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ORIGINAL PAPER

Effect of Small Interference RNA Targeting HIF-1α Mediated by rAAV Combined L-Ascorbate on Pancreatic Tumors in Athymic Mice

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Abstract To study the effect of recombinant adeno-associated virus (rAAV) vector bearing small inference RNA (siRNA) targeting hypoxia inducible factor 1α (HIF-1α) combined L-ascorbate on pancreatic tumors in athymic mice primarily. A cassette encoding siRNA targeting HIF-1α mediated by rAAV was constructed, giving rAAV-siHIF. In vitro, rAAV-hrGFP, rAAV-siHIF and L-ascorbate which were used alone or in combination were delivered to exponentially growing MiaPaCa2 cells. Then, we examined the expression of HIF-1α mRNA and protein, the secretion of VEGF in MiaPaCa2 cells under hypoxic condition with Real-time PCR, Western Blot, ELISA, respectively. In vivo, MiaPaCa2 cells were inoculated subcutaneously on the back of nude mice. Nude mice with xenograft tumor were randomly divided into equal groups and were injected with rAAV-hrGFP or rAAV-siHIF or were fed with L-ascorbate. Then, we measured the size of tumor every 3 days and drew a tumor growth curve. After 30 days, all mice were sacrificed and the tumors were dissected. At last, we examined the expression of HIF-1α, VEGF and CD34 by immunohistochemistry and counted micro-vessel density (MVD). In vitro, we found that rAAV-siHIF could inhibit the expression of HIF-1α mRNA and protein in MiaPaCa2 human pancreatic cancer cells but L-ascorbate could only restrain the expression of HIF-1α protein. Moreover, rAAV-siHIF and L-ascorbate could all inhibit the secretion of vascular VEGF. In vivo, we found that rAAV-siHIF could inhibit the growth of nude mice xenograft tumor and the expression of HIF-1α and VEGF and MVD while L-ascorbate can only inhibit the growth of xenograft tumor in the early and middle stage. These results suggest that rAAV-siHIF and L-ascorbate can inhibit the growth of nude mice xenograft tumor and HIF-1α could be a target of pancreatic cancer genetic and pharmacological therapy.

Keywords Recombinant adeno-associated virus (rAAV) · Hypoxia inducible factor (HIF) · Small interference RNA (siRNA) · L-Ascorbate

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Introduction

Pancreatic adenocarcinoma is a highly malignant digestive tumor 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 and chemotherapy [2, 3]. Recently, it is reported that hypoxia adaptation of tumor cells are regulated by hypoxia inducible factor 1 (HIF-1) which is the key transcription factor of oxygen homeostasis and is related to tumor angiogenesis [4], while angiogenesis is a critical step for invasive tumor growth and metastasis. However, vascular endothelial growth factor (VEGF) is a major regulator of angiogenesis and vasculogenesis [5]. Indeed, many evidences have demonstrated HIF-1 is positively related to the activity of VEGF transcription in a variety of human malignancies such as pancreatic adenocarcinoma [6, 7]. At present, inhibition of angiogenesis is an important and

potent therapeutic strategy for tumor [8]. Pancreatic adenocarcinoma, as a solid tumor, can express HIF-1α richly [9], so it might be an effective measure to treat pancreatic adenocarcinoma through inhibiting the expression of HIF-1α.

HIF-1, as a heterodimer, consists of HIF-1α and HIF-1β subunit [10]. Under normoxia conditions, HIF-1α is quickly hydroxylated by oxygen-activated HIF-1 prolyl hydroxylase (PHD) while oxygen-stimulated PHD is inactivated under hypoxic conditions. It is interesting that L-ascorbate, as a cheap and convenient medicine, can employ Fe (II) to raise the activity of HIF-1 PDH which may promote HIF-1α protein to degrade [11].

Studies have recently manifested that adeno-associated virus (AAV) is a highly efficient vector and can infect both dividing and non-dividing cells and achieve long-term gene expression [12, 13]. At present, RNA interference (RNAi), as a powerful tool of gene therapy, can repress the expression of target gene, which can achieve the effect of gene knockout [14]. So, AAV-based vector for the delivery of siRNA targeting HIF-1α will be an efficient vector to inhibit the expression of HIF-1α in tumor cells.

In this study, we will use recombinant adeno-associated virus (rAAV) bearing siRNA of HIF-1α (rAAV-siHIF) combined L-ascorbate to observe the impact on the growth of xenograft tumor in nude mice. The aim is to explore primarily if HIF-1α could act as the target of treating pancreatic adenocarcinoma.

Materials and Methods

The Construction of rAAV Vector Mediating Small Interference RNA Targeting HIF-1α

AAV Helper-Free System including pAAV-hrGFP, pAAV-RC and pHelper was bought from Stratagene (the feature of all plasmids is available from http://www.stratagene.com). The vector plasmid of pAAV-hrGFP has a monoclonal site, namely *Mlu* I, which locates the starting point of 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. A pair of primers for PCR was A-*Mlu* I-forward: 5′-ATC *ACGCGT* CCATGG AATTCG AACGCT GA-3′ and A-*Mlu* I-*Xba* I-*Mun* I-reverse: 5′-GCT *ACGCGT TCTAGA CAATTG* GTGGTC TCATAC AGAACT TATAAG-3′. Human H1 promoter was inserted into the *Mlu* I site of pAAV-hrGFP, giving pAAV-H1-hrGFP. Then, we confirmed the orientation of pAAV-H1-hrGFP by restriction and sequencing. A pair of complementary oligonucleotides with 58 bp (sense: 5′-*AAT TG*ATGG AACATG ATGGTT CACTTC AAGAGA GTGAAC CATCAT GTTCCA TTTTTT *T*-3′ and anti-sense: 5′-*CTA GA*AAAA AATGGA ACATGA TGGTTC ACTCTC TTGAAG TGAACC ATCATG TTCCAT *C*-3′) was designed according to the gene of HIF-1α (GenBank No. U22431) and 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) were cultured in Dulbecco's modified Eagle's medium (DMEM) including 10% fetal calf serum (FCS). The flasks were incubated at 37°C in 95% air and 5% CO_2. When the cells were 90% confluent, pAAV-H1-siHIF-hrGFP or pAAV-hrGF, pAAV-RC and pHelper were co-transfected into HEK293 cells using ViraPack™ Transfection Kit (Stratagene). We could see the expression of GFP in HEK293 cells by fluorescence microscopy after 24 h and purify and collect rAAV by the method of chloroform-PEG8000/NaCl-chloroform after 72 h [15]. Then, the shape of rAAV was identified with electronic microscope and the purity and titer of it was assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and AVSachTM ELISA (Vector Gene Technology Company Ltd., China) respectively.

Grouping and Hypoxic Incubation and Administration In Vitro

This experiment includes five groups, namely the control group (1), the rAAV-hrGFP group (2), the rAAV-siHIF group (3), the L-ascorbate group (4) and the rAAV-siHIF combined L-ascorbate group (5). The control group is MiaPaCa2 cells cultured in DMEM containing 10% FCS. The rAAV-hrGFP group and the rAAV-siHIF group are MiaPaCa2 cells transfected rAAV-hrGFP and rAAV-siHIF with 1×10^7 v.p/cell respectively and cultured in DMEM containing 10% FCS. The L-ascorbate group is MiaPaCa2 cells cultured in DMEM containing 10% FCS and 25 μMol/L L-ascorbate (SIGMA). Over 95% of MiaPaCa2 cells transfected by rAAV had the expression of GFP by fluorescence microscopy. All groups were cultured at 37°C in 1% O_2, 94% N_2 and 5% CO_2. Then, we tested the expression of HIF-1α mRNA and protein and the secretion of VEGF in MiaPaC2 cells after 24 and 48 h.

Real-time PCR to Detect the Expression of HIF-1α mRNA

Total RNA was extracted from MiaPaCa2 cells. cDNAs were synthesized by AMV reverse transcriptase at 42°C for 10 min and 95°C for 2 min. We used SYBR ExScript™ RT-PCR Kit (TaKaRa, Japan) and ABI Prism 7900HT sequence detection system. The reagents went through a phase of 95°C for 30 s and were then cycled 40 times of 95°C for 5 s and 60°C for 15 s and 72°C for 30 s. The primers of HIF-1α were 5′-TCATCC AAGAAG CCCTAA CGTG-3′ as forward primer and 5′-TTTCGC TTTCTC TGAGCA

TTCTG-3′ as reverse primer. The primers of β-actin were 5′-TGGCAC CCAGCA CAATGA A-3′ as forward primer and 5′-CTAAGT CATAGT CCGCCT AGAAGC A-3′ as reverse primer. The relative quantity results of Real-Time PCR were analyzed by ABI 7900HT software system.

Western Blot to Detect the Expression of HIF-1α Protein

Total proteins were extracted from MiaPaCa2 cells. HIF-1α monoclonal antibody was purchased from BD Biosciences. β-Actin polyclonal antibody was purchased from Santa Cruz. Total protein concentrations were determined using a BCA protein assay kit (PIERCE) and by Biophotometer (Eppendorf, Germany). Total proteins were separated on 8% SDS-PAGE gel for HIF-1α and 12% for β-actin, transferred to polyvinylidene difluoride membranes by Mini Trans-Blot (Bio-Rad). The blot membrane was then incubated with primary and secondary antibodies and treated with enhanced chemiluminescence detection reagents (Amersham, UK). The specific blotting band was recorded on film. The results were analysed by ImageJ software (available from the NIH at http://rsb.info.nih.gov/ij/).

ELISA to Detect the Secretion of VEGF

MiaPaCa2 cells of five groups were seeded in 96-well culture plate (10^4 cells/well) and five different wells were used for each experimental condition. Then, culture medium was assayed for VEGF in five groups using the human VEGF ELISA kit (BOSTER, China) according to the manufacturers' instructions. Absorbance in every well was measured in spectrophotometer at a wavelength of 450 nm.

Xenograft of MiaPaC2 Cells in Nude Mice and Grouping and Administration In Vivo

A total of 40 male athymic BALB/c nude mice (4-week-old, weighting 20.1±0.4 g) were purchased from the Chinese Academy of Sciences. The rodents had free access to water and standard diet under special pathogen-free (SPF) conditions. The present study was approved by the local animal ethics committee. The 5.0×10^6 MiaPaCa2 cells were inoculated subcutaneously on the back of nude mice. Then, 25 nude mice with xenograft tumor which were selected after 10 days and were randomly divided into five equal groups (five mice per group), as grouping in vitro. rAAV with 1.0×10^{11} v.p was injected into the right hind leg anterior tibial muscle, while mice were fed on L-ascorbate 100 mg per kilogram a day. The longest (a) and the smallest (b) diameters of subcutaneous tumors were measured every 3 days and tumor volume was determined by equation of $V=ab^2/2$ and a tumor growth curve was drew. After 30 days, all mice were sacrificed and the tumors were dissected.

Immunohistochemistry to Assess the Expression of HIF-1α and VEGF and Microvessel Density (MVD)

Paraffin-embedded tumor tissues were cut at 4 μm serial sections and processed as usual. The sections of all specimens were stained with hematoxylin and eosin (H&E) routinely. Immunohistochemistry was carried out in accordance with the instructions of SP kit and diaminobenzidine tetrahydrochloride kit. Some sections were stained by immunohistochemistry to show cells that expressed HIF-1α and VEGF and the endothelial cell marker CD34. The expression of HIF-1α and VEGF were analysed by ImageJ software. MVD was measured according to the method as described previously [16]. HIF-1α monoclonal antibody, VEGF muticlonal antibody and rabbit anti-CD34 antibody were purchased from BD Biosciences, Santa Cruz and DAKO (Denmark), respectively.

Statistical Analysis

Data were expressed as means±SD and statistical analysis was done with SPSS11.5 using Least-significant difference (LSD). Differences were considered statistically significant when $P<0.05$.

Results

The Construction and Identification of rAAV-siHIF

H1 promoter was gained by PCR from human genomic DNA which was extracted from human blood cells and subcloned into pAAV-hrGFP, giving pAAV-H1-hrGFP (Fig. 1). The orientation of pAAV-H1-hrGFP was determined through

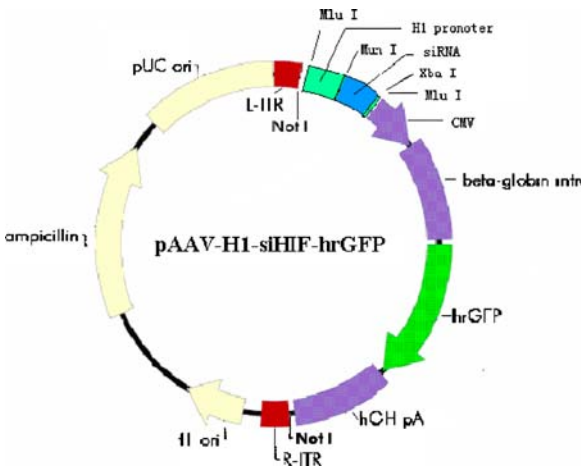

Fig. 1 Diagram of the construction of the vector plasmid, pAAV-H1-siHIF-hrGFP, bearing a cassette encoding siRNA targeting HIF-1α driven by H1 promoter

sequencing and restriction. A pair of complementary oligonucleotides with 58 bp annealed in vitro and the annealing siRNA targeting HIF-1α was subcloned into pAAV-H1-hrGFP, giving pAAV-H1-siHIF-hrGFP (Fig. 1), which was identified by restriction and sequencing and the insert was correct and had no any base mutation. Three plasmids were co-transfected into HEK293 cells to pack rAAV using AAV Helper-Free System. It was satisfactory for the purity of AAV and the ratio of AAV structure protein by SDS-PAGE and the shape of AAV identified with electronic microscope. The titer of AAV amounted to 1×10^{11} v.p/ml through AVSachTM ELISA.

The Expression of HIF-1α in MiaPaCa2 Cells

From Real-time PCR, we found that rAAV-siHIF could inhibit the expression of HIF-1α mRNA in MiaPaCa2 cells after 24 h under hypoxia and the expression of HIF-1α mRNA after 48 h decreased 90% nearly, while the control group, rAAV-hrGFP group and L-ascorbate group did not have these effects ($P>0.05$) (Fig. 2a). From Western Blot, we found that rAAV-siHIF and L-ascorbate could successfully suppress the expression of HIF-1α protein after 24 h under hypoxia and the expression of HIF-1α protein after 48 h declined over 90% in the combined group but the control group and rAAV-hrGFP group did not have these effects ($P>0.05$) (Fig. 2b,c,d).

The Secretion of VEGF in MiaPaCa2 Cells

From ELISA, we found that rAAV-siHIF and L-ascorbate could all inhibit the VEGF secretion of MiaPaCa2 cells under hypoxia and the effect of combined group after 48 h was the most obvious. Moreover, the effect of rAAV-siHIF on inhibiting the VEGF secretion was more profound than that of L-ascorbate ($P<0.05$) (Fig. 3).

The Growth of Xenograft Tumors and the Expression of HIF-1α and VEGF and MVD

From the graph of tumor growth curve, we found that there was no difference about tumor growth between the control group and the rAAV-hrGFP group, while there was difference between the control group and the rAAV-siHIF group or the combined group. Moreover, it was interesting that L-ascorbate could inhibit the growth of xenograft tumor in the early and middle stage such as the sixth day, the ninth day, twelfth day, the fifth day, the eighth day and the 21st day and there was significant difference between the control group and the L-ascorbate group ($P<0.01$ or $P<0.05$) (Fig. 4a). With fluorescence microscope, we may see the expression of GFP in tumors dissected from nude mice injected with rAAV. From routine H&E staining, we found that the rAAV-siHIF group and the combined group had obvious central necrosis while other groups only had minor

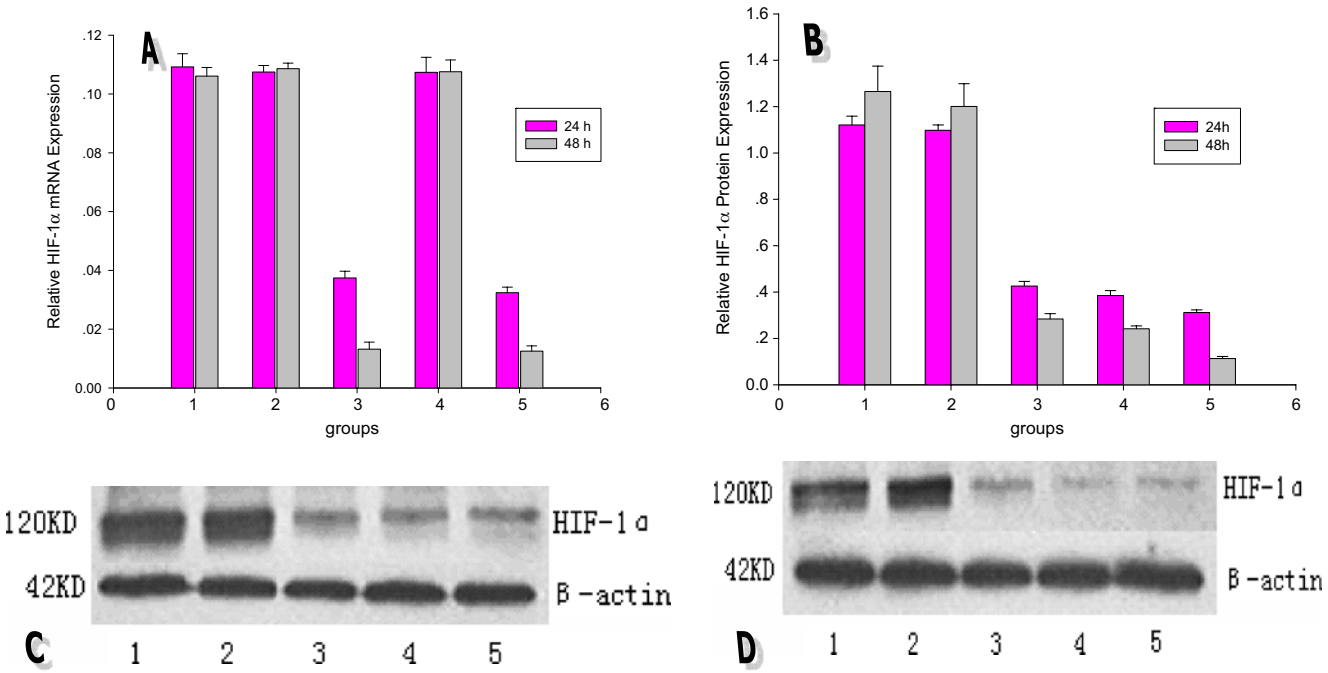

Fig. 2 Expression of HIF-1α mRNA and protein in five groups after 24 and 48 h under hypoxic conditions. **a** Expression of HIF-1α mRNA was determined by real-time PCR. **b** Expression of HIF-1α protein was determined by Western blotting. **c** Expression of HIF-1α protein by Western blotting in five groups after 24 h. **d** Expression of HIF-1α protein by Western blotting in five groups after 48 h

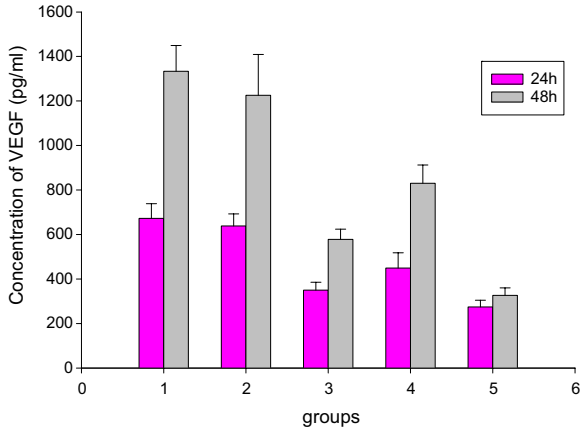

Fig. 3 Secretion of VEGF was determined by ELISA in five groups after 24 and 48 h under hypoxic conditions

central necrosis or no necrosis. From immunohistochemistry, we found that there was no difference between the control group and the rAAV-hrGFP group or the L-ascorbate group about the expression of HIF-1α and VEGF in pancreatic xenograft tumor ($P>0.05$) while there was significant difference between the control group and the rAAV-siHIF group or the combined group ($P<0.01$) (Fig. 4b). Moreover, microvessels were less numerous in the rAAV-siHIF group and the combined group than other groups (Fig. 4b).

Discussion

Hypoxia, as a characteristic feature of solid tumors, has emerged as a pivotal factor of the tumor since it can promote tumor progression and resistance to therapy [17]. The transcriptional regulator HIF-1 is an essential mediator of oxygen homeostasis and plays a key role in the pathophysiology of cancer [18]. 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 [9]. HIF-1 consists of HIF-1α and HIF-1β subunit [10]. Under normoxia conditions, HIF-1α is quickly hydroxylated by oxygen-activated HIF-1 PDH while oxygen-stimulated PHD is inactivated under hypoxic conditions. After HIF-1α accumulates and associates with HIF-1β subunit, it will bind to its downstream genes, such as the gene of angiogenesis, glycolysis and so on and make them express. As it has been reported currently, HIF-1 is relative to the glycolysis of tumor cells, tumor angiogenesis and so forth [19, 20]. The association of over-expression of HIF-1 with up-regulating of VEGF pathway has been shown in pancreatic adenocarcinoma [21], because HIF-1 is the key role in the activation of VEGF transcription in hypoxic cells. Levels of expression of VEGF mRNA and protein are markedly up-regulated in the majority of human tumors and VEGF is a major regulator of angiogenesis and vasculogenesis, which plays a crucial role in tumoral growth and metastasis dissemination [22]. Evidence has shown that solid tumors would not grow beyond the volume of 2–3 mm^3 in the absence of neo-angiogenesis because of the insufficient oxygen and nutrients diffusion from blood vessels [23]. Suppressing the activity of HIF-1 could block the angiogenesis processes, which can inhibit the growth and metastasis of tumor [24]. So, genetic and pharmacological modulation of HIF-1 activity could represent a novel therapeutic approach to cancer. Pancreatic adenocarcinoma, as a highly malignant digestive solid tumor, can migrate easily, resist therapy and tolerate nutrient scarcity [19]. The main reason may be that pancreatic adenocarcinoma can express HIF-1α richly. So, inhibition of HIF-1 activity might be an important therapeutic pathway approach for pancreatic cancer.

In this study, we used rAAV-siHIF and L-ascorbate, which were used alone or in combination, to affect the expression of HIF-1α gene and protein in MiaPaCa2 human pancreatic

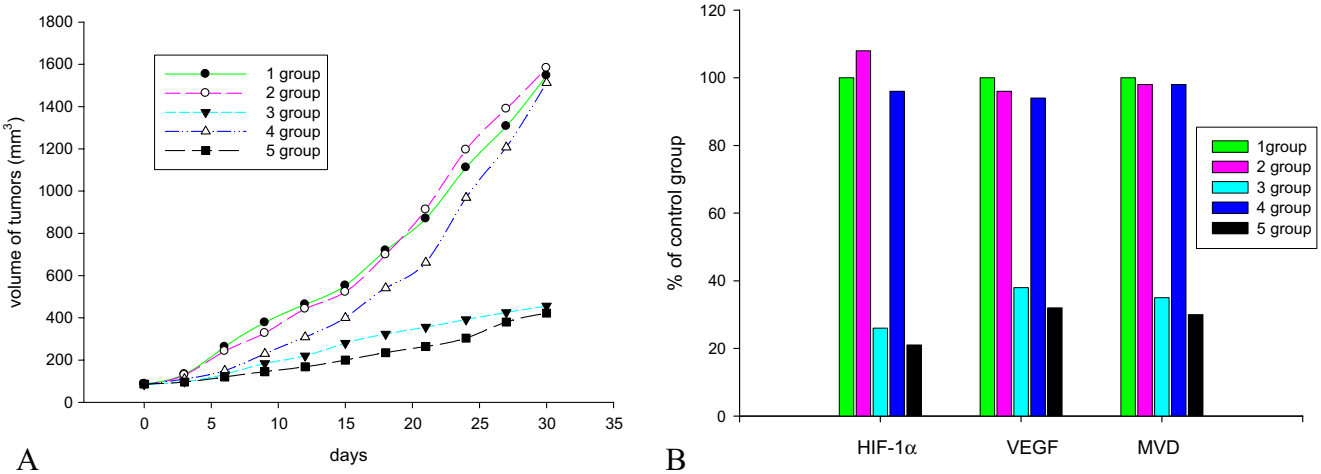

Fig. 4 The growth of xenograft tumors and the expression of HIF-1α and VEGF and MVD in five groups. **a** Xenograft tumors growth curve in five groups. **b** Expression of HIF-1α and VEGF and MVD was determined by immunohistochemistry in five groups

cancer cells under hypoxia. We found that rAAV-siHIF could rapidly inhibit the expression of HIF-1α mRNA but L-ascorbate could not do so. Furthermore, rAAV-siHIF and L-ascorbate could quickly suppress the expression of HIF-1α protein and the effect of the combined group was more efficient than that seen when rAAV-siHIF or L-ascorbate was used separately. It indicated that rAAV-siHIF could realize its function of inhibiting gene expression of HIF-1α and L-ascorbate could really promote HIF-1α protein to degrade in MiaPaCa2 cells. Moreover, rAAV-siHIF and L-ascorbate could all inhibit the secretion of VEGF in MiaPaCa2 cells and the effect of the combined group was most obvious. The reason might be that rAAV-siHIF and L-ascorbate can all inhibit the expression of HIF-1α protein while HIF-1α is a key role for the expression of VEGF transcription. In animal experiment, we found that rAAV-siHIF could inhibit the growth and MVD of xenograft tumors and the expression of HIF-1α and VEGF while L-ascorbate can only repress the growth of xenograft tumors in the early and middle stage. The reason might be that xenograft tumors in the late stage are short of blood vessels so L-ascorbate can not diffuse into tumor tissues and cells, which result in having no any effect on tumors. Moreover, the central necrosis in the rAAV-siHIF group and the combined group was related to the decline of MVD. Because normal tissues can little express HIF-1 while hypoxic tissues such as tumor tissues can express HIF-1 richly, rAAV-siHIF has impact on only tumor tissues, which shows its function of relative tropism in vivo. The mechanism of rAAV-siHIF inhibiting the growth of xenograft tumors might be that rAAV-siHIF can inhibit the expression of HIF-1α while HIF-1α is the key factor in the activation of VEGF, which is a major mediator of angiogenesis in tumors. The result is rAAV-siHIF can inhibit angiogenesis of tumors, which restricts the growth of tumors. However, L-ascorbate has the same function as rAAV-siHIF in the early and middle stage of tumor. So, HIF-1α could become an important target of pancreatic cancer genetic and pharmacological therapy.

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