Original Article
LDHA is a feedback activator of hypoxia inducible factor 1-alpha in ovarian cancer

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Abstract: Hypoxia inducible factor 1-alpha (HIF-1α) is commonly up-regulated in multiple human malignancies, and plays an important role in tumor metabolism through regulating glycolytic enzymes, including glucose transporter 1 (GLUT1), hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA). However, little is known about the possible roles of glycolytic enzymes in regulation of HIF-1α expression. In current study, by immunohistochemical analysis, we found that HIF-1α and LDHA were consistently up-regulated in different pathological types (serous, mucinous and endometrioid) of human ovarian cancer tissues compared to normal ovary tissues. By spearman correlation analysis, we revealed that HIF-1α expression in serous cystadenocarcinoma was closely correlated with LDHA expression at protein level. Under hypoxic condition, HIF-1α and LDHA protein were markedly increased in SKOV3 and HO8910 cells; genetic silencing of HIF-1α expression reduced LDHA level. Specially, knockdown of LDHA significantly attenuated HIF-1α protein accumulation, indicating a feedback role of LDHA on HIF-1α expression. Taken together, our findings reveal a novel circuit between LDHA and HIF-1α, and support that HIF-1α/LDHA axis might be a potential therapeutic target in ovarian cancer.

Keywords: HIF-1α, LDHA, feedback, ovarian cancer

Introduction

Ovarian cancer, as one of the most gynecologic malignancies, is the fourth cause of cancer-related death in women [1]. Due to more than half of patients are diagnosed at advanced stages, the overall 5-year survival rate is only approximately 33%. When ovarian cancer is confined to the ovary, the rate is about 90% [2]. Because deficiency in effective screening programs and lack of obvious clinical symptoms, however, it is hard to make an early diagnosis [3-5]. Therefore, underlying the molecular mechanisms involved in ovarian cancer may benefit the treatment of this deadly disease.

Ovarian cancer is a type of solid tumor, of which accompanied with hypoxic microenvironment [6, 7]. Hypoxia plays a crucial role in the initiation and progression of ovarian cancer [8]. Hypoxia inducible factor 1 (HIF-1), as the key regulator of oxygen homeostasis, consists two subunits: an oxygen-labile α-subunit (HIF-1α) and a constitutively expressed β-subunit (HIF-1β) [9]. Through binding to hypoxia response elements (HRE) in the promoter of multiple targeted genes involved in angiogenesis, cell survival and glucose metabolism, HIF-1α mediates the adaptive alternations to hypoxic conditions [10, 11]. Aerobic glycolysis, known as the Warburg effect, is widely believed to arise as a result of a hypoxic tumor microenvironment [12, 13]. Lactate dehydrogenase A (LDHA), which catalyzes the inter-conversion of pyruvate and L-lactate, is a direct target of HIF-1α and frequently deregulated in human malignancies, including pancreatic cancer [14, 15], colorectal cancer [16], glioblastoma [17], and non-small cell lung cancer [18]. Serum LDH is also up-regulated in gynecologic and breast tumors and this is partly related to elevated LDHA expression [19]. In ovarian cancer, a selective small-molecule BET bromodomain (BRDs) inhibitor, JQ1, has been demonstrated to suppress tumor growth through down-regulating LDHA [20]. Specially, up-regulated HIF-1α levels have been evaluated in numerous studies [21]. However, limited knowledge is known about the correla-
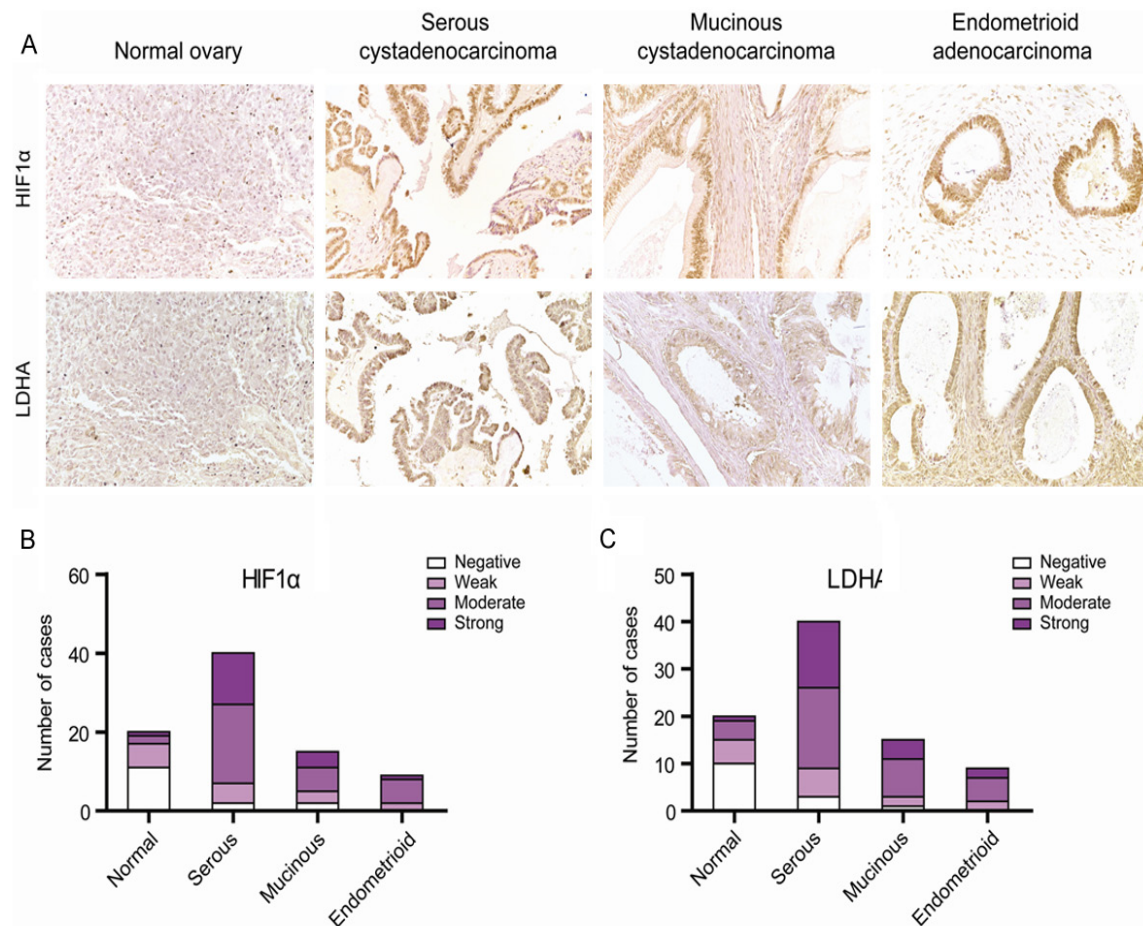
Figure 1. HIF-1α and LDHA expression are commonly up-regulated in ovarian cancer tissues. The expression of HIF-1α and LDHA in different pathological types of ovarian epithelial carcinoma tissues was assessed using IHC staining. (A) Representative images were captured at × 200 magnification. HIF-1α (B) and LDHA (C) IHC staining scores in different types of ovarian cancer tissues.

tion between HIF-1α and LDHA in ovarian cancer.

In this study, by a large scale of immunohistochemical analysis, we determined the expression pattern of HIF-1α and LDHA in different pathological types and demonstrated that LDHA expression was closely correlated with HIF-1α level. Furthermore, a feedback role of hypoxia-induced LDHA on HIF-1α expression was revealed. Thus, we identified a novel HIF-1α/LDHA circuit as potential therapeutic target for ovarian cancer.

Materials and methods

Cell culture

Human ovarian cancer cell lines SKOV3 and HO8910 were all purchased from Cell Bank of Chinese Academy of Sciences (Shanghai,

China). Cells were cultured in RPMI-1640 medium (Gibco, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin (Invitrogen, USA) in 5% CO_2 at 37°C. For hypoxic condition, cells were cultured in a modular incubator chamber (Thermo Electron, MA, USA) that was infused with a mixture of 1% O_2, 5% CO_2 and 94% N_2.

Immunohistochemical staining (IHC)

The commercial ovarian cancer tissue microarray (TMA) was purchased from Xi-an Alenabio Biotech Inc (OV1005a, Xi-an, Shanxi, China). For immunohistochemical staining, sections were dewaxed, rehydrated, and antigen retrieval was performed by incubating in citric acid (pH 6.0) for 10 min at 98°C, and endogenous peroxidase was blocked with 0.3% H_2O_2 for 15 min. Then sections were blocked with 10%
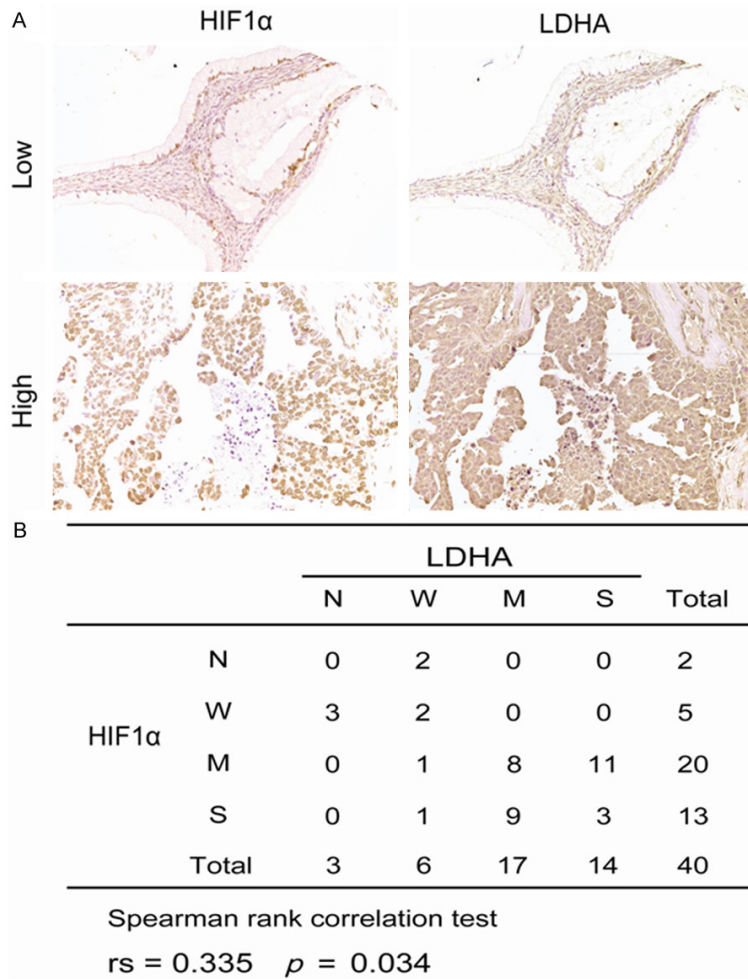
Figure 2. Correlation between HIF-1α and LDHA. A. IHC analysis showed representative low (up) and high (down) expression of HIF-1α and LDHA in consecutive sections; images were captured at × 200 magnification. B. Statistical analysis of the correlation between HIF-1α and LDHA. N, negative; W, weak; M, moderate; S, strong. *P* values were calculated by the Spearman correlation test.

Figure 2B table:

		LDHA				
		N	W	M	S	Total
HIF1α	N	0	2	0	0	2
	W	3	2	0	0	5
	M	0	1	8	11	20
	S	0	1	9	3	13
	Total	3	6	17	14	40

Spearman rank correlation test

rs = 0.335   *p* = 0.034

Quantitative real-time PCR

Total RNA from cell lines was extracted by RNA Extraction Kit (SLNco, Cinoasia, China). cDNA for RT-PCR was synthesized using the qScript cDNA Synthesis Kit (Quanta Biosciences, MD, USA) according to manufacturer's protocol. The mRNA expression of HIF-1α or LDHA was conducted on an ABI Prism 7300 PCR Detection System (Applied Biosystems, Foster City, CA, USA) with SYBR Green Real-time PCR Master Mix (QPK-201, TOYOBO, Japan). The primers used in this study are as follows: HIF-1α, forward 5'-ATCCATG TGACCATGAGGAAATG-3', reverse, 5'-TCGGCTAGTTAGGG-TACACTTC-3'; LDHA, forward 5'-ATGGCAACTCTAAAGGAT-CAGC-3', reverse, 5'-CCAACCC-CAACAACTGTAATCT-3'; β-actin, forward 5'-GCACAGAGCCTC-GCCTT-3', reverse, 5'-GTTGTC-GACGACGAGCG-3'. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method normalized to the β-actin mRNA levels.

Small interfering RNA (siRNA) transfection

Specific target sequence for small interfering RNA was synthesized from (GenePharma, Shanghai, China) and the control nucleotide sequence of small interfering RNA was 5-GTACATAGGGACGTAA-CG-3. SKOV3 and HO8910 cells were seeded into two 6-well plates. After incubation for 24 h, cells were transfected with indicated siRNA using Lipofectamine reagent (Invitrogen, USA) as recommended by the manufacturers. After 8 h incubation, the medium was replaced with RPMI 1640 containing 10% FBS.

Western blotting

Total protein was extracted from ovarian cancer cells using RIPA buffer (Thermo Fisher, MA, USA) and quantified with the BCA assay kit
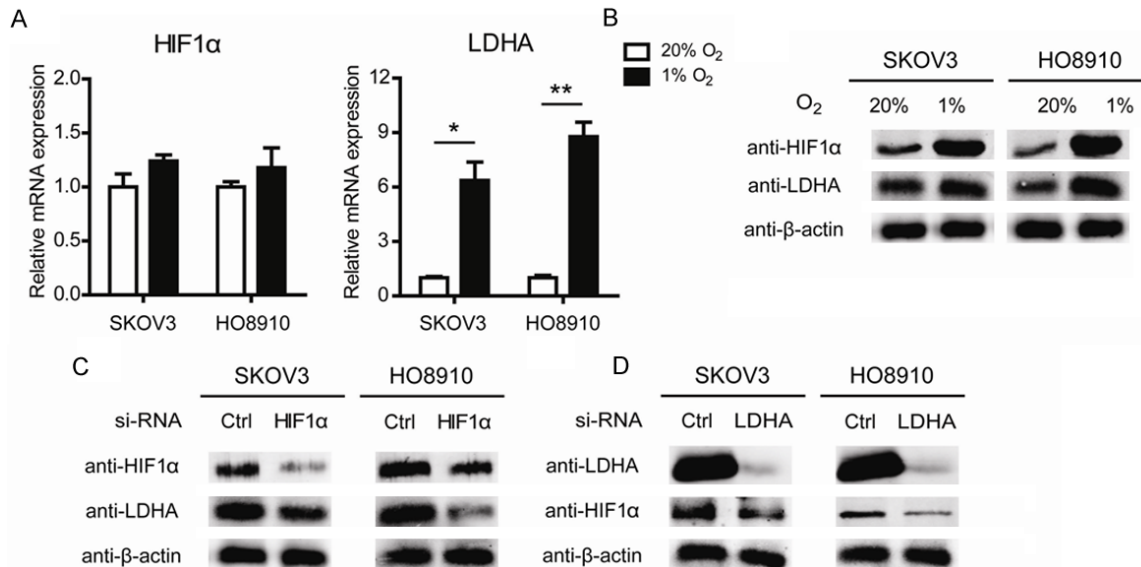
bovine serum albumin (BSA) for 30 min (Sangon, Shanghai), and incubated with 1:200 rabbit anti-HIF-1α (ab113642, Abcam, USA), 1:200 rabbit anti-LDHA (ab125683, Abcam, USA) in PBST for 1 h. Secondary antibody labeled by HRP (rabbit) (Proteintech, US) was incubated for 1 h and staining was visualized by incubation in 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and subsequently counterstained by hematoxylin. Scoring was calculated according to the positive area: less than 5% scored negative; 6-30% scored weak; 31-60% scored moderate; more than 60% scored strong. Negative and weak staining was defined as low expression, while moderate and strong staining was defined as high expression.

Figure 3. LDHA is a feedback activator of HIF-1α. The mRNA (A) and protein level (B) of HIF-1α and LDHA was measured under hypoxia. (C) Effects of genetic silencing of HIF-1α on LDHA expression. (D) Effects of knockdown of LDHA on HIF-1α expression. 20% O_2 versus 1% O_2, *P < 0.05, **P < 0.01.

(Thermo Fisher, MA, USA). Equal amounts of total protein were loaded and separated by 10% SDS-PAGE, then transferred to PVDF membrane, blocked in 5% non-fat milk and incubated with the primary antibodies overnight at 4°C. Subsequently, the membranes were incubated with the appropriate secondary antibodies for 1 h at room temperature. The signal was then detected by chemiluminescence with SuperSignal kit (Pierce, Rockford, IL, USA). β-actin was loaded as the endogenous control.

Statistical analysis

Data were presented as the means ± standard deviations (SDs). All statistical analyses were performed using the SPSS 16.0 (SPSS Inc.; Chicago, USA) and GraphPad Prism 5 (San Diego, CA) software. Correlation between HIF-1α and LDHA expression was analyzed by spearman correlation test. Statistical significance was defined to be P < 0.05.

Results

HIF-1α and LDHA expression are commonly up-regulated in ovarian cancer tissues

Elevated HIF-1α expression in epithelial ovarian cancer has been demonstrated in numerous previous studies. Consistent with this, by IHC analysis of a TMA, which contains 20 cases of

normal ovary tissues, 40 cases of serous cystadenocarcinoma, 15 cases of mucinous cystadenocarcinoma and 9 cases of endometrioid adenocarcinoma, we observed that HIF-1α expression was prominently located in the nucleus and remarkably up-regulated in ovarian cancer tissues compared to normal ovary tissues regardless of pathological types (**Figure 1A** and **1B**). As a direct target of HIF-1α in metabolic regulation, LDHA was widely expressed in the cytoplasm of ovarian cancer cells, whereas weakly expressed in normal ovary tissues (**Figure 1A** and **1C**).

Correlation between HIF-1α and LDHA

Although intense studies have revealed the regulatory roles in glycolytic enzymes, including GLUT1 [22], HK2 [23] and LDHA [24], little data is available to observe their expression pattern together in ovarian cancer tissues. By IHC analysis of two consecutive TMA, we found that HIF-1α expression in serous cystadenocarcinoma was closely correlated with LDHA protein level (**Figure 2**). As shown in **Figure 2B**, HIF-1α and LDHA were co-down-regulated in 17.5% (7/40) serous cystadenocarcinoma, and co-up-regulated in 17.5% (7/40) serous cystadenocarcinoma, 77.5 (31/40) serous cystadenocarcinoma, indicating the regulatory function of HIF-1α on LDHA expression.

LDHA is a feedback activator of HIF-1α

To further determine the correlation between HIF-1α and LDHA, we cultured ovarian cancer cells SKOV3 and HO8910 under hypoxic condition. As shown in **Figure 3A**, LDHA mRNA was markedly up-regulated with 1% oxygen compared to 20% oxygen, while HIF-1α mRNA remained unaltered. We next detected HIF-1α and LDHA expression at protein level. The result showed that both HIF-1α and LDHA protein were induced by hypoxic condition (**Figure 3B**). To demonstrate whether up-regulation of LDHA is HIF-1α-dependent, we measured LDHA level after genetic silencing of HIF-1α. As shown in **Figure 3C**, specific siRNA targeting HIF-1α resulted in a markedly decrease in HIF-1α expression and compromised LDHA expression induced by hypoxia, indicating that up-regulation of LDHA in ovarian cancer was HIF-1α-dependent. As LDHA plays critical roles in glycolysis and contributes to acidified microenvironment, we reasoned whether LDHA can regulate HIF-1α through uncovered signaling. To test this hypothesis, we suppressed LDHA in SKOV3 and HO8910 cells, and detected HIF-1α expression. To our surprise, knockdown of LDHA significantly attenuated HIF-1α protein level under hypoxia, indicating that a feedback was existed between LDHA and HIF-1α (**Figure 3D**).

Discussion

Hypoxia is a common characteristic of many solid malignancies and is associated with aggressive phenotype with increased proliferation, invasiveness and metastases [25, 26]. Under hypoxic condition, several modulations have been demonstrated to regulate HIF-1α stability and transcriptional activity via post-transnational modifications, including hydroxylation, ubiquitination, and acetylation, which ultimately contribute to accumulation of HIF-1α protein [23, 27]. It is widely reported that HIF-1α expression in ovarian cancer or borderline tissues was significantly up-regulated than that in benign tissues [28-32]. Notably, HIF-1α expression is related to FIGO stage, histological type, lymph node metastasis and 5-year survival rate of ovarian cancer [21]. In this study, we observed that HIF-1α expression was universally expressed in ovarian cancer regardless of pathological type, while merely detected in normal ovary tissues. Due to unavailable to clinical follow-ups, however, we failed to test the prognostic value of HIF-1α in current cohort.

Reprogrammed glucose metabolism is a key hallmark of cancer cells [33]. As noted by Warburg, in contrast to normal cells, tumor cells could rapidly metabolize glucose to lactate under aerobic conditions despite this process (glycolysis) being far less efficient in ATP production [34]. LDHA exhibits a critical branch point in aerobic glycolysis of tumor cells. Here we first demonstrated that LDHA expression was up-regulated in different pathological types of ovarian cancer tissues in relative to normal ovary tissue, indicating the potential oncogenic functions of LDHA in ovarian cancer as revealed in other malignancies [16, 35-38]. Many known oncogenes, such as Myc, HIF-1α, forkhead box protein M1 (FOXM1), and Krüppel-like factor 4 (KLF4) are involved in the transcript expression and post-transcriptional modification of LDHA [14, 37, 39]. By IHC analysis in consecutive sections, we revealed a close correlation between HIF-1α and LDHA. Then, we found that hypoxia-induced HIF-1α was responsible for LDHA expression as demonstrated by genetic silencing of HIF-1α compromised LDHA protein level under hypoxic condition. Indeed, silencing of LDHA also attenuated HIF-1α accumulation. It has been reported that lactate can enhance the stabilization of HIF-1α, and activate NF-κB and PI3K pathway in endothelial cells [40-43]. However, whether the feedback role of LDHA on HIF-1α accumulation is mediated by extracellular lactate remains further investigation.

Taken together, our study reveals the expression pattern of HIF-1α/LDHA axis and identifies a novel circuit between HIF-1α and LDHA. This finding, as a proof of principle, provides new insight into understanding the complicated interaction between HIF-1α and cancer metabolism.

Disclosure of conflict of interest

None.

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