HIF-1α inhibits IDH-1 expression in osteosarcoma

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Abstract. Recently, hypoxia inducible factor-1 (HIF-1) was reported to be correlated with isocitrate dehydrogenase 1 (IDH-1) in several types of tumors. However, the expression and significance of HIF-1 and IDH-1 in osteosarcoma is still unknown. In the present study, the expression levels of IDH-1 and HIF-1α in 35 formalin-fixed paraffin-embedded sections from osteosarcoma patients were investigated by immunohistochemistry. The expression levels of IDH-1 and HIF-1α in human osteosarcoma cell lines (MG-63 and 143B) were further detected by western blotting under normal and hypoxic conditions. In addition, HIF-1α was downregulated via lentiviral vector-mediated RNA interference (RNAi) in the MG-63 human osteosarcoma cell line. The results revealed that HIF-1α was negatively correlated with IDH-1 in the osteosarcoma tissues. Both in MG-63 and 143B cell lines, the expression of HIF-1α was increased while IDH-1 was decreased under a hypoxic condition compared to normal conditions. HIF-1α downregulation promoted IDH-1 expression in the MG-63 cell line under either normal or hypoxic conditions. In conclusion, our findings suggest that HIF-1α inhibits IDH-1 in osteosarcoma and consequently increases the incidence of osteosarcoma.

Introduction

Osteosarcoma is the most common malignant bone tumor in children and adolescents. Its 5-year overall survival rate has increased to 70-80% (1,2) due to the development of effective diagnostic and treatment strategies for osteosarcoma in recent years (3). However, the survival rate has now reached a plateau. One important reason is the lack of precise targets and related mechanisms.

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Numerous solid tumors, including osteosarcoma, require a hypoxic microenvironment. A hypoxic state aids tumor growth and metastasis by inducing the release of various cytokines that promote angiogenesis (4). Hypoxia-inducible factor-1 (HIF-1) was first discovered by Wang and Semenza (5) in the study of hypoxia-induced gene expression. It is composed of two subunits, HIF-1α and HIF-1β, in which HIF-1α is the main unit and its expression is regulated by oxygen partial pressure. Hypoxia contributes to processes critical for cancer progression, such as resistance to chemotherapy and radiation therapy (6). HIF-1α is overexpressed in many types of malignant tumors (7-9), and is reported to be a critical and highly specific factor responding directly to hypoxia (10,11). HIF-1α is highly expressed in tumors under hypoxic conditions. It promotes the transcription of downstream target genes to adapt to the changes in the tumor microenvironment and to enhance the proliferation and metastasis of tumor cells and their tolerance to radiotherapy and chemotherapy (12).

Isocitrate dehydrogenase (IDH) is a type of enzyme family that plays an important role in many types of tumors. These enzymes are involved in the tricarboxylic acid (TCA) cycle and produce α-ketoglutarate (α-KG) via the oxidative decarboxylation of isocitrate (13). There are 5 human IDH genes (IDH-1 and IDH2 are homologous genes), encoding a total of 3 different IDH enzymes, in which IDH-1 is located in the cytoplasm and peroxidase and acts as the main source of non-mitochondrial NADPH required by multiple metabolic pathways (14). IDH-1 is regulated by cholesterol and fatty acid biosynthetic pathways, suggesting that IDH-1 provides cytosolic NADPH required by these pathways (15). Memon *et al* (16) found that in bladder cancer, the IDH-1 protein expression in lowly differentiated cancer cell lines was significantly lower than that in highly differentiated cancer cell lines, and the IDH-1 expression level was also significantly decreased in advanced cancer tissues. The IDH-1 gene may play a role as a tumor-suppressor gene to maintain the steady state of cells; thus, the occurrence of malignancies may be greatly increased after its mutation (17).

In recent years, numerous studies have been conducted on HIF-1α and IDH-1 expression in osteosarcoma (18-21) while studies on the correlation of the two have rarely been reported. The present study was designed to investigate the expression interaction of the two genes under different conditions to explore their relationship and to further understand the mechanism of HIF-1α and IDH-1 in osteosarcoma.

Materials and methods

Tissue specimens and clinical data. Thirty-five formalin-fixed, paraffin-embedded osteosarcoma specimens (before the administration of neo-adjuvant chemotherapy) were collected according to Chinese National Ethical Guidelines ('Code for Proper Secondary Use of Human Tissue', Chinese Federation of Medical Scientific Societies). All of the patients exhibited the diagnostic criteria for osteosarcoma as defined by the World Health Organization classification. Written informed consent was obtained from each patient before entering the present study, and all study protocols were approved by the Ethics Committee for Clinical Research of Wuhan University (Wuhan, China). The clinical data of IDH-1 were described in our previous study (22). Osteosarcoma specimens were graded using Rosen classification (23) (grade 1, tumor necrosis <50%; grade 2, tumor necrosis 50-90%; grade 3, tumor necrosis >90%; grade 4, tumor necrosis 100%; grade 1 and 2 are low histological Rosen grades, and grade 3 and 4 are high histological Rosen grades). We obtained 4 cases of grade 1, 12 cases of grade 2, 13 cases of grade 3, and 6 cases of grade 4. All of the osteosarcoma specimens were sliced into 4-μm serial sections followed by immunohistochemical staining.

Immunohistochemistry and evaluation of immunohistochemical results. All of the sections were obtained from formalin-fixed, paraffin-embedded osteosarcoma tissues and baked at 65°C for 2 h. After dewaxing, the sections were washed with phosphate-buffered saline (PBS) 3 times, and then placed in EDTA buffer for microwave antigen retrieval, boiled at medium heat and 10 min later, boiled again at low heat. After cooling, the sections were washed again with PBS 3 times, and then placed in 3% hydrogen peroxide solution and incubated for 10 min at room temperature in the dark. Anti-IDH-1 rabbit polyclonal antibody (Protein Tech Group, Chicago, IL, USA) or anti-HIF-1α rabbit polyclonal antibody (Abcam, Cambridge, MA, USA) was added to each section to cover the tissue and kept at 4°C overnight. Then, a goat anti-rabbit secondary antibody (KPL, Gaithersburg, MD, USA) was added to each section and incubated at 37°C for 50 min. The sections were washed with PBS 3 times for 5 min each time. Following the removal of PBS, 50-100 μl fresh DAB solution (Zymed, South San Francisco, CA, USA) was added to each section. Chromogenic reactions were controlled with a microscope, and hematoxylin staining was performed. A brown or tan zone was considered positive staining. The percentage of positive staining cells in the specimens was used to score the immunohistochemistry staining results (24,25) (for each section, two double-blinded pathologists independently observed each slide, 10 fields were randomly selected, and the mean positive cell percentage was used as the final result), and the specific classification was as follows: 0, no positively stained cells; 1 point, a positive cell percentage (i.e., cell staining rate) ≤10%; 2 points, a positive cell percentage of 10-25%; 3 points, a positive cell percentage of 26-50%; 4 points, a positive cell percentage of 51-75%; and 5 points, a positive cell percentage of >75%. A staining rate of 10% was chosen as the critical value of the negative and positive protein expression, and a staining rate >10% was considered positive protein expression and ≤10% was negative. Zero to 3 points denoted a low expression level (cell staining rate ≤50%), and 4-5 points represents a high protein expression level (cell staining rate >50%).

Western blotting. Human osteosarcoma cell lines MG-63 and 143B were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) through LGC Promochem (Wesel, Germany). Osteosarcoma cells were exposed to hypoxia (oxygen content of 1%) and normal conditions (oxygen content of 20%) (26). The cells were thoroughly lysed with an appropriate amount of RIPA lysis buffer (Aspen, USA), and the lysate was centrifuged at 4°C at 12,000 rpm for 5 min to collect the total protein solution. Using a BCA protein assay kit (Aspen), the protein concentration of the sample was determined. According to the concentration of each sample, 40 μg of total protein of each sample was used as a loading sample, in which the appropriate amount of 5X protein loading buffer was added, and then heated in a 95°C water bath for 5 min and loaded on a gel. After the electrophoresis was complete, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The transferred membranes were blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween-20 (TBST) and washed 6 times in TBST. IDH-1 and HIF-1α proteins were detected by the rabbit polyclonal antibody for IDH-1 (Protein Tech Group) or HIF-1α (Abcam). β-actin proteins were recognized by the β-actin-specific monoclonal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibodies were diluted according to the manufacturer's instructions, and were incubated overnight at 4°C followed by incubation with peroxidase-conjugated goat anti-rabbit immunoglobulin (1:2,000; Santa Cruz Biotechnology) in TBST for 1 h. Signals were developed using enhanced chemiluminescent reagent (Pierce Biotechnology, Rockford, IL, USA). β-actin was used as the internal loading control. The band intensity was analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA). Relative expression was calculated as the intensity ratio of target protein to that of β-actin.

Establishment of stable HIF-1α-knockdown osteosarcoma cells. To downregulate HIF-1α, a small interfering RNA sequence inserted into the pLL3.7 shRNA lentivector (Genesil, Wuhan, China) to reduce HIF-1α expression was 5'-CUGAUG ACCAGCAACUUGAdTdT-3' (sense) and 5'-UCAAGUUGCU GGUCAUCAGdTdT-3' (antisense) (27). The empty lentivector (Genesil) was used as a negative control. Non-lentivector infection was used as an additional control. The lentivector stocks were added to the osteosarcoma cell line MG-63. The cells were infected with the lentivector, and the GFP-positive cells were counted before G418 selection, using 800 μg/ml for MG-63. The efficiency of the highest infection, as determined by directly counting GFP-positive cells and G418 selection, was obtained at a multiplicity of infection (MOI) of 50 for MG-63. The frequency of infection rose to 95% for MG-63 cells. Cells infected with knockdown lentivector (KD) were called KD cells. Cells without lentivector infection were called NT cells, and the cells with empty lentivector infection were called NC cells. After selection, the efficiency of infection was verified by western blotting.

Table I. Clinical features of the 35 osteosarcoma tumor samples.

Clinical features	n (%)	P-value	HIF-1α protein Low (%)	High (%)	P-value
Total	35 (100.0)		21 (60.0)	14 (40.0)	
Age (years)		0.688			0.437
≤20	20 (57.1)		13 (65.0)	7 (35.0)	
>20	15 (42.9)		8 (53.3)	7 (46.7)	
Sex		0.225			0.985
Male	27 (77.1)		16 (59.3)	11 (40.7)	
Female	8 (22.9)		5 (62.5)	3 (37.5)	
Primary tumor site		0.146			0.864
Femur	14 (40.0)		8 (57.1)	6 (42.9)	
Tibia	10 (28.6)		6 (60.0)	4 (40.0)	
Other	11 (31.4)		7 (63.6)	4 (36.4)	
Histological type		0.245			0.364
Osteoblastic	15 (42.9)		9 (60.0)	6 (40.0)	
Small cell	3 (8.6)		2 (66.7)	1 (33.3)	
Chondroblastic	3 (8.6)		2 (66.7)	1 (33.3)	
Acinous	1 (2.9)		0 (0.0)	1 (100.0)	
Telangiectatic	2 (5.7)		1 (50.0)	1 (50.0)	
Fibroblastic	8 (22.9)		4 (50.0)	4 (50.0)	
Mixed	3 (8.6)		3 (100.0)	0 (0.0)	
Histological Rosen grade[a]		0.005			0.032
1	4 (11.4)		3 (25.0)	1 (75.0)	
2	12 (34.3)		10 (83.3)	2 (16.7)	
3	13 (37.1)		6 (46.2)	7 (53.8)	
4	6 (17.1)		2 (33.3)	4 (66.7)	
Low-Rosen grade (1+2)	16 (45.7)		13 (81.3)	3 (18.7)	
High-Rosen grade (3+4)	19 (54.3)		8 (42.1)	11 (57.9)	

There were no significant differences in HIF-1α expression levels between groups in terms of sex, age, primary tumor location and tumor tissue type (P>0.05).

MTT assay. A total of 3.5×10^3 cells were seeded in each test well into a 96-well plate to detect cell growth. The cells were washed with PBS after cultured for 1-6 days. MTT (5 mg/ml) was then added to each well, and the mixture was incubated at 37˚C for 4 h. The culture medium was then replaced with an equal volume of dimethyl sulfoxide (DMSO). After shaking the plate at room temperature for 10 min, the absorbance of each well was determined at 570 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical processing. All of the data were analyzed using SPSS 20.0 statistical software. Numerical variable data with homogeneity of variance were analyzed by an independent samples t-test, and non-normal numerical variable data were analyzed using the Mann-Whitney U test. Categorical variables were analyzed using a Chi-square or Fisher's exact probability tests while a correlation test of normal distribution data was analyzed by the Pearson correlation coefficient method, and that of the non-normal data was performed using the Spearman correlation coefficient method. The results with

P<0.05 were considered to be statistically significant and those with P<0.01 were of high statistical significance.

Results

Relationship between IDH-1 and HIF-1α protein expression in osteosarcoma and clinicopathological characteristics. There were no significant differences in HIF-1α expression levels between groups in regards to sex, age, primary tumor location and tumor tissue type (P>0.05) (Table I). The IDH-1 expression level in the low histological Rosen grade group (tumor necrosis, ≤90%) was significantly higher than that in the high histological Rosen grade group with a statistically significant difference (P<0.01), and the expression level and the tissue classification was negatively correlated (r=-0.427) (Fig. 1). In contrast, HIF-1α expression in the high histological Rosen grade group was significantly higher than that in the low histological Rosen grade group, and was positively correlated with the tissue classification (P<0.05, r=0.356) (Fig. 1). Our results also showed that the survival
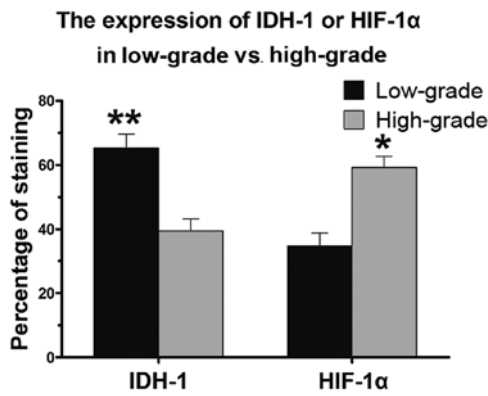
Figure 1. The percentage of immunostaining of IDH-1 and HIF-1α in low Rosen grade vs. high Rosen grade osteosarcoma tissues. IDH-1 was expressed higher in low histological Rosen grade tissues compared with that in the high histological Rosen grade tissues as determined by the percentage of immunostaining (**P<0.01), while HIF-1α was expressed higher in high histological Rosen grade tissues compared with that in low histological Rosen grade tissues (*P<0.05).
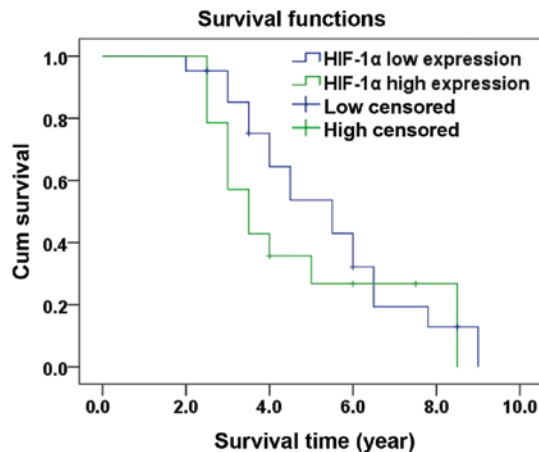

Figure 2. Relationship between HIF-1α and patient survival. The HIF-1α high expression group represents the osteosarcoma patients with >50% HIF-1α positive staining. Patients with ≤50% HIF-1α positive staining represent the low-expression group. The survival time as represented in the x-axis is expressed in years. There was no significant correlation between HIF-1α expression and overall survival (P=0.165).
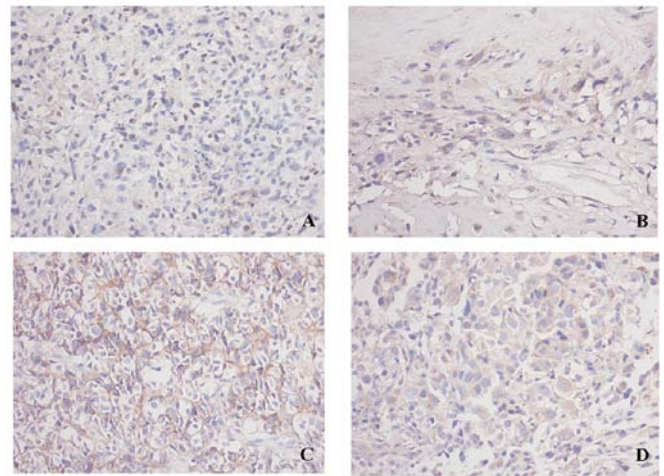

Figure 3. Expression of HIF-1α in a low histological Rosen grade biopsy and a high histological Rosen grade osteosarcoma biopsy specimen. HIF-1α expression in the high Rosen grade group was higher than that in the low Rosen grade group. (A) Expression of HIF-1α in a low histological Rosen grade biopsy specimen; magnification, x100. (B) Expression of HIF-1α in a low histological Rosen grade biopsy specimen; magnification, x200. (C) Expression of HIF-1α in a high histological Rosen grade biopsy specimen; magnification, x100. (D) Expression of HIF-1α in a high histological Rosen grade biopsy specimen, magnification, x200.
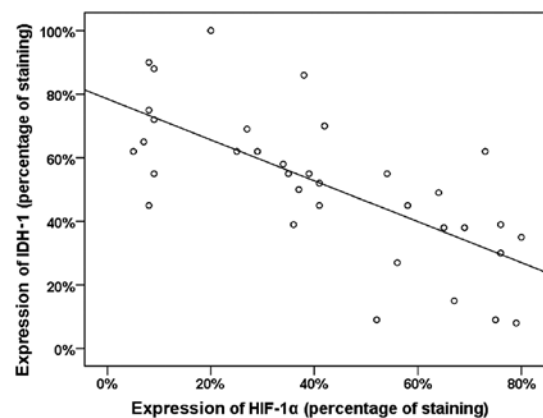

Figure 4. Relationship between the expression of HIF-1α and IDH-1 proteins. The expression of HIF-1α and IDH-1 proteins in osteosarcoma tissues was significantly negatively correlated (P<0.01, r=-0.70) through a Pearson correlation coefficient analysis.

rate was not significantly correlated with the expression of HIF-1α (P>0.05) (Fig. 2).

Expression of IDH-1 and HIF-1α in osteosarcoma in vivo by immunohistochemistry. We already described that IDH-1 is mainly located in the cytoplasm (22). In the present study, we found that HIF-1 protein was also significantly expressed in the cytoplasm (Fig. 3). The HIF-1α protein staining rate ranged from 5 to 80%, with a mean value of 41.46% and an SD of 24.81%, and the corresponding positive staining score was 1-5, with a mean value of 3.00 and an SD of 1.33. The positive expression rate of HIF-1α protein was 77.1% (27/35) with a high expression rate of 40% (14/35). The correlation test between the IDH-1 and the HIF-1α expression levels in 35 samples revealed P<0.01 and r=-0.700 (Fig. 4), indicating that the IDH-1 protein expression level was negatively correlated with that of the HIF-1α protein.

Expression of IDH-1 and HIF-1α in osteosarcoma in vitro under normal and hypoxic conditions by western blot assay. Our results showed that the expression of IDH-1 protein in 143B and MG-63 cells under normal conditions was higher than that under hypoxia, and the differences were statistically significant (P<0.05) (Fig. 5A and C). In contrast, HIF-1α proteins in 143B and MG-63 cells under hypoxic conditions were both highly expressed compared to that in normal conditions (P<0.05) (Fig. 5B and C).

HIF-1α downregulation decreases the growth rate of MG-63 cells. HIF-1α downregulation decreased the cell growth rate of MG-63 KD cells by 0.6- and 0.5-fold on day 6 compared to the MG-63 NC cells under normal conditions and hypoxic conditions, respectively (P<0.01) (Fig. 6). There was no signifi-
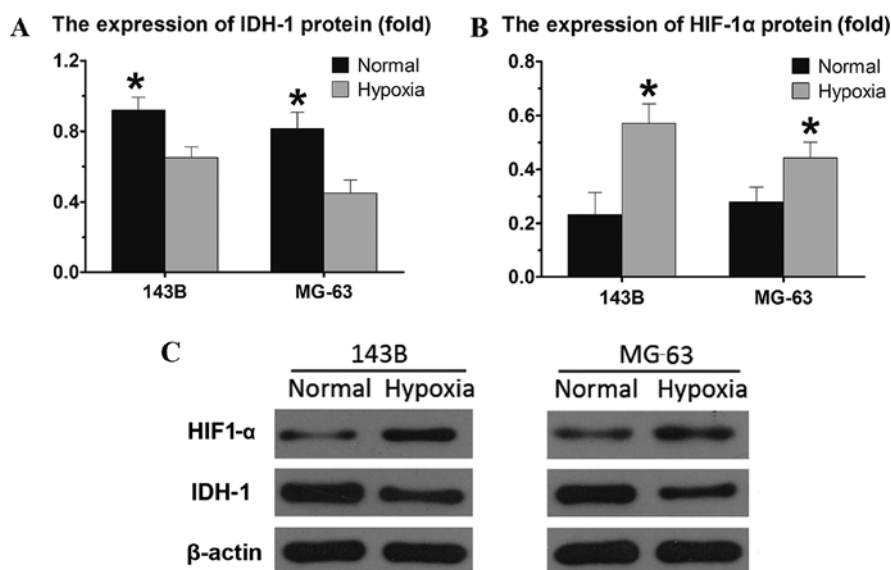
Figure 5. Expression of HIF-1α and IDH-1 in 143B and MG-63 cells. (A) Expression levels of IDH-1 protein in 143B and MG-63 cells under normal conditions were significantly higher than that under hypoxia (*P<0.05). (B) Expression levels of HIF-1α protein in 143B and MG-63 cells under hypoxia were significantly higher than under normal conditions (*P<0.05). (C) Representative western blot results are shown.
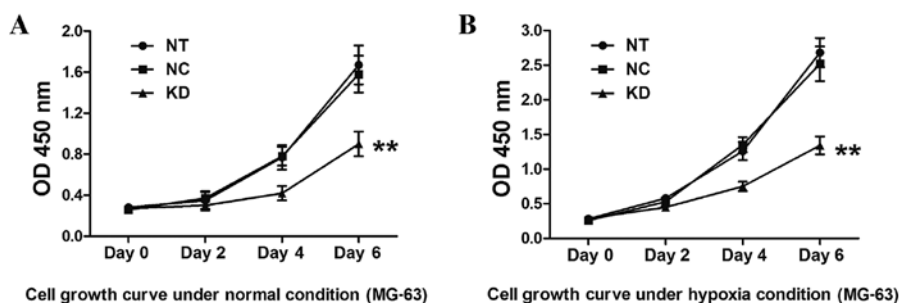

Figure 6. Downregulation of HIF-1α inhibits cell proliferation of MG-63 cells. HIF-1α downregulation inhibited cell proliferation in the MG-63 KD cells (**P<0.01) compared to the NT and NC cells under either (A) normal or (B) hypoxic conditions. HIF-1α expression did not differ between the NT and NC cells.
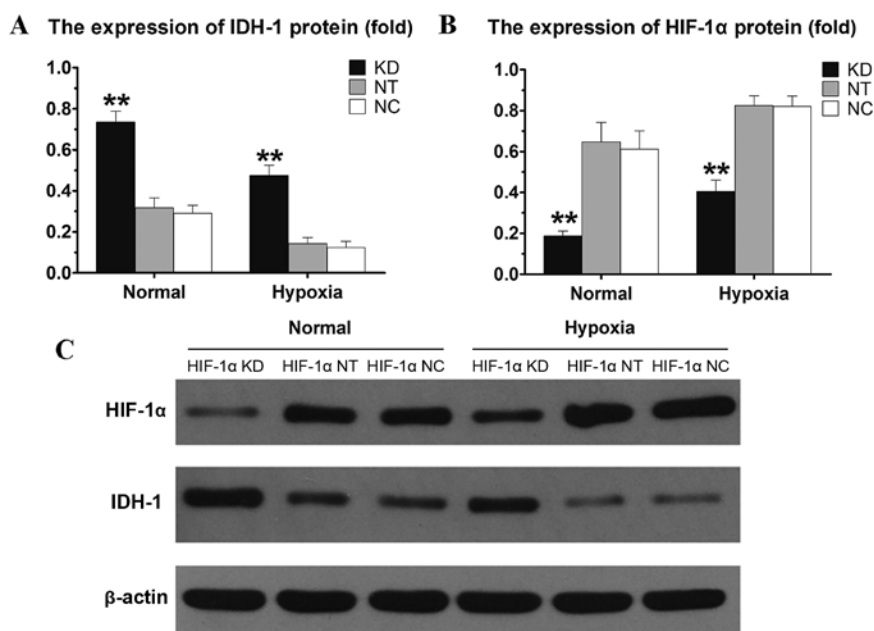

Figure 7. Expression of HIF-1α and IDH-1 in MG-63 KD (for HIF-1α), NT and NC cells under normal conditions and hypoxia. (A) IDH protein expression in MG-63 KD cells was significantly higher than that in NT and NC cells under normal conditions or hypoxia (**P<0.01). (B) HIF-1α was decreased significantly in the MG-63 KD cells, compared with NT and NC cells (**P<0.01) both in the hypoxic and normal group. (C) Representative western blot results are shown.

cant difference in the cell growth rate between MG-63 NT and MG-63 NC cells either under normal conditions or hypoxic conditions. The MG-63 cell growth rate was suppressed under hypoxic conditions.

Downregulation of HIF-1α increases the expression of IDH-1 under normal and hypoxia conditions. Lentivectors were used to downregulate HIF-1α and the infection efficiency was confirmed by western blotting. HIF-1α was significantly decreased in the MG-63 KD cells, compared with the NT and NC cells (P<0.01) both in the hypoxic and normal group (Fig. 7B and C). There was no significant difference in HIF-1α protein expression between NT and NC cells (P>0.05). Meanwhile, IDH-1 protein expression in the MG-63 KD cells was significantly higher than that noted in the NT and NC cells either under normal or hypoxic conditions (P<0.01) (Fig. 7A and C).

Discussion

The expression of IDH-1 and HIF-1α has been reported in multiple types of tumors (10,16,28). However, there is still a lack of in-depth study on their expression in osteosarcoma either *in vivo* or *in vitro*. In the present study, the results showed that the average HIF-1α protein staining rate in the osteosarcoma tissue samples of 35 patients was 41.46% and the positive expression rate was 77.1%. IDH-1 sustains loss-of-function mutations in certain human tumors, which likewise contribute to tumor growth via stimulating the HIF-1α pathway and mutationally altering metabolic enzymes (29,30) while human tumors without IDH-1 mutations have rarely been studied. Through Pearson correlation coefficient analysis, we first discovered that the expression of IDH-1 and HIF-1α proteins in osteosarcoma tissues was significantly negatively correlated with each other, suggesting that there may be some correlation between them. We also conducted research on the relationship between HIF-1α protein expression and the clinicopathological features in osteosarcoma, and found that the HIF-1α expression levels were not significantly correlated with sex, age, primary tumor location or tumor tissue type. IDH-1 protein expression in the low histological Rosen grade group was significantly higher than that in the high histological Rosen grade group while the HIF-1α expression level showed opposite trends.

The IDH-1 protein is localized in the cytoplasm and peroxisomes and plays an important role in glucose and lipid metabolism, antioxidative damage against ROS, and radiation injury (31). IDH-1 expression in the osteosarcoma tissue can inhibit the proliferation and metastasis of osteosarcoma (18). Yang *et al* (32) found that the elevated IDH-1 converted hypoxia-induced melanoma cell invasion and metastasis to apoptosis, following downregulation of HIF-1α. However, whether HIF-1α expression affects IDH-1 remains unknown. We performed a western blot assay to detect IDH-1 and HIF-1α proteins in 143B and MG-63 cells under hypoxic and normal conditions. We observed that under normal conditions, HIF-1α bands were almost invisible in the 143B cells and weakly expressed in the MG-63 cells while IDH-1 protein was highly expressed in both cell lines, which suggested a negative correlation between the expression of

IDH-1 and HIF-1α *in vitro*, in accordance with our previous *in vivo* results by immunohistochemistry. Notably, HIF-1α expression in both osteosarcoma cell lines was significantly increased under hypoxic conditions, and the expression of IDH-1 was decreased. HIF-1α activates the transcription of genes whose protein products function either to increase O_2 delivery or to provide metabolic adaptation under conditions of hypoxia, further inducing the proliferation and metastasis of tumor cells (33). The IDH-1-dependent pathway is active in most cell lines under normal culture conditions (34) but is inhibited under hypoxia possibly due to the overexpression of HIF-1α to some extent.

HIF-1α was found to enhance the proliferation and metastasis of tumor cells under hypoxia by promoting the transcription of hypoxia adaptation genes (8,35). Qian *et al* (36) found that knockdown of HIF-1α inhibited cell proliferation and invasion of lung cancer cells. In a further experiment, we first downregulated the expression of HIF-1α in MG-63 cells via lentiviral vector-mediated RNA interference. The western blot assay showed that downregulation of HIF-1α promoted the expression of IDH-1 protein in osteosarcoma cells under either hypoxic or normal conditions. The results strongly supported our previous observation from osteosarcoma patients and cells. Suppression of HIF-1α also decreased the proliferation of osteosarcoma cells as shown via MTT assay. Our previous study showed that IDH-1 downregulation exacerbated osteosarcoma cell proliferation (18). We, therefore, conclude that HIF-1α may reduce the expression of IDH-1 protein and consequently enhance the incidence of osteosarcoma. In addition, we found that cell proliferation may be promoted under conditions of hypoxic stress. This suggests that overexpression of HIF-1α under hypoxia increases the transcription of downstream genes, which contributes to cancer cell proliferation (37,38).

To further investigate the relationship of IDH-1 and HIF-1α expression with the survival of patients with osteosarcoma, we performed a survival correlation analysis on 35 patients with osteosarcoma, and the results showed that the survival of patients with osteosarcoma was not significantly correlated with the expression of HIF-1α. From the survival curve, however, we found that the 2- to 6-year survival of patients in the HIF-1α low-expressing group was higher than that in the HIF-1α high-expressing group. Therefore, we considered that the expression of HIF-1α was correlated with the survival of patients with osteosarcoma to some extent, while no significant results were shown due to the limitation of the number of cases and the influence of cases lost to follow-up.

In summary, the present study highlights that HIF-1α inhibits IDH-1 expression in osteosarcoma *in vitro* and *in vivo*, which further clarifies the role of HIF-1α in osteosarcoma pathogenesis.

Acknowledgements

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