Research Article

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Overexpression of von Hippel–Lindau protein synergizes with doxorubicin to suppress hepatocellular carcinoma in mice

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Background & Aims: Hypoxia-inducible factors (HIFs) and nuclear factor-κB (NF-κB) regulate genes involved in carcinogenesis and progression of cancers including hepatocellular carcinoma (HCC). The von Hippel–Lindau (VHL) protein (pVHL) targets HIFα subunits for destruction and participates in modulating the activity of NF-κB. The present study aimed to investigate whether the overexpression of pVHL synergizes with doxorubicin in the treatment of HCC.

Methods: Overexpression of pVHL was induced by infecting mouse HCC Hepa1–6 and H22 cells, or injecting subcutaneous Hepa1–6 tumors in C57BL/c mice, with adenoviral vectors encoding mouse *VHL* gene. Cell proliferation, apoptosis, tumoral angiogenesis, and gene expression and DNA-binding activity of NF-κB were examined. The therapeutic effects of pVHL were also evaluated in orthotopic Hepa1–6 tumors by intraportal delivery of Ad-VHL.

Results: Ad-VHL enhanced the anti-tumor activity of doxorubicin by inhibiting cell proliferation, and causing cell cycle arrest and apoptosis. The Ad-VHL infection downregulated HIF-1α and HIF-2α expression, and inhibited NF-κB activity and the expression of genes involved in apoptosis, proliferation, angiogenesis, invasion, and metastasis. Injection of Ad-VHL into HCC tumors augmented doxorubicin-induced suppression of tumor growth by inhibiting cell proliferation and tumor angiogenesis, and by inducing cell apoptosis. Effects on the expression of HIFαs, activity of NF-κB, and their downstream genes were in accordance with the *in vitro* findings. Intraportal injection of Ad-VHL enhanced the efficacy of doxorubicin to suppress the growth of orthotopic liver tumors.

Conclusions: By targeting HIF and NF-κB, overexpression of pVHL enhances the efficacy of doxorubicin, and warrants consideration as a potential therapeutic strategy for treating HCC.

Keywords: von Hippel–Lindau protein; Hepatocellular carcinoma; Hypoxia-inducible factor α; Nuclear factor-κB.

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Abbreviations: HCC, hepatocellular carcinoma; HIF, hypoxia inducible factor; HRE, hypoxia-response-element; VHL, von Hippel–Lindau gene; pVHL, VHL tumor suppressor protein; VEGF, vascular endothelial growth factor; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; MMP-9, matrix metalloproteinase 9; CXCR4, CXC chemokine receptor 4; TGF-β, transforming growth factor-β; MOI, multiplicity of infection.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide and has a very poor prognosis [1]. Despite extensive exploration for novel therapeutic drugs and strategies, there has been little success in improving the survival of HCC patients. A recent clinical investigation indicates that none of the adjuvant protocols is particularly effective in the treatment of HCC after surgery [2]. Sorafenib is the only drug which has been recommended as the first line treatment for HCC patients who can no longer be treated with potentially more effective therapies including arterial chemoembolization (TACE) [3], but it has not been widely accepted since it is cost-prohibitive. Doxorubicin is arguably the most commonly used traditional chemotherapeutic drug for treating HCC, especially by means of TACE [3]. Unfortunately, systemic administration of doxorubicin provided a response rate only of 4% in a recent clinical trial involving a large number of HCC patients [4]. Therefore, new strategies to enhance the efficacy of doxorubicin to treat HCC are needed.

Hypoxic microenvironments are common within solid tumors including HCC, and associated with failure of treatment and poor prognosis [5]. Over a hundred genes are regulated by hypoxia-inducible factor (HIF), including those controlling tumor angiogenesis, energy metabolism and cell proliferation [6]. The discovery of HIF-1α subunit was followed by the discovery of the HIF-2α and HIF-3α subunits [7]. While HIF-3α is estranged from the other two subunits, HIF-2α shares 48% similarity in amino acid sequence with HIF-1α. Both HIF-1α and HIF-2α activate transcription of hypoxia-inducible genes in an HRE (hypoxia-response-element)-dependent manner [8]. HIF-1α is widely expressed in all tissues, while HIF-2α is intensely expressed in the liver and intestine, and more closely related to the prognosis of HCC [8,9]. Conditional inactivation of HIF-2α suppressed the development of VHL-associated liver hemangiomas, suggesting that liver angiogenesis is predominantly regulated by HIF-2α [10].

Cancer

Germline mutations in the von Hippel–Lindau (*VHL*) gene and somatic inactivation or loss of the wild-type *VHL* allele are linked to the development of tumors. The VHL protein (pVHL) mediates an important control mechanism by targeting HIFα subunits for ubiquitin-mediated degradation [5]. The α domain of pVHL binds elongin C, which recruits additional proteins to form a ubiquinating complex; and its β domain binds the HIFα subunits for ubiquitination and proteasomal destruction in a VHL-dependent manner [7]. Therefore, inactivation of pVHL by mutation leads to the accumulation of HIFα subunits, and reintroduction of VHL into VHL-deficient cells suppresses the capacity of the cells to form tumors *in vivo*, an effect attributed to HIFα degradation [11].

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor which has recently emerged as a cancer target. Activated NF-κB translocates to the nucleus and binds to cognate sequences in the promoter region of over 60 genes involved in cell proliferation (cyclin D1), angiogenesis (VEGF), apoptosis (Bcl-2 family), and chemoresistance [12]. Inhibition of NF-κB activity enhances the efficacy of doxorubicin against various types of cancer [13,14]. NF-κB plays a key role in carcinogenesis and the progression of HCC [15]. It has been demonstrated that VHL modulates the activity of NF-κB, in addition to regulating HIFs [16,17]. The activity of NF-κB is increased in VHL-deficient renal cancer cells, and overexpression of pVHL sensitizes renal cancer cells to cytotoxicity induced by tumor necrosis factor-α (TNF-α) or bortezomib through a NF-κB-dependent mechanism [18,19]. VHL has also been shown to inhibit the expression of survivin [18], Bcl-2 [19], cyclin D1 [20], matrix metalloproteinase 9 (MMP-9) [21] and CXC chemokine receptor 4 (CXCR4) [22], to activate the transcription of p53 [23], and to regulate the turnover of the extracellular matrix and intracellular junctions [24], which are involved in tumor progression and metastasis.

All the above studies indicate that VHL may be a promising target for HCC therapy as it regulates genes involved in different aspects of cancer. We have previously demonstrated that overexpression of pVHL synergizes with antisense HIF-1α to suppress the growth of lymphoma [25] and glioma [26]. Recently we demonstrated synergistic effects between antisense HIF-1α and doxorubicin [27]. Here, we investigated whether overexpression of pVHL may have a therapeutic benefit by enhancing the efficacy of doxorubicin to combat HCC.

Materials and methods

Mice, cells, and reagents

Male C57BL/6 mice (6–8 weeks old) were obtained from the Experimental Animal Research Center, the First Affiliated Hospital of Harbin Medical University, China. All surgical procedures and care administered to the animals were approved by the institutional ethics committee. The human embryonic kidney cell line 293 (HEK293) and the mouse HCC cell line Hepa1–6 of C57BL/6 (H-2b) origin were purchased from the American Type Culture Collection (Rockville, MD, USA). The mouse HCC cell line H22 was purchased from China Center for Type Culture Collection (CCTCC, Wuhan, China). The cells were cultured at 37 °C in DMEM medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS). Antibodies used in this study included Antibodies against Bcl-2, caspase-3, cyclin D1, survivin, CXCR4 and GAPDH (Santa Cruz Biotechnology, CA, USA), Antibodies against Ki67, HIF-1α, HIF-2α, and CD31 (Abcam Inc., MA, USA), Antibodies against MMP-9 and COX-2 (Cell Signaling Technology, Inc., MA, USA), and an Antibody against VEGF (Millipore, CA, USA). Doxorubicin was purchased from Pharmacia, Milan, Italy.

Construction and preparation of adenovirus vectors

A cDNA encoding the mouse *VHL* gene described previously [25] and a cDNA encoding enhanced green fluorescent (*EGFP*) were inserted into a replication-deficient serotype 5 adenovirus vector, in which portions of the E1 and E3 regions were deleted [28]. The resulting constructs were designated Ad-VHL and Ad-EGFP, respectively. Expression of the exogenous genes was driven by a cytomegalovirus promoter located at the site of the E1 deletion [28]. An Ad-Null vector lacking an inserted transgene was used as a negative control. The recombinant adenoviruses were propagated in HEK293 cells, and purified by cesium chloride density gradient centrifugation. The infectious viral titer was determined by measuring the median tissue culture infective dose (TCID50).

Cell viability assay

The cells were seeded into a 96-well plate (3×10^3/well), and cultured overnight. The culture medium was replaced with fresh FCS-free media containing Ad-Null or Ad-VHL at an MOI of 100 for 90 min, followed by replacement of the culture media with 10% FCS-supplementing media containing doxorubicin at various concentrations. The cells were cultured for 72 h, and cell viability was measured with a Cell Counting Kit-8 (CCK-8) kit (Dojindo Molecular Technologies, Gaithersburg, MD, USA). Untreated cells served as controls. The cell viability index was calculated according to the formula below: experimental OD value/control OD value × 100%.

Cell cycle assay

Adenovirus-infected Hepal-6 or H22 cells were incubated with doxorubicin (80 or 40 ng/ml, respectively) as above for 48 h, and the percentage of cells at G0/G1, S and G2/M phases were determined with a cell cycle detection kit (BD Biosciences, Beijing, China) in a Beckman Coulter Epics Altra II cytometer (Beckman Coulter, California, USA).

Apoptosis assay

Cells were washed with PBS, resuspended in binding buffer, and incubated with Annexin V and PI for 15 min according to the manufacturer's instruction (BD Biosciences, San Jose, California, USA). The cells were analyzed in a cytometer to measure the apoptosis rate (%), and viewed by laser scanning confocal microscopy (LSM-510, Carl Zeiss Jena GmbH, Jena, Germany).

Electrophoretic mobility shift assay (EMSA)

The methodology has been described previously [29]. Briefly, nuclear extract (10 μg) was incubated with 1 μg of poly(deoxyinosinic-deoxycytidylic acid) in binding buffer for 30 min at 4 °C. DNA binding activity was confirmed with a biotin-labeled oligonucleotide bio-NF-κB probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) using an EMSA kit (Viagene, Beijing, China). The probe was resolved on a 4% polyacrylamide gel containing 0.25× TBE buffer, and visualized with a CoolImger imaging system (IMGR002).

Animal model and treatments

Subcutaneous HCC tumor model

Hepa1–6 cells (5×10^6) were subcutaneously injected into the backs of mice to establish tumors. Tumor volumes were estimated as described previously [27]. When tumors reached around 100 mm^3 at 2 weeks, the mice were randomly assigned to 4 groups (each group had 15 mice): Ad-Null, Ad-VHL, Ad-Null + doxorubicin, and Ad-VHL + doxorubicin. In the Ad-Null and Ad-VHL groups, mice received i.p. injection of 200 μl of PBS, and intratumoral injection of 1×10^9 TCID50 of Ad-Null or Ad-VHL in 100 μl of PBS, respectively. In the Ad-Null + doxorubicin and Ad-VHL + doxorubicin groups, mice received i.p. injection of 200 μl of doxorubicin at the dose of 12.5 mg/kg and intratumoral injection of Ad-Null or Ad-VHL at the same dose as above, respectively. Five mice from each group were randomly sacrificed 4 and 14 days after the treatment started, respectively, and their tumors excised. The remaining mice were monitored for 21 days.