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Original Article

Regulation of energy metabolism by hypoxia-inducible factor 1α in gastric cancer cells

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Abstract: Previous research by our group has demonstrated a correlation between the protein expression levels of hypoxia-inducible factor 1α (HIF-1α) and glucose transporter 1 (GLUT1) in human gastric carcinoma. In this study, the goal was to elucidate the metabolic mechanisms regulated by HIF-1α, therefore RNA interference was used to silence HIF-1α gene expression via lipofection of SGC-7901 human gastric cancer cells. Results from qRT-PCR showed that the level of HIF-1α mRNA was significantly reduced after transfection with a HIF-1α interference sequence for 24, 48 and 72 hours, thus indicating a high silencing efficiency. On Western blot analysis, protein expression of HIF-1α in SGC-7901 cells after gene silencing was markedly decreased, indicating that the RNA interference method effectively inhibited the expression of the target gene at the transcriptional and translational levels. Additionally, the rates of early apoptosis, late apoptosis, and necrosis of SGC-7901 cells subjected to hypoxia after gene silencing were much higher than those of negative control cells upon flow cytometric analysis. Furthermore, the viability of SGC-7901 cells following lipofection was inhibited in a time-dependent manner. Detection of ATP via a fluorescein-luciferase biochemiluminescence response assay also showed that the level of ATP in SGC-7901 cells gradually decreased from day 0 to day 5 after lipofection, suggesting that HIF-1α gene silencing could disturb cellular energetic metabolism. Therefore, HIF-1α may be a potential target gene involved in the endogenous hypoxic response and bioenergetic metabolism of tumor cells, and may provide a basis for novel targeting strategies in the treatment of gastric cancer.

Keywords: Hypoxia-inducible factor 1α, gastric cancer cells, apoptosis, ATP, energetic metabolism

Introduction

The occurrence and development of gastric cancer are complex processes involving multiple genes and mechanisms. During the growth of malignant tumors, the rate of tumor cell proliferation far exceeds that of vascular endothelial cells, which can lead to neovascular disorder, arteriovenous short circuits, blind-ended vessel formation, an incomplete endothelial cell layer and basement membrane and slowed or blocked blood flow. In turn, the deficiencies in oxygen and nutrient transport result in oxygen diffusion disorder, severe hypoxia and even energy metabolism imbalance in local tissue. Tumor cells adapt to a hypoxic microenvironment not only by promoting the formation of new blood vessels, but also by stimulating glucose metabolism and the glycolysis pathway. Regarding the latter processes, a number of studies have been performed to investigate the potential associated genes, with the aims of identifying the pathogenic mechanisms involved in gastric cancer, which may ultimately aid in improving diagnostic and treatment methods for the disease [1-3].

Normally, the growth of malignant tumors at a faster rate than angiogenesis leads to hypoxia and glucose deficiency in the tumor microenvironment, and can even cause a series of changes in intracellular metabolism and gene regulation [4]. Hypoxia can not only induce tumor cell mutation, but also promote the survival of malignant cell clones via hypoxia-mediated anti-apoptotic effects, which have been associated with malignant tumor cell invasion, proliferation and resistance to radiotherapy and chemo-

therapy [5]. Recent studies have shown that hypoxia-inducible factor 1 (HIF-1) is overexpressed and has enhanced activity in the hypoxic microenvironment of tumors [6, 7], and its role in the initiation and progression of tumors has attracted more and more attention. HIF-1, as a transcription factor and master regulator of oxygen homeostasis, has also been identified in tumor nuclear extracts following exposure to hypoxia [5, 8, 9]. It is composed of two subunits, namely HIF-lα and HIF-lβ, with HIF-lα serving as both the oxygen-regulated subunit and the active subunit [10]. Notably, the protein levels of HIF-1α and the binding activity of HIF-1β are upregulated during hypoxia. Additionally, overexpression of HIF-1α has been identified in numerous cancer types and precancerous lesions, but not in normal tissues or benign lesions. In the cancer microenvironment, hypoxia is a critical factor that contributes to a change in energy metabolism from aerobic to anaerobic glycolysis, which thus creates an acidic cancer microenvironment that influences HIF-1 activity [11]. Hypoxic tumor cells undergo a sequence of modifications that enable them to survive and proliferate via the activation of several survival genes, including HIF-1α [12, 13]. HIF-1 can be upregulated and combined with different hypoxia-response genes via its binding site during hypoxia. Additionally, activated HIF-1 can stimulate transcription of downstream target genes, including vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT1), heme oxygenase (HO-1), inducible nitric oxide synthase (iNOS), tyrosine hydroxylase and glycolytic enzymes [8]. Furthermore, HIF-1 is capable of inducing gene instability and selective anti-apoptotic mechanisms, and of providing basic components for intra-tumoral energy metabolism, neovascularization and cell proliferation [14].

Numerous studies have demonstrated that an increase in glucose expenditure via glycolytic metabolism is a common predictor of early-stage cancers. Like most solid cancers, gastric cancers produce energy in the form of ATP via active glycolysis, regardless of whether the conditions are aerobic or anaerobic. Glucose, as an aqueous component, is transferred through cell membranes by specific protein carriers instead of by simple diffusion, and thus the increased glucose consumption of tumor cells requires higher levels of GLUTs to be present in the cell membranes. GLUT1, a carrier of glucose in mammalian cells, mediates the transport of glucose in a bidirectional manner across cell membranes. Under hypoxic conditions, GLUT1, which is upregulated by HIF-1α, can upregulate the transport of glucose through cell membranes to compensate for the increased energy demands that arise during the process of malignant tumor proliferation [15, 16]. Many researchers have studied the relationship between GLUT1 and various tumor types, and found that abnormal expression of GLUTl and associated genes may be related to the intensive glucose metabolism in malignant cells. During hypoxia, transcription of GLUT1 is enhanced by HIF-1α and there is an increase in glycolysis [17, 18]. Additionally, both HIF-1α and GLUT l are associated with the regulation of certain oncogenes and growth factors, and thus may serve as endogenous hypoxia markers that could be used for the detection of hypoxia in tumors.

Previously, our group has shown that there is a correlation between the protein expression levels of HIF-1α and GLUT1 in human gastric carcinoma ($r=0.697$, $P<0.01$) (data not shown). Meanwhile, no positive immunohistochemical staining for the HIF-1α and GLUT1 proteins was observed in normal gastric tissues. In the present study, energetic metabolic mechanisms of glycolysis pathway were tested to determine whether they may be regulated by HIF-1α. An RNA interference technique was used to block HIF-1α expression in the SCG-7901 human gastric cancer cell line, in order to determine its potential regulatory effects on the glycolytic signaling pathway. After gene silencing, HIF-1α expression in the cells was detected by reverse transcription-quantitative polymerase chain reaction (qRT-PCR), and cell viability and apoptosis were subsequently measured. In addition, Western blot analysis and measurement of cellular ATP were performed to identify the metabolic mechanisms regulated by HIF-1α in gastric cancer cells, with the aim of investigating novel targets and therapeutic strategies for the treatment of gastric cancer.

Materials and methods

Reagents

Mouse anti-human HIF-1α monoclonal and rabbit anti-human β-actin polyclonal antibodies

were purchased from Abcam (Hong Kong, China). An UltraSensitive SP kit, liquid DAB enzyme substrate kit and poly-L-lysine were obtained from Maixin Biotechnology Co., Ltd. (Fuzhou, China). DMEM, fetal bovine serum, rhodamine 123, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and agarose gel were from Sigma-Aldrich (Gillingham, UK). FAM-labeled negative control siRNA oligos were designed and supplied by Jima Pharmaceutical Technology Co., Ltd. (Shanghai, China). Lipofectamine 2000, TRIzol reagent, a total RNA extraction kit and an ATP assay kit were from Invitrogen (Shanghai, China) and polyvinylidene difluoride (PVDF) membranes was purchased from EMD Millipore Co., Ltd. (MA, USA). All other chemicals and solvents were of analytical grade.

Cell culture

The human gastric cancer cell line SGC-7901 was provided by the immunology laboratory of West China Medical Center, Sichuan University (Chengdu, China). The cells were incubated in DMEM containing 10% fetal bovine serum, 100 U/L penicillin and 100 U/L streptomycin at 37°C and subjected to a hypoxic microenvironment induced by flushing a sealed incubator chamber (Stem Cell, Canada) with a gas mixture containing 5% CO_2, 1% O_2 and 94% N_2. Cells at passage 5 were used for the subsequent assays.

HIF-1α interference sequence and transfection

A fluorescently labeled HIF-1α interference sequence (FAM-HIF-1α-siRNA) was designed. The sequences were as follows: Upstream, 5'-CCA GCA GAC UCA AAU ACA ATT-3'; and downstream, 5'-UUG UAU UUG AGU CUG CUG GTT-3'. The sequences of the unrelated negative control siRNA oligos were as follows: Upstream, 5'-UUC UCC GAA CGU GUC ACG UTT-3'; and downstream, 5'-ACG UGA CAC GUU CGG AGA ATT-3'.

SGC-7901 cells at a density of 2×10^5 were seeded into 6-well plates with 1.5 ml DMEM to establish normal growth. On the day of transfection, HIF-1α-siRNA loaded in Lipofectamine 2000 transfection reagent was added to a subset of the SGC-7901 cells (interference group) in serum-free medium. The negative control siRNA was transfected into a different subset of the SGC-7901 cells (negative control group). Additionally, cells treated with Lipofectamine 2000 alone served as an empty vector control group, while cells left untreated served as a blank control group. The medium was replaced with fresh DMEM after transfection for 6 hours. Then 24 hours later, the cells were observed under a fluorescence microscope (Olympus, Tokyo), and transfection efficiency in the interference group was calculated by comparing the number of fluorescent cells to the total number of cells.

Reverse transcription-quantitative PCR (qRT-PCR)

Cells were harvested at 24, 48 and 72 hours after transfection. Total RNA was extracted using the TRIzol one-step extraction method and cDNA was synthesized with the RNA reverse transcription kit. The primers were designed with Primer 5 software and synthesized by Shanghai Bioengineering Co., Ltd. (Shanghai, China). The relative expression levels of target genes were analyzed with a Bio-Rad CFX manager 3.0 and calculated using the $2^{-\Delta\Delta CT}$ method. The HIF-1α silencing rate was assessed at 24, 48, and 72 hours after transfection with the following equation: HIF-1α silencing rate = $1\text{-}2^{-\Delta\Delta CT}$. Each sample was amplified in duplicate. The primer sequences used were as follows: HIF-1α forward, 5'-GCAAGACTTTCCTCAGTCGACACA-3'; HIF-1α reverse, 5'-GCATCCTGTACTGTCCTGTGGTGA3; β-actin forward, 5'-GGAGATTACTG CCCTGGCTCCTA-3'; and β-actin reverse, 5'-GACTCATCGTACT CCTGCTTGCTG-3'.

Western blotting

Transfected SGC-7901 cells were incubated under hypoxia at 37°C for the indicated times (24, 48 and 72 hours) prior to Western blotting. Following incubation, the cells were harvested and lysed in RIPA buffer [1% Triton X-100/1% sodium deoxycholate/0.1% sodium dodecyl sulfate (SDS)/150 mM NaCl/10 mM Tris HCl, pH 7.2] with protease inhibitors, and total protein concentration was determined via a BCA protein assay. Proteins were separated by 10% SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked in 5% non-fat dry milk for 1 hour and then incubated with the primary antibodies against HIF-1α and β-actin (anti-HIF-1α antibody, ab113642, dilution 1:800, Abcam, Hong Kong, China; and anti-β-actin antibody,

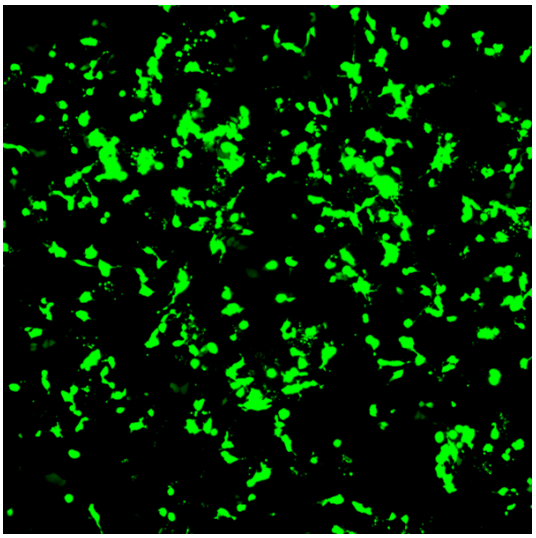

Figure 1. Fluorescence image of SGC-7901 cells after transfection of liposomal HIF-1α-siRNA. Magnification × 100.

ab16039, dilution 1:2000, Abcam, Hong Kong, China) at 4°C overnight, followed by incubation with peroxidase-linked secondary antibodies (ZDR-5307 and ZDR-5306, dilution 1:5000, ZSBio, Beijing, China). The proteins bands were visualized with enhanced chemiluminescence reagent, and ImageJ was used for quantification of the Western blotting results [19].

Cellular viability

SGC-7901 cells after transfection for 24 hours were trypsinized, resuspended and seeded into 96-well plates (Corning, NY) at a density of 1 × 10^4 cells/well with 200 μl DMEM supplemented with 10% fetal bovine serum. After incubation at the prearranged intervals in the chamber (Thermo Fisher Scientific, Inc., Waltham, MA, USA), the viability of cells was determined by MTT assay. Briefly, 20 μl MTT (5 mg/ml in PBS) and 100 μl DMEM were added into each well, and the medium was aspirated after 4 hours of incubation. The concentration of intracellular formazan solubilized with 150 μl DMSO was then quantified by measuring the optical density of each well at 490 nm (iMark microplate-reader; Bio-Rad Laboratories, Inc., CA, USA).

Apoptosis assay

Annexin V binding was used as an index of cell apoptosis according to the method by Guadall [20]. SGC-7901 cells after transfection for 48 hours were trypsinized, resuspended in 1X annexin V binding buffer, and incubated with FITC-labeled Annexin V (Annexin V-FITC) and propidium iodide (PI). The stained cells were then quantified with a FC500 flow cytometer (BD Biosciences; CA, USA) and the results were presented as the percentage of total cells. Annexin V-FITC fluorescence (abscissa) was plotted against PI uptake (ordinate). The data were gated for damaged cells (Annexin V^- and PI^+), necrotic cells (Annexin V^+ and PI^+), viable cells (Annexin V^- and PI^-) and apoptotic cells (Annexin V^+ and PI^-).

Measurement of intracellular ATP level

After transfection for 0, 1, 2, 3, 4 and 5 days, SGC-7901 cells in 96-well plates were washed once with PBS and lysed with ATP-releasing buffer. Following lysis, ATP levels were measured using the ATP assay kit according to the manufacturer's instructions. Luminescence was measured using an Lmax II Luminometer (Turner BioSystems, Sunnyvale, CA), and the data were plotted as ATP levels versus time. The same procedure was performed for the negative control, empty vector and blank control groups.

Statistical analysis

Data are presented as the mean ± standard deviation. One-way ANOVA was performed to compare the mean values of the groups, and the Fisher's least significant difference (LSD) test was performed for further multiple comparisons between the groups. $P<0.05$ was considered to indicate statistical significance.

Results

Transfection of liposomal HIF-1α-siRNA

Following transfection, SGC-7901 cells grew competently under conventional culture conditions. Fluorescence microscopy showed that there was a uniform distribution of green fluorescence in the cells, while the small extracellular fluorescent spots were considered to be adsorbed liposomes on the plate (**Figure 1**). The transfection rate was determined to be >80%.

qRT-PCR analysis

Figure 2 shows the specific amplification zones of HIF-1α (245 bp) and β-actin (114 bp), suggesting that the primers successfully amplified

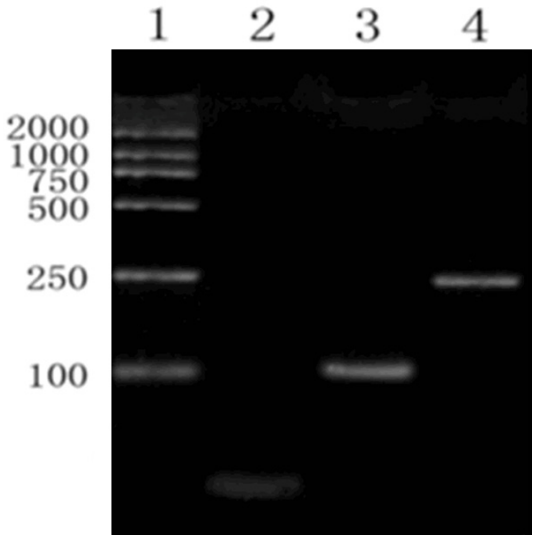

Figure 2. Primer test of PCR amplification. Lane 1: Marker; Lane 2: amplification without primer; Lane 3: amplification product (β-actin, 114 bp) using β-actin primer; Lane 4: amplification product (HIF-1α, 245 bp) using HIF-1α primer.

the target and internal reference genes specifically and generated single PCR amplification products. The PCR amplification curve was obtained by fluorescence qPCR. The $2^{-\Delta\Delta Ct}$ value and thus the silencing rate of the HIF-1α gene was significantly higher in the interference group than in the negative control and empty vector groups at 24, 48 and 72 hours after transfection (**Table 1**, P<0.05), indicating that the expression of HIF-1α mRNA was markedly suppressed after transfection with HIF-1α-siRNA.

Western blot analysis

The protein levels of HIF-1α in the different cell groups are shown in **Figure 3**. No differences in the protein levels of HIF-1α were observed among the negative control, empty vector and blank control groups (P>0.05). However, HIF-1α protein expression was significantly inhibited in the interference group (P<0.05). The inhibition rates were 84.95, 76.19 and 79.77% at 24, 48 and 72 hours after transfection, respectively, indicating that HIF-1α-siRNA markedly inhibited the protein expression of HIF-1α in SGC-7901 cells compared with control siRNA.

Cellular viability after transfection

To assess the effect of transfection on the viability of SGC-7901 cells, growth curves representing the first 5 days of transfection were evaluated (**Figure 4**). Similar to the HIF-1α expression data, there were no significant differences in the rates of cell growth among the negative control, empty vector and blank control groups (P>0.05). Additionally, cell growth was significantly inhibited in the interference group when compared with the other groups (P<0.05). Thus, HIF-1α gene silencing could suppress the growth of SGC-7901 cells.

To demonstrate whether the downregulation of HIF-1α could be related to the survival response in tumor cells, following transfection, SGC-7901 cells in each group were maintained under hypoxic conditions and cells were stained with Annexin V-FITC and PI to identify apoptotic cells. FACS analysis showed that the rate of early apoptosis for cells transfected with HIF-1α-siRNA for 48 hours was 45.36±1.32%, and that the rate of late apoptosis and necrosis was 22.92±1.69% (**Figure 5A**). No marked elevations in apoptosis were observed in the other three groups (**Figure 5B-D**). Altogether, our results indicated that HIF-1α gene silencing impeded the growth and promoted the apoptosis of SGC-7901 cells.

Intracellular ATP levels after transfection

To evaluate the effects of gene silencing on energetic metabolism, intracellular ATP levels were determined following transfection. As shown in **Figure 6**, similar trends in the levels of ATP were observed in the blank control and empty vector groups, as well as in the negative control group (P>0.05). Meanwhile, ATP levels in the interference group were significantly reduced at each time point when compared with those in the other groups (P<0.05), indicating that HIF-1α gene silencing could inhibit cellular energetic metabolism.

Discussion

The relationship between HIF expression and tumor properties has recently become a topic of interest in oncology research. Notably, a trend between rapid tumor proliferation and metastasis and positive HIF-1α expression in cancer cells has been found [21]. Additionally, some studies have demonstrated that HIF-1α may be a predictive marker of a high recurrence risk in patients with Dukes B colorectal cancers, as high-level expression of HIF-1α was strongly associated with invasive subtypes [5, 15, 18]. Furthermore, elevated expression of

Table 1. $^{\Delta\Delta}$CT values and silencing rate of HIF-1α at various time points after transfection

Group	24 h		48 h		72 h	
	$^{\Delta\Delta}$CT	Silence rate	$^{\Delta\Delta}$CT	Silence rate	$^{\Delta\Delta}$CT	Silence rate
Interference group	3.14±0.11*	88.66%*	2.77±0.09*	85.34%*	3.24±0.06*	89.42%*
Negative control	0.06±0.03	4.07%	0.04±0.02	2.73%	0.07±0.04	4.73%
Empty vector group	0.02±0.01	1.38%	0.03±0.02	2.06%	0.02±0.01	1.38%

$^{\Delta\Delta}CT = {}^{\Delta}CT_{experimental\ group} - {}^{\Delta}CT_{blank\ control\ group}$; Silencing efficiency = $1-2^{-\Delta\Delta CT}$; *p < 0.05 versus negative control or empty vector group.

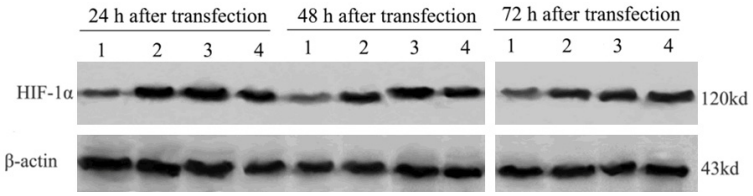

Figure 3. Electrophoretograms of HIF-1α protein detected by Western blot. Lane 1: interference group; Lane 2: negative control group; Lane 3: empty vector group; Lane 4: blank control group.

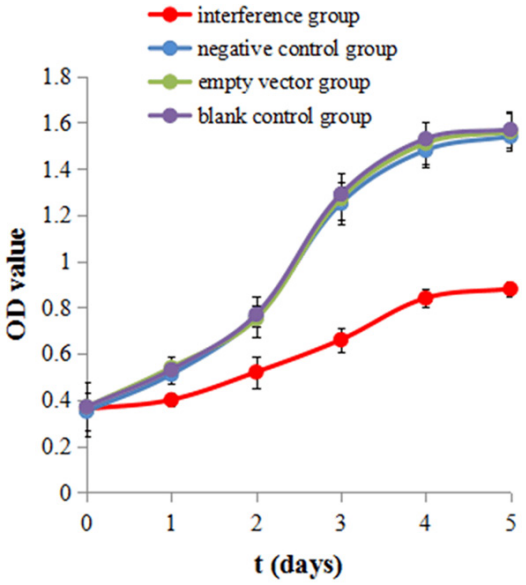

Figure 4. Growth curves of SGC-7901 cells after transfection.

HIF-1α is considered to be a response of tumor cells to hypoxia, and may be one of the factors that induces glycolysis during hypoxia [22].

HIF-1α can upregulate glucose-associated receptors, including GLUT1, GLUT4, and GLUT8 in the cellular membrane, as well as the expression of key enzyme genes, in order to facilitate the intake and metabolism of glucose in tumor cells, and even to biosynthesize more materials for the proliferation and differentiation of tumor cells via the glycolysis pathway. Moreover, HIF-1α also promotes the upregulation of PDK1 and inhibits the production and accumulation of oxygen free radicals in the mitochondria, in order to prevent apoptosis induced by oxidative stress [16, 23, 24]. In this study, the FACS data indicated that HIF-1α gene silencing promoted the apoptosis of gastric cancer cells, as indicated by the relatively high rates of early apoptosis (45.36±1.32%) and late apoptosis and necrosis (22.92±1.69%) observed after transfection with HIF-1α-siRNA. Conversely, Ward and Thompson argue that HIF-1α can inhibit the proliferation of tumor cells by disrupting mitochondrial function in tumor cells [25]. However, it has also been reported that HIF-1α could suppress the expression of c-Myc to regulate energy production in the of mitochondria of tumor cells, thus minimizing tumor cell demand for nutrients and enabling survival in a hypoxic environment at the expense of rapid proliferation. Furthermore, the high-level expression of PDK1 induced by HIF-1α is able to decrease glucose metabolism and anabolism in tumor cells, leading to the inhibition of proliferation [26]. Therefore, HIF-1α-targeted intervention therapy for the treatment of tumors may promote apoptosis rather than inhibit tumor growth [27]. Previous data for other solid cancer cells *in vitro* and from animal experiments has showed that HIF-1α suppressors, such as phosphoinositide-3-kinase (PI3K), protein kinase B (Akt), mammalian target of rapamycin (mTOR), mitogen-activated protein kinases (MAPKs) and heat shock protein 90 (HSP 90), can effectively inhibit the proliferation of tumor cells and promote their apoptosis [28, 29]. However, clinical application of these known HIF-1α suppressors is limited due to the lack of specificity.

In this study, HIF-1α-siRNA interference sequences were designed and constructed with

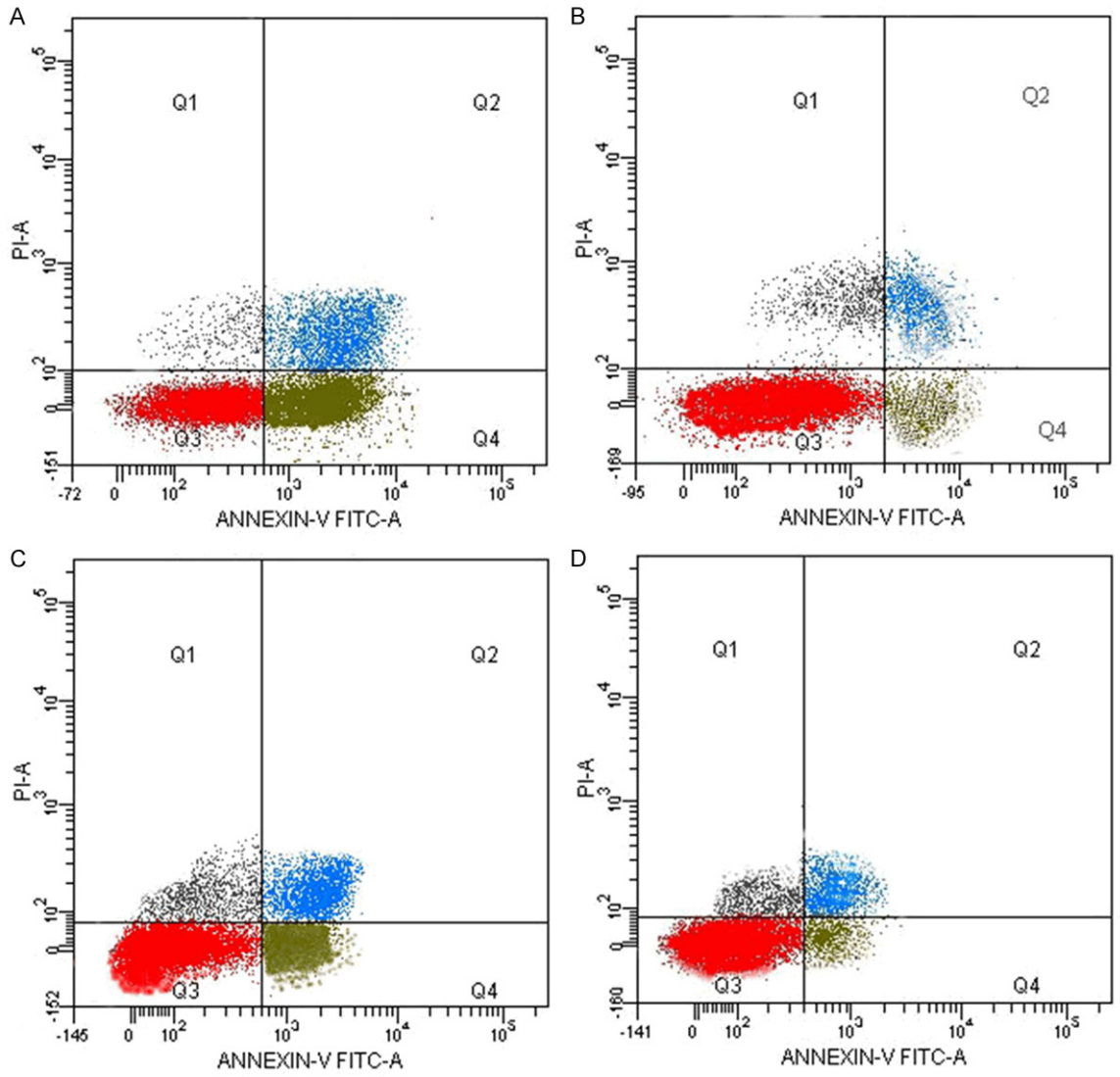

Figure 5. Apoptosis was assessed by Annexin-V/PI staining and FACS analysis. A: Interference group; B: Negative control group; C: Empty vector group; D: Blank control group.

cationic liposomes, then successfully delivered these HIF-1α-siRNA liposomes into gastric cancer cells with a high transfection efficiency. The fluorescein-luciferase biochemiluminescence response system that was used to determine ATP levels has high specificity and sensitivity and simple operation steps. Compared with the negative control transfection, HIF-1α silencing was found to significantly lower the content of cellular ATP in gastric cancer cells in a gradual time-dependent manner, which may have been due to induction of energy expenditure or cellular growth inhibition. Previously, HIF-1α and GLUT1 genes were shown to be upregulated in human gastric carcinoma, which was related to tumor size, depth of invasion, histological differentiation, clinical stage, and lymph node and remote metastasis, suggesting that HIF-1α and GLUT1 may play important roles in the tumorigenesis, progression, invasion, and metastasis of gastric carcinoma (data not shown). HIF-1α gene expression was correlated with GLUT1 expression at the mRNA and protein levels HIF-1α gene silencing was responsible for the down-regulation of downstream GLUT1 target genes, inducing the decrease of lactate converted from the glucose and pyruvate. Due to ATP formation of tumor cells mainly through the glycolysis pathway, the reduction of cellular ATP level was attributed to the inhibition of glycolysis. Although there may be other mechanisms involved in energy

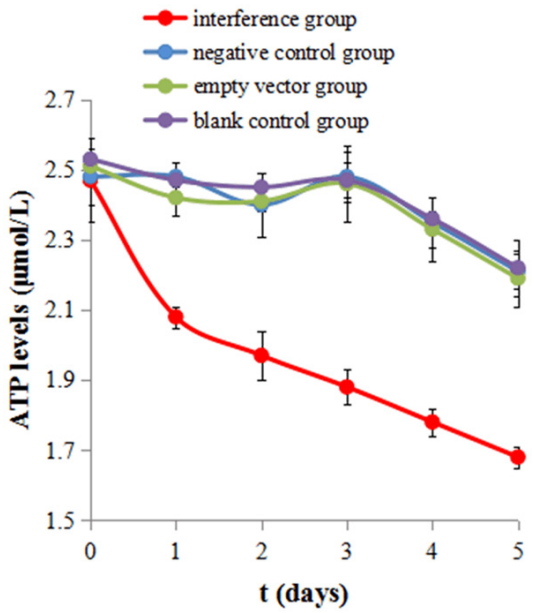

Figure 6. ATP levels in SGC-7901 cells after transfection.

metabolism, the present data suggested that overexpression of HIF-1α gene in gastric carcinoma may increase the level of energy metabolism and promote cell proliferation by upregulating glycolytic pathways according to the obtained data, which could be associated with the occurrence, development, metastasis, and poor prognosis of gastric tumors.

However, HIF-1α is not the only gene implicated in the regulation of energy balance. On targeting of the HIF-1α gene, it has been suggested that STAT3 and related pathways in tumors could be activated to exert HIF-1α-like functions [30]. Therefore, our further work will focus on identifying the pathways related to HIF-1α in gastric carcinoma, as well as key molecules in upstream or other regulatory pathways with HIF-1α-like functions, in order to elucidate the underlying mechanisms and novel therapeutic targets for the development of anti-cancer drugs.

In summary, the present study demonstrated that HIF-1α-siRNA transfected into SGC-7901 gastric cancer cells via liposomes could successfully inhibit the expression of HIF-1α at both the mRNA and protein levels. This silencing of HIF-1α expression could inhibit cell growth, reduce intracellular ATP levels, and induce apoptosis in the human gastric cancer cells. These data suggest that HIF-1α may be a potential target gene involved in the endogenous tumor response to hypoxia and inhibition of tumor energy metabolism, and thus indicate a novel therapeutic target for the treatment of gastric cancer.

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Disclosure of conflict of interest

None.

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