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# HIF-1α siRNA Leads to Apoptosis of Pancreatic Cancer Cells under Hypoxic Conditions

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**OBJECTIVE** To explore the role of hypoxic inducible factor-1α (HIF-1α) in the proliferation and apoptosis of pancreatic cancer cells under hypoxic conditions.
**METHODS** A cassette encoding small interference RNA (siRNA) targeting HIF-1α mediated by recombinant adeno-associated virus (rAAV) was constructed, giving rAAV-siHIF. rAAV-siHIF or rAAV-hrGFP was transfected into exponentially growing MiaPaCa2 cells under hypoxic conditions. Then, the expression of HIF-1α mRNA and protein, the proliferation and apoptosis of MiaPaCa2 cells were examined, using real-time PCR, Western Blot, MTT and TUNEL, respectively.
**RESULTS** Under hypoxic conditions, rAAV-siHIF inhibited the expression of HIF-1α mRNA and protein in MiaPaCa2 cells. At the same time, rAAV-siHIF decreased MiaPaCa2 cell proliferation and induced apoptosis. However, rAAV-hrGFP had no effect on the expression of HIF-1α as well as the proliferation and apoptosis of MiaPaCa2 cells under hypoxic conditions.
**CONCLUSION** Under hypoxic conditions, HIF-1α plays a key role in the proliferation of MiaPaCa2 cells, and inhibition of HIF-1α expression can lead to MiaPaCa2 cell apoptosis.

**KEY WORDS: recombinant adeno-associated virus (rAAV), hypoxia inducible factor (HIF), small interference RNA (siRNA), proliferation, apoptosis.**

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## Introduction

Pancreatic adenocarcinoma is a highly malignant tumor in digestive system with a very poor prognosis[1]. The reason is still unknown but it may be related to the existence of severe hypoxia in pancreatic adenocarcinoma because hypoxic tumor cells migrate and metastasize easily and are associated with resistance to radiotherapy, immunotherapy and chemotherapy[2,3]. Evidence has manifested that hypoxia adaptation of tumor cells are regulated by hypoxia inducible factor 1 (HIF-1)[4]. HIF-1, as a heterodimer, consists of HIF-1α and HIF-1β subunits[5]. Under normoxic conditions, newly produced HIF-1α protein is quickly hydroxylated by oxygen-activated HIF-1 prolyl hydroxylase (PHD) and degraded by the proteasomal pathway[6]. Under hypoxic conditions, oxygen-stimulated PHD is inactivated so HIF-1α accumulates and associates with HIF-1β, which a mature and functional HIF-1 comes into being[7] and plays a key role in many hypoxic responses, including the induction of mRNA synthesis of its target genes and the increase in the growth, infiltration and metastasis of tumor cells[8-10]. In fact, pancreatic adenocarcinoma, as a solid

tumor, as well as MiaPaCa2 cells, a poorly differentiated cell line of human pancreatic cancer, have a high level of expression of HIF-1α[10,11]. So, HIF-1α could play a crucial role in the pathophysiology of pancreatic cancer. However, how HIF-1α can regulate the proliferation and apoptosis of pancreatic cancer cells has not been thoroughly explored. On the basis of these facts, we selected MiaPaCa2 cells for our study, using small interference RNA (siRNA) targeting HIF-1α mediated by recombinant adeno-associated virus (rAAV), to determine the effect on the expression of HIF-1α, and to further explore the corresponding influences on the proliferation and apoptosis of MiaPaCa2 cells, and, finally, to demonstrate the role of HIF-1α in proliferation and apoptosis of pancreatic cancer cells.

## Materials and Methods

### *The construction of rAAV vector mediating siRNA targeting HIF-1α*

An AAV Helper-Free System including pAAV-hrGFP, pAAV-RC and pHelper was purchased from Stratagene (CA, USA) (the features of these plasmids are available from http://www.stratagene.com). The vector plasmid pAAV-hrGFP has a monoclonal site, namely Mlu I, which locates at the starting point of the CMV promoter. The H1 human RNA polymerase III promoter was amplified by polymerase chain reaction (PCR) from human genomic DNA which was extracted from human blood cells. The primers for the PCR was A-Mlu I forward: 5´-ATC ACG CGT CCA TGG AAT TCG AAC GCT GA-3´ and A-Mlu I-Xba I-Mun I reverse: 5´-GCT ACG CGT TCT AGA CAA TTG GTG GTC TCATAC AGA ACT TAT AAG-3´. The human H1 promoter was inserted into the Mlu I site of the pAAV-hrGFP, giving pAAV-H1-hrGFP. The orientation of pAAV-H1-hrGFP was confirmed by restriction and sequencing. A pair of complementary oligonucleotides with 58 bp (sense: 5´-AAT TGA TGG AAC ATG ATG GTT CAC TTC AAG AGA GTG AAC CAT CAT GTT CCA TTT TTT T-3´ and anti-sense: 5´-CTA GAA AAA AAT GGA ACA TGA TGG TTC ACT CTC TTG AAG TGA ACC ATC ATG TTC CAT C-3´) was designed according to the HIF-1α gene (GenBank No.U22431), annealed *in vitro* and subcloned into the pAAV-H1-hrGFP vector digested with Xba I and Mun I, giving pAAV-H1-siHIF-hrGFP. Restriction and sequencing analysis determined if the insert was correct.

Human embryonic kidney 293 cells (HEK293) (Stratagene) were cultured in Dulbecco's modified Eagle's medium (DMEM) including 10% fetal calf serum (FCS) in flasks incubated at 37°C in 95% air and 5% $CO_2$. When the cells were 90% confluent, pAAV-H1-siHIF-hrGFP or pAAV-hrGFP, pAAV-RC and pHelper were co-transfected into the HEK293 cells using a Vira-Pack™Transfection Kit (Stratagene) for packaging the rAAV-siHIF or rAAV-hrGFP. The expression of GFP in the HEK293 cells could be seen by fluorescence microscopy after 24 h and the rAAV could be purified and collected by the chloroform-PEG8000/NaCl-chloroform method[12] after 72 h. The shape of the rAAV was identified by electron microscopy and the purity and titer were assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and AVSachTM ELISA (Vector Gene Technology Co. Ltd., Beijing, China), respectively.

### *Grouping and hypoxic incubation and administration in vitro*

MiaPaCa2 cells were bought from the American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM containing 10% FCS. Three experimental groups were used. The Wt group consisted of non-transfected wild-type MiaPaCa2 cells, the Cv group of MiaPaCa2 cells transfected with rAAV-hrGFP ($1 \times 10^7$ v.p/cell) as an empty control virus vector and the Si group of MiaPaCa2 cells transfected with rAAV-siHIF ($1 \times 10^7$ v.p/cell). Over 95% of the MiaPaCa2 cells transfected by rAAV showed GFP expression by fluorescence microscopy. All the groups were cultured at 37°C in 1% $O_2$, 94% $N_2$ and 5% $CO_2$. Then, we examined the expression of HIF-1α mRNA and protein and the proliferation and apoptosis of MiaPaC2 cells after 24 h and 48 h.

### *Real-Time PCR to detect the expression of HIF-1α mRNA*

The total RNA was extracted from the MiaPaCa2 cells and cDNA was synthesized by AMV reverse transcriptase at 42°C for 10 min and 95°C for 2 min. A SYBR ExScript™ RT-PCR Kit (TaKaRa, Tokyo, Japan) and ABI (CA, USA) Prism 7900HT sequence detection system were used. The reagents were processed at 95°C for 30 s and were then cycled 40 times at 95°C for 5 s and at 60°C for 15 s and 72°C for 30 s. The primers of HIF-1α were 5´-TCA TCC AAG AAG CCC TAA CGT G-3´ as forward primer and 5´-TTT CGC TTT CTC TGA GCA TTC TG-3´ as reverse primer. The primers of β-actin were 5´-TGG CAC CCA GCA CAA TGA A-3´ as forward primer and 5´-CTA AGT CAT AGT CCG CCT AGA AGC A-3´ as reverse primer. The relative quantity results of real-time PCR were analyzed by an ABI 7900HT software system.

### *Western blot to detect the expression of HIF-1α protein*

HIF-1α monoclonal antibody was purchased from BD Biosciences (CA, USA). β-actin polyclonal antibody was purchased from Santa Cruz (CA, USA). The total proteins were extracted from the MiaPaCa2 cells and the concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) and Biophotometer (Eppendorf, Hamburg, Germany). The total proteins were separated on 8% SDS-PAGE gel for HIF-1α and

12% for β-actin, and transferred to polyvinylidene difluoride membranes by MINI TRANS-BLOT (BIO-RAD, CA, USA). The blot membrane was then incubated with primary and secondary antibodies and treated with enhanced chemiluminescence detection reagents (Amersham, Buckinghamshire, UK). The specific blotting band was recorded on film. The results were analyzed by ImageJ software (available from the NIH at http://rsb.info.nih.gov/ij/).

***TUNEL assay***

MiaPaCa2 cells were incubated on chamber slides under hypoxic conditions. Additionally one group of cells was cultured under normoxia and another group under hypoxia without TdT enzyme as negative controls. After 24 h, the endogenous peroxidase of the cells was inactivated with 30% $H_2O_2$ diluted 1:10 in methanol at room temperature for 10 min. The cells were incubated with the TdT equilibration buffer at 37°C for 60 min, and then the labeling reaction was mixed (Streptavidin-HRP) at 37°C for 30 min. The cells were further stained with diaminobenzidine at room temperature for 10 min so that the nucleus of the apoptotic cells became yellow. Then 5 fields of each slide were randomly selected and 500-1000 nuclei were counted per slide. The frequency of apoptosis was calculated as an apoptotic index (AI).

***MTT assay***

The MiaPaCa2 cells were placed in a 96-well culture plate ($10^4$ cells/well) under hypoxic conditions. Cell viability was measured after 24 h using the MTT assay. Routinely, 20 µl MTT (Sigma) stock solution (5 mg/ml) was added to each well with 200 µl media and incubated for 4 h. Then the media were removed and 200 µl DMSO (Sigma) was added to each well. The plate vibrated for 10 min. Absorbance in every well was measured in a spectrophotometer at a wavelength of 570 nm.

***Statistical analysis***

Data were expressed as means ± SD and statistical analysis was done with SPSS11.5 using analysis of variance and Bonferroni posttest. Differences were considered statistically significant when $P < 0.05$.

## Results

***The Expression of HIF-1α in MiaPaCa2 Cells***

Real-time PCR showed that rAAV-siHIF inhibited the expression of HIF-1α mRNA in the MiaPaCa2 cells after 24 h under hypoxic conditions and after 48 h the expression decreased nearly 90% compared with the unaffected Wt and Cv groups ($P > 0.05$, Fig.1A). Western blot showed that rAAV-siHIF suppressed the expression of HIF-1α protein after 24 h under hypoxic conditions and after 48 h the protein expression declined over 90% compared with the Wt and Cv groups which were not affected ($P > 0.05$, Fig.1B, C, D).

***The Apoptosis of MiaPaCa2 Cells***

Under normoxic conditions, apoptotic cells were rarely seen but there were many apoptotic cells under hypoxic conditions, which indicated that hypoxia is a key factor for apoptosis of tumor cells. In the negative control group without TdT enzyme, yellow nuclei were not found although there were many apoptotic cells, which indicated that our technique was satisfactory. The AI of the cells in the Si group after 24 h and 48 h under hypoxic conditions was significantly higher than that in the Wt group or in the Cv group ($P < 0.01$). There was no effect on the AI of the Cv group cells under hypoxic conditions compared with that in the Wt group after 24 h and 48 h ($P > 0.05$, Fig.2A).

***The Proliferation of MiaPaCa2 Cells***

From MTT assay, compared with incubation after 24 h under hypoxia, proliferative vitality of MiaPaCa2 cells after 48 h was distinctly increased ($P < 0.01$). Although MiaPaCa2 cells took on proliferative tendency within 48 h under hypoxic conditions, we found that rAAV-siHIF could inhibit the proliferation of MiaPaCa2 cells whether after 24 h or after 48 h compared with that in Wt group or Cv group ($P < 0.05$ or $P < 0.01$). However, rAAV-hrGFP had no effect on proliferative vitality of MiaPaCa2 cells under hypoxic conditions compared with that in Wt group ($P > 0.05$, Fig.2B).

## Discussion

HIF-1 is a heterodimer which consists of 2 basic helix-loop-helix proteins, namely α subunit (120 KD) and β subunit (91~94 KD), and is an essential mediator of oxygen homeostasis[13]. HIF-1β subunits are constitutive proteins, and regulation of the active complex is achieved through a multi-step process affecting the abundance and activity of HIF-1α subunits. Under normoxic conditions, HIF-1α subunits are post-translationally modified by a series of oxygen-dependent enzymatic hydroxylations at specific amino acid residues[14-17]. Prolyl-4-hydroxylation at two sites within a central degradation domain by a set of closely related Fe(2+)-and O(2)-dependent HIF-1α prolyl hydroxylase (PHD) mediates interactions with the VHL E3 ubiquitin ligase complex which targets HIF-1α for proteasomal degradation[14,15]. In fact, the von Hippel-Lindau tumor suppressor gene product, pVHL, functions as the substrate recognition component of an E3-ubiquitin ligase complex and binds HIF-1α[18,19] while PHDs are a family of enzymes that can regulate protein levels of HIF-1α under different oxygen levels. On the contrary, under hypoxic conditions, the abolition of prolyl hydroxylation results in HIF-1α stabilization, whereas the lack of asparaginyl hydroxylation allows for the accumulation and activation of HIF-

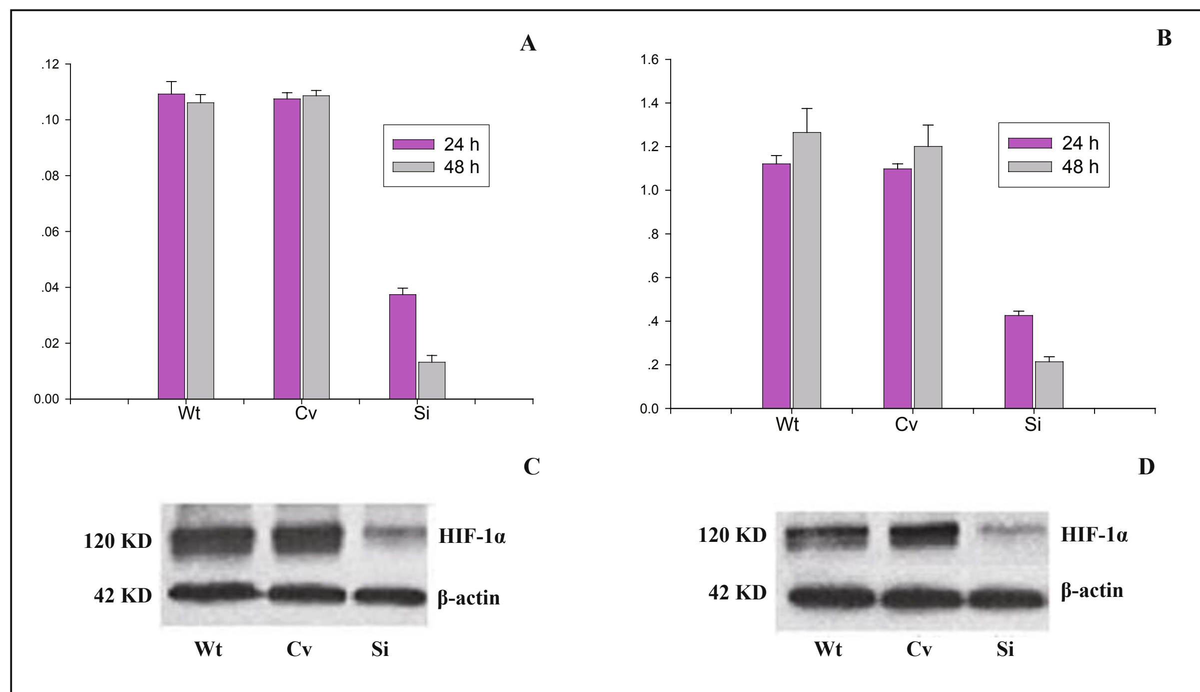


Fig.1. Expression of HIF-1α mRNA and protein after 24 h and 4 8 h under hypoxic conditions. (A) Expression of HIF-1α mRNA determined by real-time PCR. (B) Expression of HIF-1α protein determined by western blot. Western blot of HIF-1α protein expression (C) after 24 h and (D) after 48 h. Wt: non-transfected wild-type MiaPaCa2 cells; Cv: MiaPaCa2 cells transfected with rAAV-hrGFP; Si: MiaPaCa2 cells transfected with rAAV-siHIF.

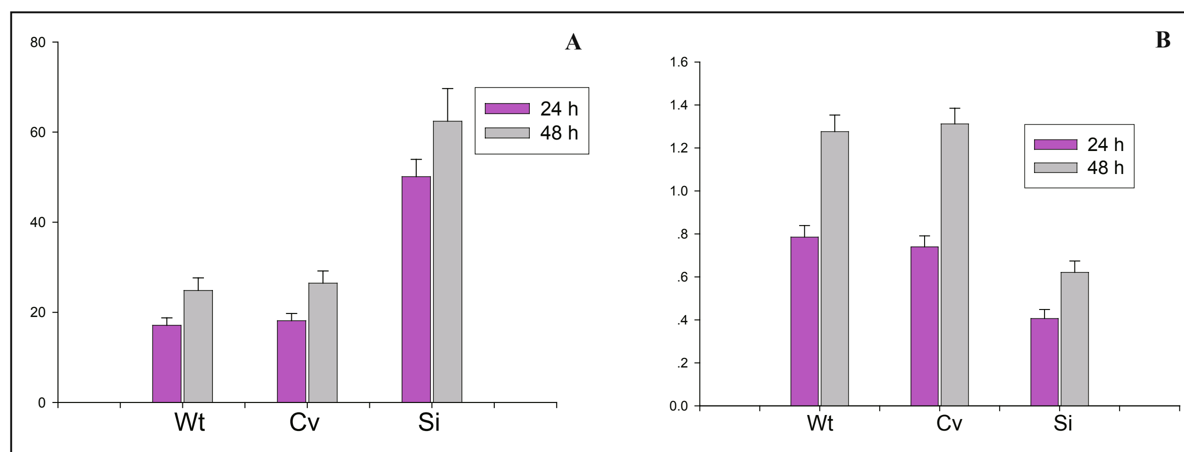


Fig.2. (A) Apoptosis of MiaPaCa2 cells after 24 h and 48 h under hypoxic conditions determined by TUNEL assay. (B) Proliferative vitality of MiaPaCa2 cells after 24 h and 48 h under hypoxic conditions determined by MTT. Wt: non-transfected wild-type MiaPaCa2 cells; Cv: MiaPaCa2 cells transfected with rAAV-hrGFP; Si: MiaPaCa2 cells transfected with rAAV-siHIF.

1α, which is then transferred to nucleus, binding with HIF-1β subunits to form the heterodimer. HIF-1 exerts the transcriptional activity by means of binding to its consensus binding site within the hypoxia-responding element in the target genes involved in energy metabolism, angiogenesis, cell survival, infiltration, metastasis and so on[8-10]. As a matter of fact, the over-expression of HIF-1 has been demonstrated in multiple types of human tumor as well as in their regional and distant metastases, as a result of adaptation of tumor cells to hypoxia[11]. However, some studies have reported that hypoxia has stimulant, inhibitory, or no effect on expression of HIF-1α mRNA in different cells[4,20,21]. Recently, Yu et al.[22] reported that HIF-1α is correlated with proliferation, but bears no relationship with the apoptosis of tumor cells in laryngeal squamous cell carcinoma (LSCC). Fan et al.[23] reported that HIF-1 α is correlated with apoptosis, but has no relationship with proliferation in non-small cell lung cancer (NSCLC). Although we can primarily know about the construction and function of HIF-1, the effect of hypoxia on HIF-1α mRNA and the role of HIF-1α in tumor cells are still very intriguing, and HIF-1α's role in

the proliferation, apoptosis of pancreatic cancer cells is elusive. RNA interference (RNAi), as a powerful tool of gene therapy, can repress the expression of target gene, which can achieve the effect of gene knock-out[24] and is a very convenient and useful measure to study the function of genes as well. Studies have recently manifested that AAV is a highly efficient vector and can infect both dividing and non-dividing cells and achieve long-term gene expression[25,26]. So, AAV-based vector for the delivery of siRNA targeting HIF-1α will be an efficient vector to inhibit HIF-1α expression in tumor cells.

In this study, we used siRNA targeting HIF-1α mediated by rAAV to inhibit HIF-1α expression in MiaPaCa2 cells under hypoxic conditions. As a result, we found that rAAV-siHIF decreased the expression of HIF-1α mRNA and protein and inhibited MiaPaCa2 cell proliferation and induced apoptosis. Moreover, we found that rAAV-hrGFP had no effect on the expression of HIF-1α in MiaPaCa2 cells. At the same time, we also found that when the expression of HIF-1α declined, the proliferation of MiaPaCa2 cells was decreased with apoptosis of MiaPaCa2 cells increased. With the change of HIF-1α expression level in different time, the proliferative vitality of MiaPaCa2 cells changed correspondingly. The higher the HIF-1α expression was, the higher the proliferative vitality of MiaPaCa2 cells was. At the same time, decline of HIF-1α expression corresponded to the increasing apoptosis of MiaPaCa2 cells. It manifested that HIF-1α could stimulate the proliferation of MiaPaCa2 cells under hypoxic conditions while inhibition of HIF-1α expression might lead to MiaPaCa2 cell apoptosis. That is to say, rAAV-siHIF can induce MiaPaCa2 cell apoptosis through inhibition of HIF-1α expression while increase of HIF-1α expression may resist MiaPaCa2 cell apoptosis. Additionally, it suggested that AAV-based vector may be a valuable tool in siRNA-based therapy of cancer.

In conclusion, HIF-1α plays a key role in the proliferation of MiaPaCa2 cells under hypoxic conditions and inhibition of HIF-1α expression leads to MiaPaCa2 cell apoptosis. Moreover, HIF-1α could act as an important target of genetic and pharmacological therapies for pancreatic cancer.

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