently, targeted therapy has been applied in colorectal cancer, breast cancer, prostate cancer, and CCRCC. Medications used in such targeted therapy are classified as follows: 1) epidermal growth factor receptor (EGFR)-targeting blockers; 2) tumor angiogenesis inhibitors such as bevacizumab, a recombinant human anti-VEGF monoclonal antibody, and endostatin, an intrinsic anti-angiogenesis factor extracted from angioendothelioma; 3) monoclonal antibody against certain cellular oncological markers, such as cetuximab and trastuzumab for human epidermal receptor 2 (HER-2), and rituximab for CD20; 4) kinase inhibitors such as Bcr-Abl1 tyrosine kinase inhibitor imatinib and dasatinib, the insulin-like growth factor receptor-1 (IGFR1) kinase inhibitor, hydrochloride (NVP-AEW541), the mTOR kinase inhibitor temsirolimus (CCI-779), and the aurora kinase inhibitor histone deacetylase (HDACs); and 5) the ubiquitin-proteasome inhibitor bortezomib.

For CCRCC, the main drugs used for targeted therapy are VEGF inhibitors, which are tumor angiogenesis inhibitors. In addition to VEGF that is extensively expressed and is a known oncogenic gene in CCRCC, platelet derived growth factor (PDGF) is also expressed in CCRCC. Moreover, VEGF and PDGF domains are critical components for angiogenesis and tumor cell proliferation. One of the downstream effects of the VHL-HIF-EPO pathway is the up-regulation of VEGF production and an increase in inflammation reactions. There are two common groups of targeted therapy medications: small molecule tyrosine kinase inhibitors and VEGF monoclonal antibody.

Small molecule tyrosine kinase inhibitors, such as sunitinib, sorafenib and pazopanib, exert their functions by suppressing intracellular protein kinase activity of VEGF receptors [52]. In recent studies of CCRCC treatment using tyrosine kinase inhibitors of VEGFR and PDGRF (sunitinib, sorafenib), the mortality associated with CCRCC was reported to decrease [44, 53, 54]. The anti-tumor effect can be significantly improved by combined treatment with crizotinib and axitinib. Combination treatment also prolongs patient survival and significantly inhibits tumor growth by inhibiting angiogenesis [55].

In addition, as a tyrosine kinase inhibitor, glivec exerts its anti-tumor effects through two mechanisms: 1) directly attenuates tumor growth by suppressing the RAF/MEK/ERK signal transduction pathway; 2) inhibits tumor angiogenesis by blocking the receptors of VEGF and PDGF, resulting in the indirect attenuation of tumor growth. Although glivec has commonly used for gastrointestinal stromal tumor and chronic myelogenous leukemia, it can give us a few insights on the drug’s possible application in CCRCC targeted treatment.

The second group of targeted therapy medications used is VEGF monoclonal antibodies such as bevacizumab, which suppresses tumor growth by blocking the interaction between VEGF and the intrinsic VEGF receptors [56]. A retrospective review from 2005 to 2008 to identify RCC patients who had undergone debulking nephrectomy followed by VEGF-targeted therapy with sunitinib, sorafenib, or bevacizumab showed a significant improvement in the progression-free survival of the patients [57].

**Summary and perspectives**

In summary, increasing number of studies have provided evidence that HIF plays an important
role in CCRCC tumorigenesis, progression, and metastasis, and that it is involved in cellular proliferation, angiogenesis, invasion, and resistance to radiotherapy and chemotherapy. Clinical data also indicate that HIF-1α overexpression is associated with poor prognosis in CCRCC [58]; therefore, HIF-α is strongly suggested as a target for CCRCC treatment. The HIF-1α overexpression observed in CCRCC may be regarded as a proposing signaling or an indicator for the potential of HIF-α targeted therapy in CCRCC. Accordingly, some small molecule inhibitors of the HIF pathway have been used in combination with other therapeutic drugs to improve anticancer efficacy [59]. However, these small molecule inhibitors are currently used only for late stage cancer patients; if this medication could be used for early stage cancer patients as well, it may improve anticancer efficacy and the overall survival rate. Further studies are needed to investigate this possibility.

Although different approaches to identify HIF pathway inhibitors that effectively inhibit renal cell carcinoma tumorigenesis have been applied, the application of the developed agents have some limitations such as the undesirable side effects, relatively low specificity, less obvious antitumor effects, and the lack of an evaluation system for clinical efficacy. Thus, further studies are needed to optimize the application of the existing therapeutic agents targeting HIF to improve clinical outcomes. Furthermore, it is an urgent task to search for novel targets for gene therapy for CCRCC.

The increasing number of studies on gene targeting treatments for RCC has provided us the directions for future studies in the following aspects: 1) as biomarkers being used to screen first-line medications for individualized therapy; 2) as targeting medication prescribed reasonably, which may achieve the better outcome when therapy combined with other targeting medications; 3) further studies may focus on reducing side effects and the tolerance of targeting medications; 4) under the current health system, appropriate therapy needs to be developed for specific populations to get maximum-effectiveness with minimum cost; 5) the right time point for starting targeted therapy should be determined to achieve the ideal treatment effect.

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Disclosure of conflict of interest

None.

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