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Original contribution

Histone deacetylase-1 as a prognostic factor and mediator of gastric cancer progression by enhancing glycolysis☆

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Summary Histone deacetylase 1 (HDAC1) has been shown to be closely associated with tumor development. We investigated its effects on survival and biological behavior in gastric cancer (GC). HDAC1 expression and glycolysis activity were analyzed in a cohort of 252 samples of primary GC tumors and in vitro study. High HDAC1 ($HDAC1^{High}$) staining was seen in 60.7% patients with GCs, which was significantly greater than was seen in normal epithelial cells (19.4%; $P < .005$). $HDAC1^{High}$ expression was associated with larger tumor size ($P = .001$), advanced T stage ($P = .001$), lymph node metastases (N stage; $P < .001$), and lymphovascular invasion ($P = .005$). Univariate and multivariate survival analyses showed HDAC1 expression to be an independent prognostic factor for both disease-free survival and overall survival ($P < .05$). In vitro studies showed a notably decreased glycolysis rate in *HDAC1* knockdown cells. In patients' samples, $HDAC1^{High}$ expression was always accompanied with high Maximal standardized uptake value (SUVmax) value ($P < .05$). A hypoxia-inducible factor (HIF)-1α response element–luciferase reporter system showed HDAC1 to affect HIF1α activity in a dose-dependent manner. In conclusion, HDAC1 promotes glycolysis in GC and affects HIF-1α activity in tumor progression and metastasis. $HDAC1^{High}$ expression was also an independent adverse prognostic factor for overall survival and disease-free survival.

1. Introduction

Gastric cancer (GC) is one of the most common malignancies in Eastern Asia [1,2], and its incidence has increased with the aging population [3]. In China, about 3 million new GC diagnoses and 2 million deaths from GC occur each year [4,5]. Standard curative treatment is resection with lymph node dissection; prognosis is mainly determined by TNM stage. However, the TNM staging system is clinically limited, in that even patients diagnosed at the same stage are split into recurrent and non-recurrent groups. Identifying underlying mechanisms for tumor progression and metastasis could facilitate earlier diagnosis and personalized therapy strategies.

Histone modification through reversible acetylation is a crucial event in gene expression. Histone acetylation is controlled by histone acetyltransferase and histone deacetylase (HDAC) [6,7]. HDACs have been widely associated with cancer development and treatment [8]. For example, HDAC1

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is overexpressed in many cancers, including breast cancer, esophageal cancer, lung cancer, renal cell cancer, prostate cancer, and classical Hodgkin lymphoma [9-13]. Moreover, HDAC1 overexpression often correlates with clinicopathological features that predict poor prognosis in breast cancer, prostate cancer, and lung cancer [9,10,12]. In addition, whereas silencing *HDAC1* with siRNA or shRNA results in cell-cycle arrest, cell growth inhibition, chemosensitivity and induction of apoptosis in breast and colon cancer cells, or esophageal cancer [13-15], HDAC1 overexpression leads to increased cell proliferation in prostate and breast cancers [12,16,17], which indicates that HDAC1 promotes cancer progression. HDAC1 is reportedly overexpressed in GC [7], but its prognostic value and underlying mechanism have not been fully investigated.

The present study focused on HDAC1 expression in GC tissues, its clinical significance, and their underlying mechanisms, especially its influence on glycolysis.

2. Materials and methods

2.1. Patient specimens

The study subjects were 252 patients with GC, which has been described previously [18]. Their pathologic diagnoses were reconfirmed by examining hematoxylin and eosin–stained slides and reviewing pathological reports and clinical records (representative slide: Supplementary Fig. S1). All patients were restaged according to the eighth Union for International Cancer Control/American Joint Committee on Cancer TNM system. This study was approved by the institutional review board of Yancheng Hospital. Every patient gave signed informed consent for research use of their clinical samples.

2.2. Immunohistochemical staining

Slides were deparaffinized, rehydrated, and then incubated with rabbit polyclonal antibody against HDAC1 (10197-1-AP, 1; 100; Proteintech, Wuhan, China) at 4°C overnight after heat-induced epitope retrieval. Staining detection was performed using the GTVision III Kit (GK500705; Gene Tech, Shanghai, China) detection kit according to the manufacturer's instructions. Phosphate-buffered saline was used as a negative control. Staining was semiquantitatively scored [19] by evaluating both staining intensity (0, no stain; 1+, weak stain; 2+, moderate stain; 3+, strong stain) and percentage of stained cells (0, <5%; 1, 5%-25%; 2, 26%-50%; 3, 51%-75%; and 4, >75%) and multiplying intensity and percentage positivity scores; a resulting product of ≥4 indicated a sample with high HDAC1 expression ($HDAC1^{High}$) and <4 indicated a sample with low HDAC1 expression ($HDAC1^{Low}$) [20].

2.3. Cell culture and reagents

Human gastric adenocarcinoma cell lines, AGS and MGC-803, were originally obtained from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in the Dulbecco modified Eagle medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 37°C incubator supplied with 5% CO_2.

2.4. Stable transfection of GC cells

Biologically active short hairpin RNA (shRNA) was generated using the lentiviral expression vector pLKO.1-puro. The shRNA target sequence for human *HDAC1* was 5′-GAAGT CCGAGGCATCTGGC-3′. PLKO.1-scramble shRNA with limited homology with any known sequences in the human, mouse, or rat genomes was used as a negative control. AGS and MGC-803 cells were transfected with the pLKO.1-shHDAC1 knockdown plasmid or pLKO.1 scramble. The stably transfected cells were isolated using puromycin selection to obtain stable *HDAC1* knockdown cells.

2.5. RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was obtained by reverse transcription using a TaKaRa PrimeScript RT reagent Kit (RR036A; TaKaRa, Dalian, China). Expression statuses of candidate genes and *β-actin* were determined by quantitative real-time polymerase chain reaction (PCR) using an ABI 7900HT Real-Time PCR system (Applied Biosystems, Carlsbad, CA). Primers used were human *HDAC1*: 5′-TTCAAGCTCCACATCAGTCCTTC-3′ (forward) and 5′-CTCTTCCTCACAGGCAATTCGTT-3′ (reverse), and human *β-actin*: 5′-CTACGTCGCCCTGGACTTCGAGC-3′ (forward) and 5′-GATGGAGCCGCCGATCCACACGG-3′ (reverse). All reactions were run in triplicate.

2.6. Western blot analysis

Equal amounts of cell lysates were subjected to 10% SDS-PAGE; proteins were transferred onto polyvinylidene difluoride membranes. The membranes were probed overnight with specific primary antibodies, which were detected with corresponding secondary antibodies (Cell Signaling Technology, Danvers, MA). Immunoreactive bands were visualized using enhanced chemiluminescence (Thermo Scientific, Carlsbad, CA). The following primary antibodies were used: HDAC1 (10197-1-AP, 1; 100; Proteintech) and β-actin (14395-1-AP; Proteintech). β-Actin served as a loading control.

2.7. Glycolysis analysis

Glucose Uptake Colorimetric Assay Kit (Biovision, Milpitas, CA) and Lactate Colorimetric Assay Kit (Biovision) were used to examine glycolysis processes in GC cells, according to the manufacturer's protocol. Real-time PCR was performed to test expression of glycolytic enzymes. All reactions were run in triplicate.

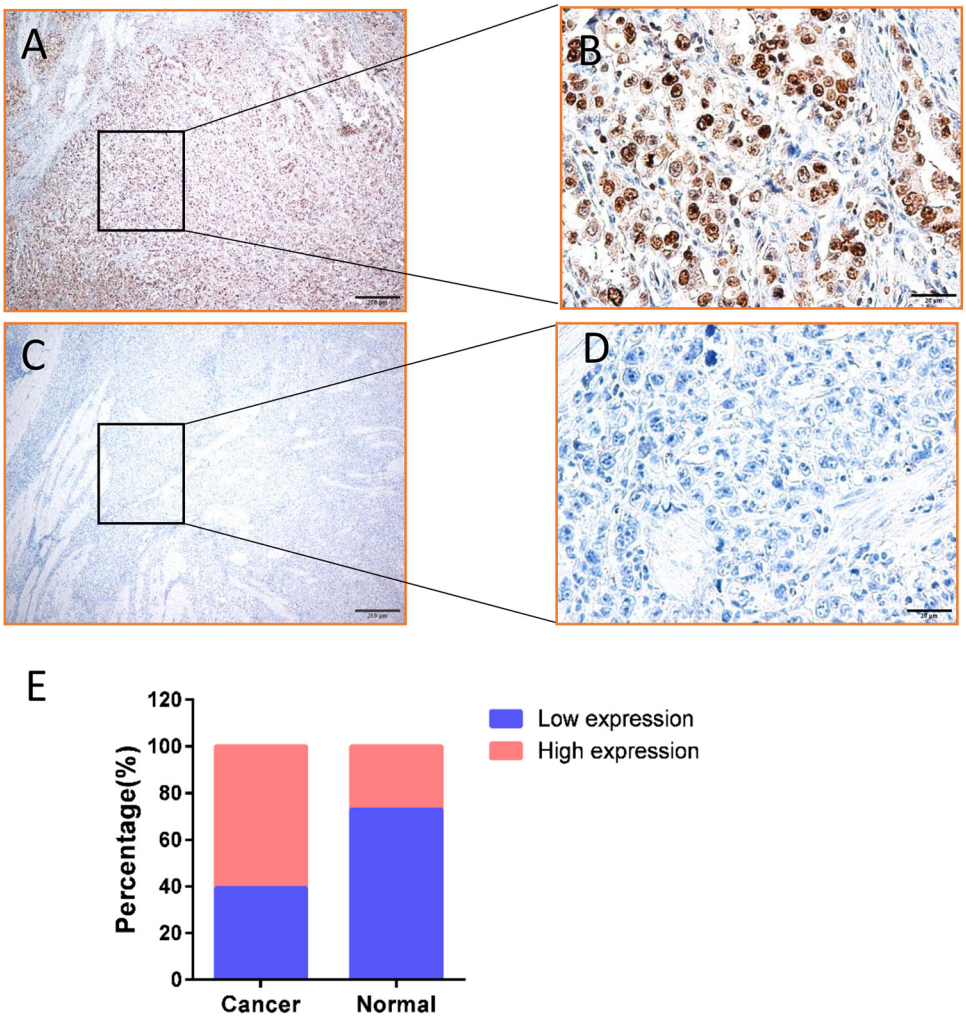

Fig. 1 HDAC1 expression in GC. A–D, Examples of HDAC1^{High} (A and B) and HDAC1^{Low} (C and D) expression in GC, as determined by immunohistochemical staining (A and C, original magnification ×100; B and D, ×400). E, HDAC1 expression was significantly higher in GC than in matched normal controls ($P < .05$).

2.8. Luciferase analysis

HIF-1α response element (HRE) sequence basic was cloned into pGL3-Basic Luciferase Reporter Vectors (Promega). The HRE promoter activity was normalized by cotransfection the cells with a Renilla luciferase reporter containing a full-length Renilla luciferase gene. Both firefly and Renilla luciferase activities were quantified using a Dual-Luciferase Reporter Assay System (Promega, Wisconsin, USA) 48 hours after transfection as described previously [20].

2.9. Statistical analysis

Statistical associations between clinical parameters and immunohistochemical staining were tested using χ^2 test to compare the expected and observed frequencies in the high and low HDAC1 expression groups. Five-year overall survival (OS) and disease-free survival (DFS) were calculated using the Kaplan-Meier method to estimate survival and hazard functions of censored data. The difference in survival between the groups at each observed point was compared using the log-rank test. Variables for which $P < .05$ with respect to survival in univariate analysis were included into multivariate analysis, which used the Cox proportional hazard model to relate survival to other risk factors. All in vitro experiments were performed independently with 3 replications. $P < .05$ was considered significant. All statistical analyses were carried out on SPSS for Windows, version 21.0 (SPSS, Chicago, IL).

3. Results

3.1. HDAC1 expression in GC

HDAC1 immunostaining was predominantly localized in GC cell nuclei. Fig. 1A-D shows representative images of positive and negative HDAC1 staining in cancer tissues. HDAC1^{High} staining was seen in 60.7% (153/252) of

Table 1 Associations between HDAC1 expression and clinicopathological factors in patients with GCs

Characteristics	Total	HDAC1 expression		*P*
		Low expression, n (%)	High expression, n (%)	
Sex				.100
Male	134	59 (44.0)	75 (56.0)	
Female	118	40 (33.9)	78 (66.1)	
Age (y)				.371
≥60	131	48 (36.6)	83 (63.4)	
<60	121	51 (42.1)	70 (57.9)	
Primary site				.060
Antrum/Distal	95	32 (33.7)	63 (66.3)	
Cardia/Proximal	86	38 (44.2)	48 (55.8)	
Fundus/Body	48	24 (50.0)	24 (50.0)	
Gastroesophageal junction	23	5 (21.7)	18 (78.3)	
Diameter (cm)				.001 *
<4	160	63 (39.4)	97 (60.6)	
≥4	92	36 (39.1)	56 (60.9)	
Histologic grade				.146
G1/G2	108	48 (44.4)	60 (55.6)	
G3	144	51 (35.4)	93 (64.6)	
Histological type				.643
Adenocarcinoma/mucinous adenocarcinoma	221	88 (39.8)	133 (60.2)	
Signet-ring cell cancer	31	11 (35.4)	20 (64.5)	
T stage				.001 *
T1/2	40	25 (62.5)	15 (37.5)	
T3	111	46 (41.4)	65 (58.6)	
T4	101	28 (27.7)	73 (72.3)	
N stage				<.001 *
N0	69	41 (59.4)	28 (40.6)	
N1	52	21 (40.4)	31 (59.6)	
N2	58	17 (29.3)	41 (70.7)	
N3	73	20 (27.4)	53 (72.6)	
Lymphovascular invasion				.005 *
Negative	199	87 (43.7)	112 (56.3)	
Positive	53	12 (22.6)	41 (77.4)	
Perineural invasion				.700
Negative	185	74 (40.0)	111 (60.0)	
Positive	67	25 (37.3)	42 (62.7)	

* $P < .05$.

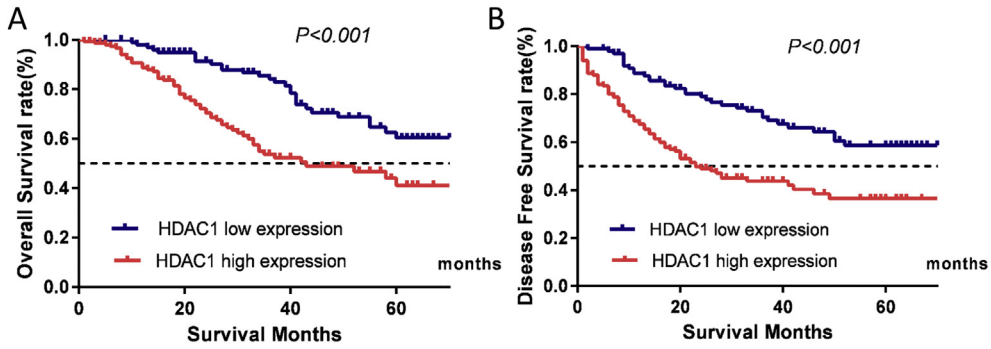

Fig. 2 High HDAC1 expression correlates with shorter survival in patients with GC. A, Five-year OS: $HDAC1^{High}$, 40.5%; $HDAC1^{Low}$, 59.9%; $\chi^2 = 14.311$, $P < .001$. B, Five-year DFS: $HDAC1^{High}$, 35.9%; $HDAC1^{Low}$, 58.4%; $\chi^2 = 19.706$, $P < .001$.

Table 2 Univariate and multivariate Cox proportional hazard analyses of *HDAC1* gene expression and OS for patients with GC

Factor	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Sex	0.780 (0.514-1.183)	.242		
Age	1.262 (0.832-1.915)	.273		
Diameter	1.254 (0.810-1.941)	.309		
T stage	2.029 (1.479-2.782)	<.001 *	1.227 (0.805-1.870)	.342
N stage	1.652 (1.365-2.000)	<.000 *	1.417 (1.107-1.813)	.006 *
Grade	1.694 (1.094-2.625)	.018 *	1.184 (0.753-1.863)	.465
Lymphovascular invasion	2.193 (1.404-3.426)	.001 *	1.688 (1.066-2.673)	.026 *
Perineural invasion	1.835 (1.193-2.822)	.006 *	1.912 (1.224-2.989)	.004 *
Tumor location	1.149 (0.957-1.379)	.137		
HDAC1	2.339 (1.487-3.681)	<.001 *	1.670 (1.039-2.685)	.034 *

* $P < .05$.

interpretable GCs, but only 27.0% of normal epithelial cells ($P < .05$; Fig. 1E).

Relationships between HDAC1 expression and patients' clinicopathological characteristics are shown in Table 1. $HDAC1^{High}$ expression was associated with larger tumor size ($P = .001$), advanced T stage ($P = .001$), lymph node metastasis (N stage; $P < .001$), and lymphovascular invasion ($P = .005$).

3.2. HDAC1 was an independent prognostic factor for survival in GC

The median follow-up time for this cohort was 35 months. Of the 252 patients, tumors recurred in 119 patients (47.2%), of whom 89 (35.3%) patients died of the disease. Five-year OS rates differed significantly between $HDAC1^{High}$ patients (40.5%) and $HDAC1^{Low}$ patients (59.9%; $\chi^2 = 14.311$, $P < .001$, log-rank analysis; Fig. 2A), as did 5-year DFS rates ($HDAC1^{High}$: 35.9%, $HDAC1^{Low}$: 58.4%, $\chi^2 = 19.706$, $P < .001$; Fig. 2B). Furthermore, multivariate Cox regression analysis showed HDAC1 expression to be an independent predictive factor for both OS (hazard ratio [HR], 1.670; 95% confidence interval [CI], 1.039-2.685; $P = .034$; Table 2) and DFS (HR, 1.748; 95% CI, 1.154-2.646; $P = .008$; Table 3). These data imply that HDAC1 is a potential prognostic factor for GC.

3.3. Silencing HDAC1 expression impaired glycolysis in GC cells

Switching from oxidative phosphorylation to glycolysis as a glucose metabolism pathway is a critical step in carcinogenesis. Because HDAC1 expression correlated with tumor size and advanced tumor stage, we hypothesized that silencing *HDAC1* could impair glycolysis in GC cells. We established stable *HDAC1* knockdown AGS and MGC803 cells, which was verified by RT-PCR and western blot analyses that showed mRNA and protein levels to be silenced (Fig. 3A and B). We then examined glucose consumption, lactate, and ATP production in *HDAC1* knockdown cells. As anticipated, silencing *HDAC1* strongly decreased glucose consumption, lactate, and ATP production in AGS and MGC803 cells (Fig. 3C-E). SUVmax in positron-emission tomography/computed tomography (PET/CT) scan reflects the Warburg effect. In a cohort of 32 patients with GC, we observed that $HDAC1^{High}$ expression was always accompanied by high SUVmax values (Fig. 3F). Glycolysis is a multienzyme

Table 3 Univariate and multivariate Cox proportional hazard analyses of *HDAC1* gene expression and DFS for patients with GC

Factor	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Sex	0.949 (0.662-1.361)	.775		
Age	1.132 (0.789-1.624)	.500		
Diameter	1.187 (0.814-1.729)	.373		
T category	2.068 (1.568-2.728)	<.001 *	1.450 (1.028-2.045)	.034 *
N stage	1.477 (1.260-1.732)	<.001 *	1.226 (1.003-1.475)	.041 *
Grade	1.917 (1.302-2.822)	.001 *	1.336 (0.899-1.986)	.152
Lymphovascular invasion	2.205 (1.497-3.248)	<.001 *	1.775 (1.188-2.651)	.005 *
Perineural invasion	1.503 (1.022-2.211)	.038 *	1.429 (0.963-2.121)	.076
Tumor location	1.069 (0.910-1.257)	.416		
HDAC1	2.408 (1.611-3.598)	<.001 *	1.748 (1.154-2.646)	.008 *

* $P < .05$.

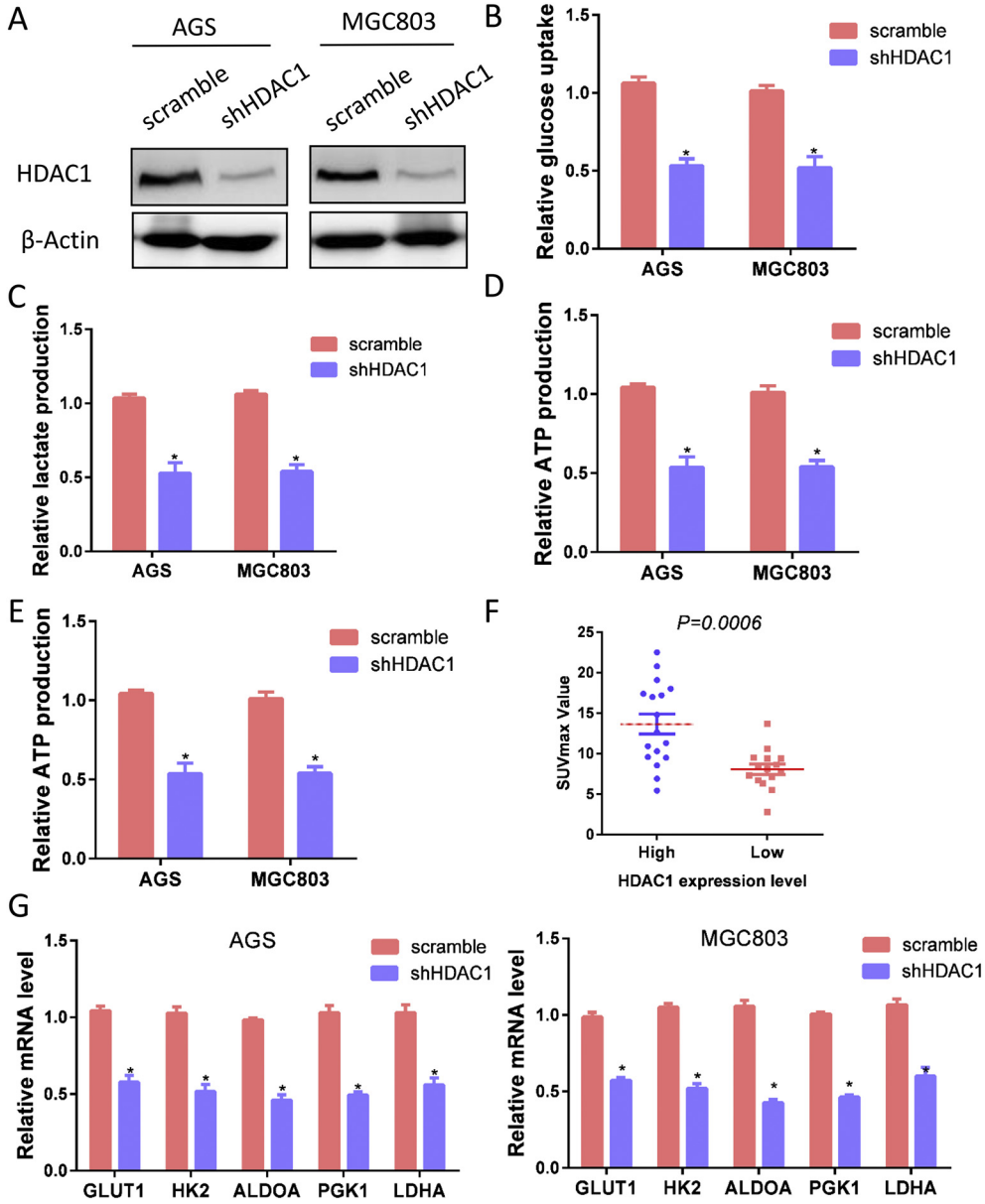

Fig. 3 HDAC1 expression correlates with glycolysis activity in GC tissues. Western blot (A) and RT-PCR analysis (B) show the effect of *HDAC1* knockdown in AGS and MGC803 cells (loading control: β-actin). Silencing HDAC1 decreased glucose consumption (C), lactate production (D), and ATP production (E). F, HDAC1 expression correlated with SUVmax in patients with GC (data shown as mean ± SD from 3 independent experiments). G, *HDAC1* knockdown upregulated or downregulated several rate-limiting enzymes in glycolysis, most significantly GLUT1, HK2, ALDOA, PGK1, and LDHA. *$P < .05$.

reaction with several rate-limiting steps. Knockdown *HDAC1* expression upregulated or downregulated several rate-limiting glycolytic enzymes—most significantly, GLUT1, HK2, ALDOA, PGK1, and LDHA (Fig. 3G).

3.4. HDAC1 affected hypoxia-inducible factor-1α activity in GC

Hypoxia-inducible factor (HIF)-1α is a transcription factor that mediates glycolysis. Because stabilized HIF-1α can promote expression of glycolysis enzymes, we considered that HDAC1 might affect HIF-1α activity to promote glycolysis in GC. HIF-1α acts by binding to the HRE in hypoxic environments. Therefore, we used an HRE-luciferase reporter system to assess whether HDAC1 affected HRE activity. As expected, HDAC1 increased HRE-luciferase activity in a dose-dependent manner (Fig. 4A), and HRE activity decreased by silencing HDAC1 (Fig. 4B). This finding indicates that HDAC1 indeed affects HIF-1α activity to promote glycolysis.

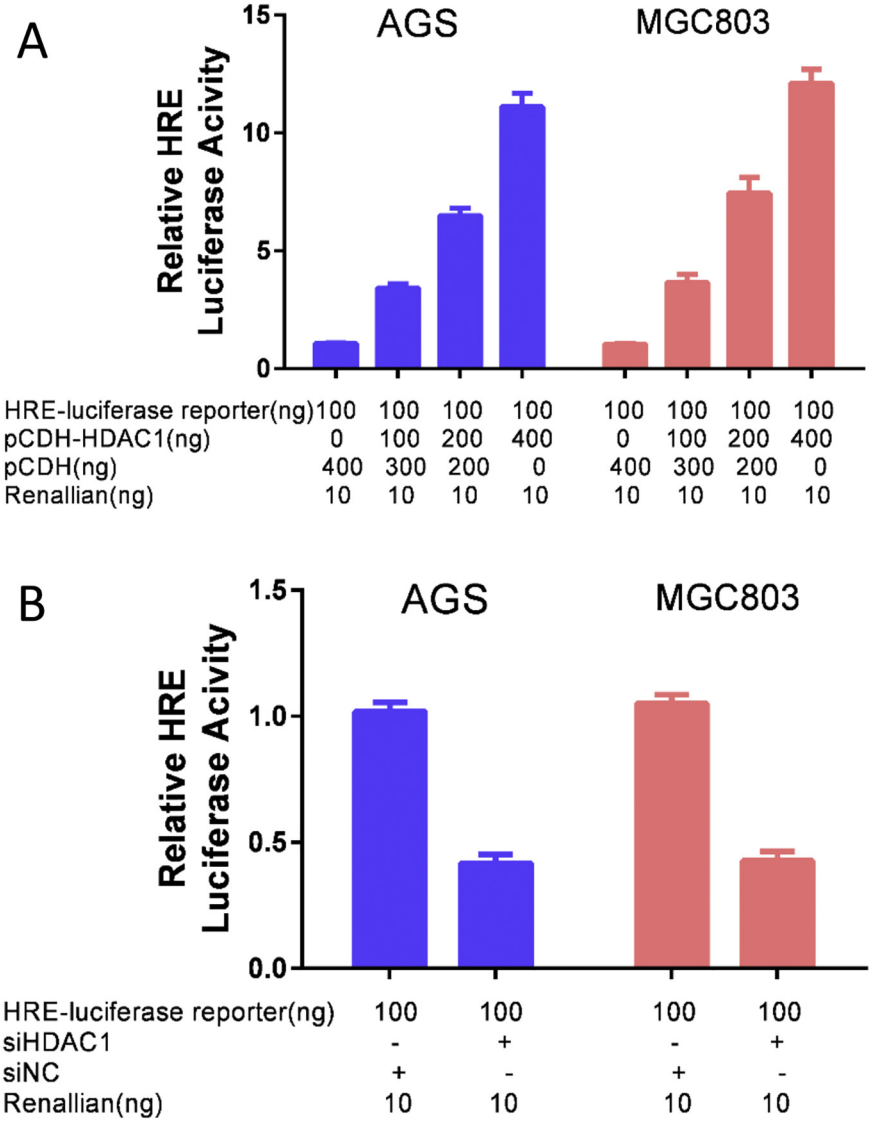

Fig. 4 HDAC1 affected the HIF-1α activity in GC. A, HDAC1 dose-dependently increased HRE-luciferase activity ($P < .05$). B, Silencing HDAC1 significantly expression significantly decreased HRE activity.

4. Discussion

HDAC1 expression has been reported in different cancers [7,9,10,13-15,17], and its high expression has been shown to correlate with advanced stage, uncontrolled tumor cell proliferation, and poor prognosis in various tumors, which implies that HDAC1 has a critical role in carcinogenesis. In the present study, we first confirmed that HDAC1 expression was lower in normal gastric tissues than in GC tissues, and that high HDAC1 expression was associated with larger tumors, advanced T stage, lymph node metastases, and lymphovascular invasion. HDAC1 was also shown to be an independent prognostic factor. These data strongly suggest that HDAC1 is an oncoprotein in GC tumorigenesis.

Tumor cells proliferate at a rapid rate, which tends to create a hypoxic microenvironment, which in turn causes cancer cells to switch metabolically from oxidative phosphorylation to glycolysis [21,22]. This phenomenon—called the Warburg effect or aerobic glycolysis—is a basic characteristic of carcinogenesis and progression [23-25]. Thus, our findings for the effect of HDAC1 on glycolysis both underscore the effect of HDAC1 on GC and imply a mechanism for this function. Silencing *HDAC1* expression significantly decreased the glycolysis rate in vitro. High 18-labeled 2-fluoro-2-deoxy-D-glucose(18-FDG) uptake by tumors is thought to reflect the Warburg effect, and PET/CT imaging is based on it [20]. Among the patients in our cohort who underwent PET/CT scans before gastrectomy, SUVmax values were significantly higher in $HDAC1^{High}$ patients than in $HDAC1^{Low}$ patients. Thus, both in vitro functional experiments and in vivo evaluations using patients' samples indicate that HDAC1 regulates glycolysis.

HIF-1α promotes cancer cell survival and tumor metastasis under hypoxic stress, in part by upregulating glycolytic

enzymes during anaerobic glycolysis, thus increasing glycolysis and ATP production [26-28]. Conversely, silencing *HDAC1* downregulated several glycolytic enzymes that are transcribed down-target from HIF-1α. HDAC1 is a key epigenetic regulator, and its aberrant recruitment is a key mechanism of gene misregulation [29,30]. We therefore hypothesized that HDAC1 regulates glycolysis in GC by increasing HIF-1α activity. A luciferase study confirmed that HDAC1 dose-dependently increased HRE activities, indicating that HIF-1α and HDAC1 interact in mediating glycolysis.

In conclusion, our study supports the role of HDAC1 as a crucial mediator in GC by promoting glycolysis and increasing HIF-1α activity. HDAC1 may therefore be considered an oncoprotein. Its high expression also predicts shorter survival for patients with GC. Further investigation is required to elucidate the details of the mechanism by which HDAC1 promotes progression and metastases of GC.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2018.10.031.

Compliance with Ethical Standards

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

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