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ORIGINAL ARTICLE

Inhibition of HDAC6 as a Therapeutic Strategy for Cervical Cancer Mediated by the p21 Signaling Pathway

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ABSTRACT

Background: **This study aimed to investigate the role of HDAC6 in cervical cancer by exploring its impact on cell proliferation, apoptosis, and interaction with the p21 signaling pathway.**
Methods: **A retrospective analysis of cervical cancer tissue samples obtained from 40 patients (20 recurrent and 20 non-recurrent) was conducted. Immunohistochemistry was performed to assess HDAC6 and p21 expression levels in the clinical samples. HeLa cells were transfected with HDAC6-targeted lentivirus for knockdown experiments. The cell proliferation rate was evaluated using the Cell Counting Kit-8 (CCK-8) assay, while apoptosis was measured by flow cytometry. Protein expression levels of HDAC6, p21, Bcl-2, and Bax were determined by western blotting. Cisplatin was applied to investigate its combined effect with HDAC6 knockdown on cell proliferation and apoptosis.**
Results: **In clinical samples, elevated HDAC6 expression level was associated with advanced International Federation of Gynecology and Obstetrics (FIGO) stage and poor prognosis, while reduced p21 expression level was correlated with disease recurrence. In HeLa cells, HDAC6 knockdown led to a significant reduction (67.3%) in cell proliferation compared with controls ($p < 0.05$) and increased apoptosis rates ($p < 0.05$). The combination of HDAC6 knockdown and cisplatin further enhanced the inhibition of cell proliferation and promoted apoptosis more effectively than cisplatin or HDAC6 knockdown alone ($p < 0.01$). Western blot analysis revealed the reduced Bcl-2 expression level and the increased Bax and p21 expression levels in the HDAC6 knockdown group.**
Conclusions: **HDAC6 knockdown, as demonstrated by both clinical sample analysis and in vitro HeLa cell experiments, could inhibit cervical cancer progression by decreasing cell proliferation and promoting apoptosis. Its combination with cisplatin could be a promising therapeutic strategy.**
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KEYWORDS

HDAC6, cervical cancer, p21 signaling pathway, apoptosis, cisplatin, cell proliferation

INTRODUCTION

Cervical cancer is closely associated with persistent infection by high-risk human papillomavirus (HR-HPV), which is considered as the primary etiological factor [1]. HR-HPV infection, particularly types 16 and 18, accounts for the majority of cervical cancer cases worldwide [2]. The oncogenic potential of these viruses stems primarily from the activity of their E6 and E7 proteins,

which inactivate key tumor suppressor proteins p53 and pRB, respectively, driving uncontrolled cellular proliferation and malignant transformation [3]. Early screening of cervical cancer, primarily through HPV testing and cytology (Pap smears), plays a crucial role in reducing mortality by identifying precancerous lesions before their progression to invasive cancer [4]. However, the economic burden of widespread screening programs presents challenges, especially in low- and middle-income countries, where the cost-effectiveness of such strategies is a critical factor [5,6].

In clinical practice, cervical cancer patients exhibit varied responses to neoadjuvant chemotherapy or radical chemoradiotherapy, which are key treatments, especially for patients with advanced-stage disease [7]. This variability is partly related to differences in tumor biology, including gene expression profiles that may affect treatment sensitivity [8]. Identifying specific genetic markers associated with chemotherapy or radiotherapy resistance has the potential to optimize treatment strategies and improve outcomes [9,10]. This underscores the importance of personalized treatment plans that consider individual tumor characteristics and patient-specific factors, such as genetic predisposition and molecular targets [11].

The underlying mechanisms of cervical carcinogenesis involve a complex interplay of genetic and epigenetic factors [9,12]. Disruptions in key regulatory pathways, including those governing cell cycle progression, apoptosis, and DNA repair, contribute to tumor development and progression [13,14]. One of the novel therapeutic targets identified in recent years is histone deacetylase 6 (HDAC6), a critical regulator of cellular processes including protein degradation, cell motility, and stress responses [15,16]. HDAC6 has emerged as a promising target owing to its overexpression in cervical cancer tissues, where it is associated with more aggressive tumor phenotypes and poorer patient outcomes [17]. Its role in promoting cancer cell proliferation and resistance to standard therapies makes it a potential candidate for targeted intervention [18,19]. In particular, HDAC6 has been found to regulate the expression levels of proteins involved in cell survival and apoptosis, making it a promising target for cancer therapy [20].

The primary objective of this study was to investigate the mechanism by which HDAC6 could inhibit the proliferation of HeLa cells via the p21 signaling pathway. Additionally, this study aimed to evaluate the potential synergistic effect of combining HDAC6 knockdown with the chemotherapy agent cisplatin for the treatment of cervical cancer.

MATERIALS AND METHODS

Sample collection

A retrospective analysis was conducted on 485 patients who underwent type C radical hysterectomy and pelvic lymph node dissection for cervical cancer at the Second Hospital of Jilin University (Jilin, China) between February 2014 and December 2016. This study was approved by the ethics committee of the Second Hospital of Jilin University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. In this study, 20 patients who experienced recurrence within 18 months (as of July 31, 2018) and 20 non-recurrent patients were selected. The selection criteria for both groups included matching with disease stage and treatment regimen, aiming to minimize differences in clinical characteristics. Paraffin-embedded tissue samples from both groups, accounting for a total of 40 cases, were prepared as 4-micron-thick paraffin sections for further analysis.

Experimental reagents

The human cervical cancer cell line HeLa was obtained from the American Type Culture Collection (ATCC). Cisplatin for injection was sourced from Shandong Luoxin Pharmaceutical Group Co., Ltd. (China). Recombinant lentiviral particles for HDAC6 transfection and negative control lentiviral particles were provided by GeneChem Co., Ltd. (Shanghai, China). Monoclonal antibodies against HDAC6, p21, Bcl-2, Bax, P53, and β-actin were purchased from Cell Signaling Technology (CST; Danvers, MA, USA). The protein pre-stained marker was obtained from Thermo Fisher Scientific (Waltham, MA, USA), and the CCK8 assay kit was acquired from Seven Sea Futai Biotechnology Co., Ltd. (Shanghai, China). RNA extraction kits were purchased from Meiji Biotechnology Co., Ltd. (Guangzhou, China). The apoptosis detection kit was sourced from Seven Sea Futai Biotechnology Co., Ltd.

Dataset for screening chemoradiotherapy resistance genes

Gene expression microarray data related to chemoradiotherapy resistance in cervical cancer were retrieved from the NCBI GEO database (https://www.ncbi.nlm.nih.gov/gds/). Differential gene expression analysis was conducted for genes significantly associated with chemoradiotherapy resistance using the limma package to normalize microarray data and identify differentially expressed genes (DEGs). Genes were ranked based on log fold-change values, with statistical significance determined by adjusted p-values ($p < 0.05$, corrected using the Bonferroni method). Genes consistently ranked at the top across all experiments were considered significant. The Gene Ontology (GO) enrichment analysis of DEGs was performed using DAVID (false discovery rate (FDR) < 0.05). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was also conducted (corrected $p < 0.05$) to identify key signaling pathways associated with chemoradiotherapy resistance. The regulatory networks and associated pro-

tein expression were analyzed to identify genes involved in chemoradiotherapy resistance.

Immunohistochemistry
Immunohistochemistry was performed on tumor tissues from 40 cervical cancer patients to assess the expression levels of proteins encoded by genes identified in section 2.3. Comparative analysis was conducted between the recurrent and non-recurrent groups. Protein expression was evaluated by selecting five random high-power fields (200 ×) from each tissue section. The staining intensity was scored as follows: 0 (no staining), 1 (light yellow), 2 (yellow), and 3 (brown). The percentage of positive cells was scored as follows: 0 (≤ 5%), 1 (5% - 25%), 2 (26% - 50%), 3 (51% - 75%), and 4 (> 75%). The final score for each field was calculated by multiplying the staining intensity score by the positive cell percentage score. Expression levels were classified as low (0 - 3 points) or high (4 - 12 points).

Lentiviral design and synthesis
To achieve stable downregulation of HDAC6 in HeLa cells, lentiviral vectors carrying HDAC6-targeted shRNA were constructed. Both the RNA interference lentivirus and negative control lentivirus were packaged by GeneChem Co., Ltd., and sequences are presented in Supplementary Table 1.

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)
RT-qPCR was employed to assess HDAC6 mRNA expression in HeLa cells following transfection with lentiviral vectors. Primers are listed in Supplementary Table 2.

Cell grouping
HeLa cells were divided into several experimental groups according to the different treatments to be applied: the control group, which consisted of cells without any treatment; the HDAC6 knockdown group, where cells were transfected with lentivirus carrying HDAC6-shRNA for stable gene knockdown; the negative control group, where cells were transfected with lentivirus carrying the negative control vector; the cisplatin treatment group, where cells were treated with cisplatin to induce cellular stress; and the combination group, where cells were transfected with HDAC6-shRNA and treated with cisplatin to assess the combined effect.

Cell proliferation assay
The cell proliferation rate was measured using the Cell Counting Kit-8 (CCK8) assay. After grouping and applying the respective treatments, HeLa cells were seeded into 96-well plates at an appropriate density. At specified time points post-treatment, 10 μL of CCK8 solution was added to each well and incubated for 1 - 4 hours at 37°C. The absorbance was measured at 450 nm using a microplate reader. The proliferation rate was calculated by comparing the absorbance of the treated groups with that of the control group.

Cell apoptosis detection
Cell apoptosis was evaluated using flow cytometry after staining with annexin V and propidium iodide (PI). Cells from the various experimental groups were harvested, washed with phosphate-buffered saline (PBS), and stained according to the manufacturer's instructions. Flow cytometry was performed, and the proportion of cells in the early and late stages of apoptosis was determined. This allowed for the comparison of apoptotic rates across the different treatment groups, particularly in response to HDAC6 knockdown and cisplatin treatment.

Western blotting
Protein expression levels of key markers (e.g., HDAC6, p21, Bcl-2, Bax, P53, and β-actin) were determined by western blotting. Total protein was extracted from HeLa cells in each treatment group, and protein concentration was measured using a BCA assay kit. Equal amounts of protein were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferring onto polyvinylidene fluoride (PVDF) membranes. After blocking, the membranes were incubated with primary antibodies against the target proteins overnight at 4°C. After washing, the membranes were incubated with HRP-conjugated secondary antibodies. The proteins were visualized using an enhanced chemiluminescence (ECL) detection system. Band intensities were quantified using image analysis software, and β-actin served as the loading control.

Data processing and statistical analysis
Statistical analysis was performed using SPSS 19.0 software (IBM SPSS Statistics for Windows; IBM Corp., Armonk, NY, USA). All data were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare mean values between groups, with standard deviations calculated for each group, and $p < 0.05$ was considered statistically significant.

RESULTS

Gene chip analysis and screening of DEGs
Gene expression profiling was performed on cervical cancer tissue samples collected from patients before and after undergoing chemoradiotherapy using data from the GSE3578 dataset in the Gene Expression Omnibus (GEO) database. The analysis included 20 patients with 40 paired tissue samples (collected pre- and post-radiotherapy), and 19 patients with 38 paired tissue samples (collected pre- and during chemoradiotherapy with cisplatin). Each sample was tested in duplicate.
Using the limma package in R software, DEGs were identified and ranked based on log Fold-Change values.

Table 1. Correlation between HDAC6 expression level and cervical cancer patients' clinicopathological characteristics.

Clinical characteristics	HDAC6				
	Number of patients	Low expression	High expression	χ^2	p
Age					
> 50	17	4 (0.2)	13 (0.8)	5.507	0.019
≤ 50	23	14 (0.6)	9 (0.4)		
FIGO Stage					
IB1	6	3 (0.5)	3 (0.5)	7.354 (Fisher)	0.045
IB2	14	8 (0.6)	6 (0.4)		
IIA1	17	4 (0.2)	13 (0.8)		
IIA2	3	3 (1.0)	0 (0)		
LNM					
Negative	28	12 (0.4)	16 (0.6)	0.173	0.677
Positive	12	6 (0.5)	6 (0.5)		
Stromal invasion					
> 50%	31	14 (0.5)	17 (0.5)	0.000	1.000
≤ 50%	9	4 (0.4)	5 (0.5)		
LVSI					
Negative	5	4 (0.8)	1 (0.2)	1.443	0.230
Positive	35	14 (0.4)	21 (0.6)		

Fisher's exact test. LVSI: lymphovascular space invasion, FIGO: International Federation of Gynecology and Obstetrics, LNM: lymph node metastasis.

Table 2. Correlation between p21 expression level and cervical cancer patients' clinicopathological characteristics.

Clinical characteristics	p21				
	Number of patients	Low expression	High expression	χ^2	p
Age					
> 50	17	8 (0.5)	9 (0.5)	0.753	0.385
≤ 50	23	14 (0.6)	9 (0.4)		
FIGO Stage					
IB1	6	4 (0.7)	2 (0.3)	2.342 (Fisher)	0.582
IB2	14	9 (0.6)	5 (0.4)		
IIA1	17	7 (0.4)	10 (0.6)		
IIA2	3	2 (0.7)	1 (0.3)		
LNM					
Negative	28	17 (0.6)	11 (0.4)	1.231	0.732
Positive	12	5 (0.4)	7 (0.6)		
Stromal invasion					
> 50%	31	18 (0.6)	13 (0.4)	0.117 (adjusted chi-squared value)	0.732
≤ 50%	9	4 (0.4)	5 (0.6)		
LVSI					
Negative	5	2 (0.4)	3 (0.6)	1.443 (adjusted chi-squared value)	0.230
Positive	35	20 (0.6)	15 (0.4)		

Fisher's exact test. LVSI: lymphovascular space invasion, FIGO: International Federation of Gynecology and Obstetrics, LNM: lymph node metastasis.

Table 3. Statistical analysis of HDAC6 protein expression level in cervical tissues of recurrent and non-recurrent patients.

Non-recurrent	Recurrent		χ^2	p
	HDAC6 positive	HDAC6 negative		
HDAC6 positive	2 (20%)	12 (80%)	5.7857	p < 0.05
HDAC6 negative	2 (70%)	4 (30%)		

Table 4. Statistical analysis of p21 protein expression level in tissues of recurrent and non-recurrent patients.

Non-recurrent	Recurrent		χ^2	p
	p21 positive	p21 negative		
p21 positive	2 (65%)	0 (35%)	9.09	p < 0.05
p21 negative	11 (10%)	7 (90%)		

A total of 352 DEGs were identified, showing a fold-change greater than five with an adjusted p-value of less than 0.01 (Bonferroni correction) (Figure 1A). To prioritize the most promising candidates for functional validation, the analysis concentrated on the top 50 genes with the highest fold-change values. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses revealed significant enrichment in pathways associated with cell cycle regulation, apoptosis, and response to chemotherapeutic stress. Among them, HDAC6 was one of the most significantly upregulated genes and was directly involved in these critical pathways. In contrast, p21 (CDKN1A), a well-established tumor suppressor and key regulator of the cell cycle and apoptosis, was identified as one of the most significantly downregulated genes. Given their central and opposing roles in pathways directly relevant to cervical cancer progression and treatment resistance, HDAC6 and p21 were selected as primary candidates for further investigation. These genes were identified as being critical to pathways involved in cell death and proliferation (Figure 1B). These results indicate significant changes in gene expression level following chemoradiotherapy, particularly in pathways that are essential for regulating cell survival and apoptosis.

Correlation between expression levels of HDAC6 and p21 and clinicopathological characteristics

The data presented in Table 1 indicated that HDAC6 expression level in cervical cancer tissues was significantly associated with specific clinicopathological characteristics. HDAC6 expression level was significantly associated with age, in which a higher HDAC6 expression level was found in patients aged over 50 years ($p = 0.019$). Analysis of the correlation between HDAC6 expression level and clinicopathological characteristics revealed a borderline significant association with FIGO stage ($p = 0.045$). Despite the limited sample size in each subgroup, which might affect statistical power, a clear trend of increasing HDAC6 expression level was found with advancing disease stage. This trend is consistent with the proposed role of HDAC6 in tumor progression and aggressiveness, and it also aligns with the more robust findings linking high HDAC6 expression level to disease recurrence and poor prognosis (Table 3). However, no significant associations were found between HDAC6 expression level and other parameters, such as lymph node metastasis ($p = 0.677$), depth of stromal invasion ($p = 1.000$), and lymphovascular space invasion (LVSI) ($p = 0.230$).

The analysis of p21 expression level in cervical cancer tissues indicated no significant correlation with most clinicopathological characteristics (Table 2). There was no significant association between p21 expression level and patient age ($p = 0.385$) or FIGO stage ($p = 0.582$). Similarly, no significant correlations were identified between p21 expression level and lymph node metastasis ($p = 0.267$), depth of stromal invasion ($p = 0.732$), and LVSI ($p = 0.810$). These results suggest that p21 expression level may not be strongly influenced by these clinicopathological factors in cervical cancer patients.

Relationship between expression levels of HDAC6 and p21 and patient prognosis

HDAC6 was found to be primarily expressed in the cytoplasm, with some expression observed in the nucleus. Patients with promising prognosis exhibited lower HDAC6 expression level (Figure 2A & B), while higher HDAC6 expression level was found in patients with poor prognosis (Figure 2C & D). Statistical analysis of HDAC6 expression level in recurrent and non-recurrent cervical cancer patients is summarized in Table 3. The results revealed a significant difference, in which HDAC6 expression level was higher in recurrent pa-

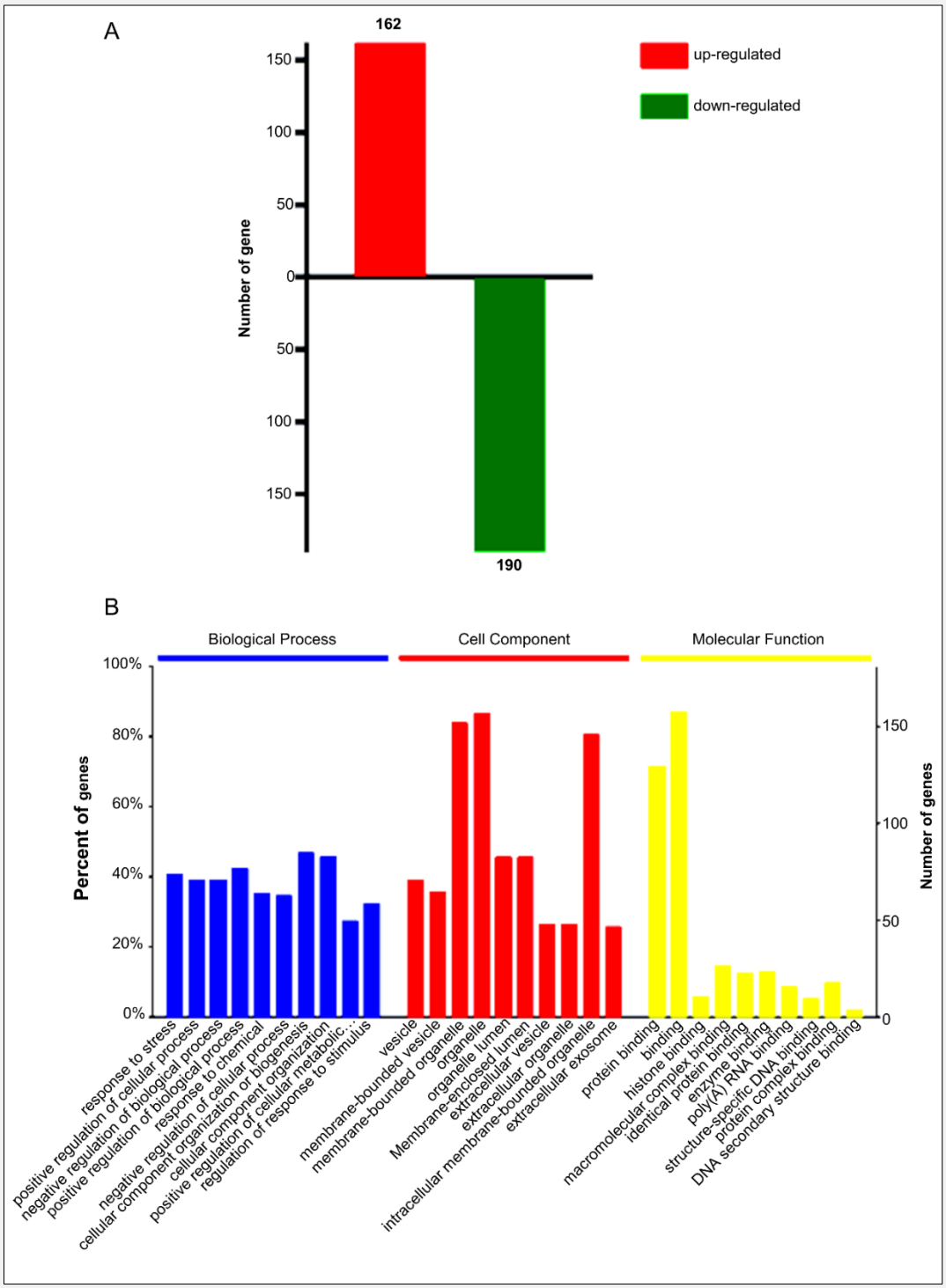


Figure 1. Analysis of differentially expressed genes and associated enriched signaling pathways in cervical cancer tissues pre- and post-chemoradiotherapy.

A: Differentially expressed genes identified through gene expression analysis of cervical cancer tissues before and after chemoradiotherapy. The analysis included tissue samples from 20 patients before and after radiotherapy and from 19 patients before and during chemoradiotherapy (a combination of cisplatin and radiotherapy). Genes with a log fold-change greater than five and a corrected p-value of less than 0.01 (Bonferroni correction) were considered significantly differentially expressed. B: Enriched signaling pathways associated with the differentially expressed genes identified in Panel A, displaying the signaling pathways significantly enriched in the differentially expressed genes, highlighting pathways involved in key biological processes, such as cell proliferation and apoptosis.

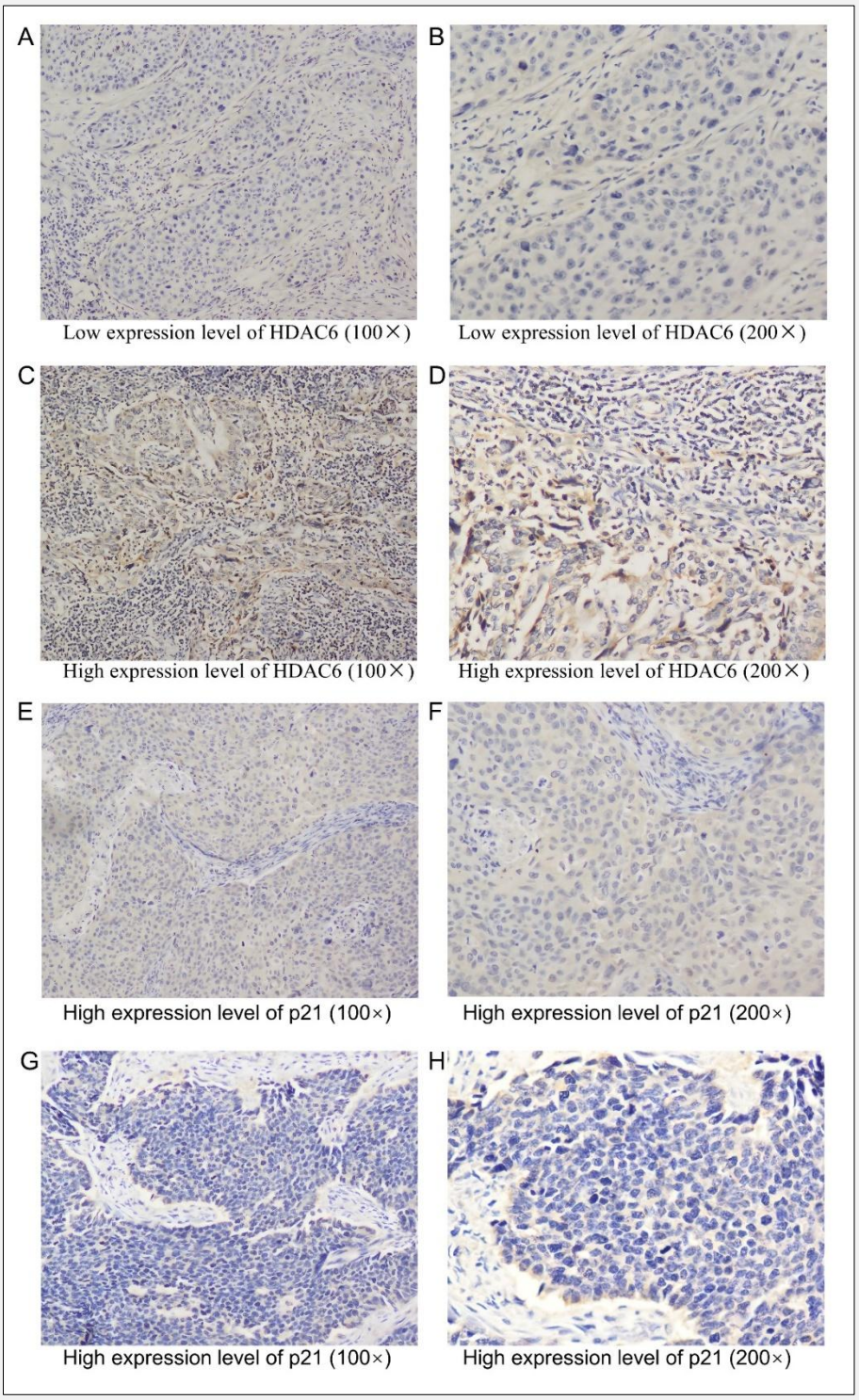


Figure 2. HDAC6 and p21 expression levels in cervical cancer patients with different prognoses.

A: Low expression level of HDAC6 in patients with promising prognosis (× 100). B: Low expression level of HDAC6 in patients with promising prognosis (× 200). C: High expression level of HDAC6 in patients with poor prognosis (× 100). D: High expression level of HDAC6 in patients with poor prognosis (× 200). E: High expression level of p21 in patients with promising prognosis (× 100). F: High expression level of p21 in patients with promising prognosis (× 200). G: Low expression level of p21 in patients with poor prognosis (× 100). H: Low expression level of p21 in patients with poor prognosis (× 200).

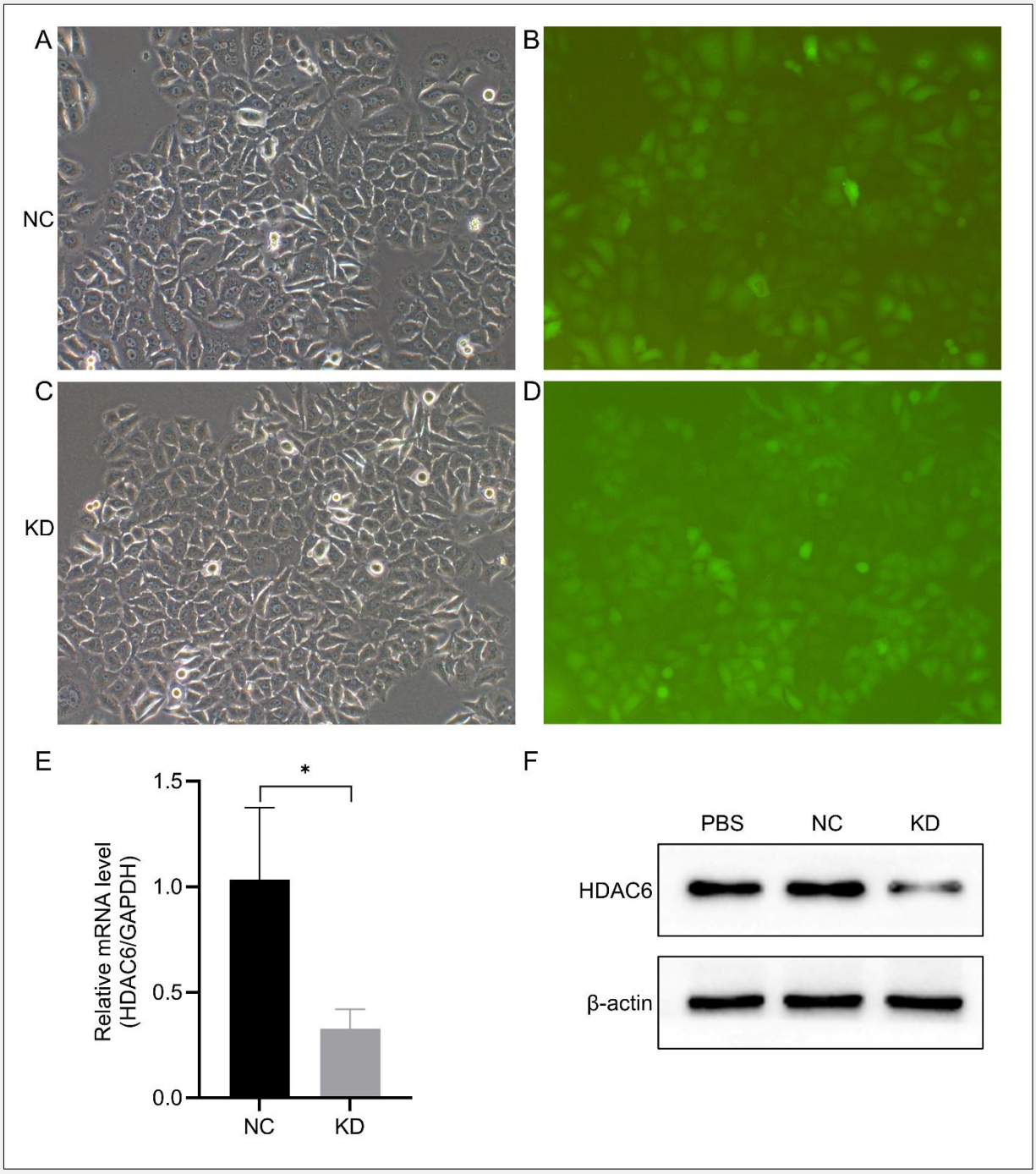


Figure 3. Efficiency of lentiviral infection and HDAC6 knockdown in HeLa cells.

A: Bright-field microscopy image of HeLa cells transfected with control lentivirus (NC) at 100 × magnification. B: Fluorescence microscopy image of HeLa cells transfected with control lentivirus (NC) at 100 × magnification, indicating infection efficiency. C: Bright-field microscopy image of HeLa cells transfected with HDAC6 knockdown lentivirus (KD1) at 100 × magnification. D: Fluorescence microscopy image of HeLa cells transfected with HDAC6 knockdown lentivirus (KD1) at 100 × magnification, showing infection efficiency exceeding 70% at 72 hours. E: Quantitative PCR analysis showing a 67.3% reduction in HDAC6 mRNA levels in the knockdown (KD) group compared to the control (NC) group. F: Western blot analysis of HDAC6 protein expression in three groups: HeLa cells treated with PBS, HeLa cells transfected with control lentivirus (NC), and HeLa cells transfected with HDAC6 knockdown lentivirus (KD). A significant reduction in HDAC6 protein expression is observed in the KD group compared to NC (* $p < 0.05$).

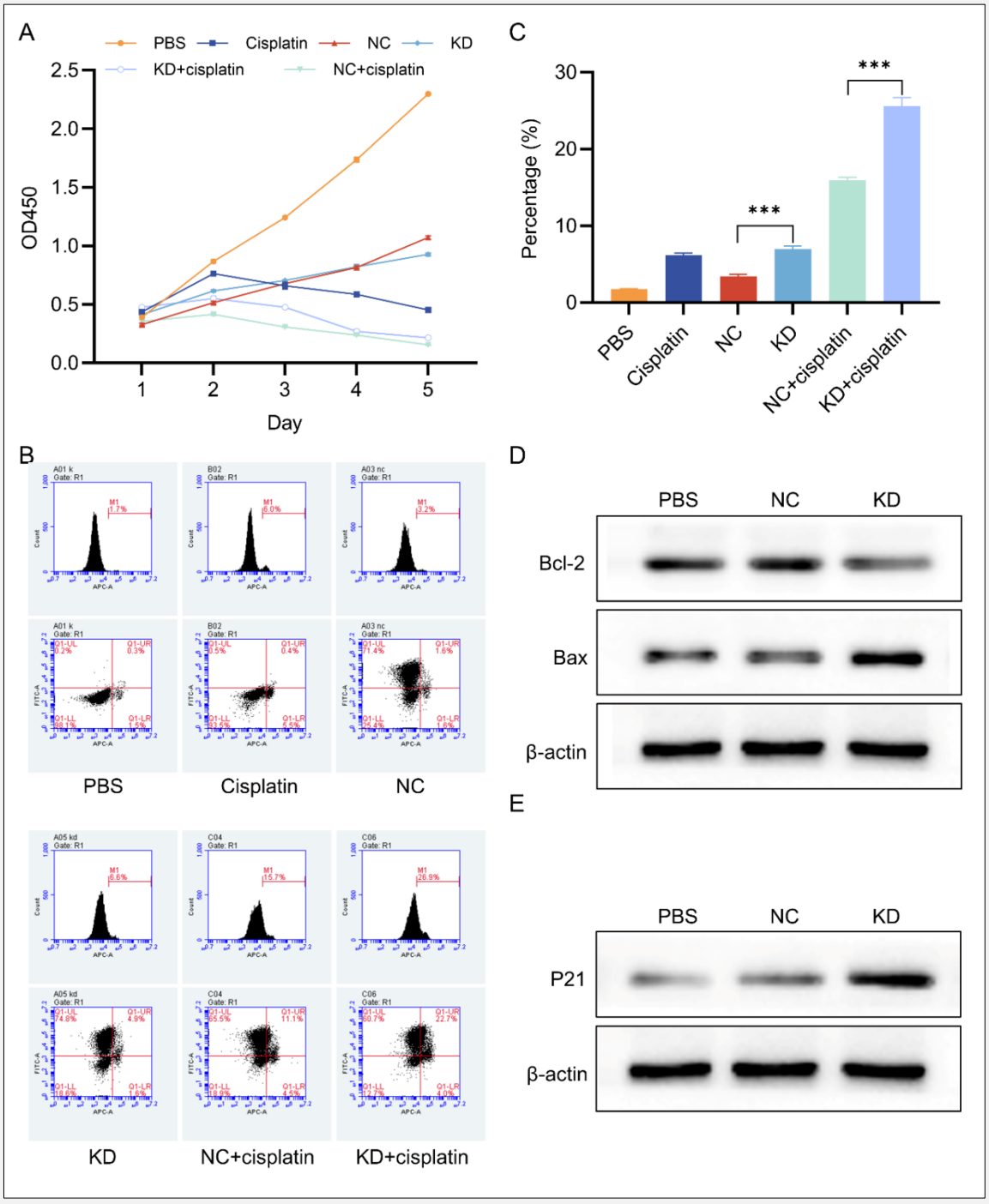


Figure 4. Effect of HDAC6 knockdown and cisplatin on HeLa cell proliferation, apoptosis, and protein expression.

A: Proliferation inhibition of HeLa cells following HDAC6 knockdown and cisplatin treatment. Groups: 1, HeLa cells + PBS; 2, HeLa cells + cisplatin; 3, NC (HeLa cells + control lentivirus); 4, KD (HeLa cells + HDAC6 knockdown lentivirus); 5, KD + cisplatin (HeLa cells + HDAC6 knockdown lentivirus + cisplatin); 6, NC + cisplatin (HeLa cells + control lentivirus + cisplatin). B: Flow cytometry results showing apoptosis induction in HeLa cells treated with HDAC6 knockdown lentivirus and/or cisplatin. C: Quantitative analysis of apoptotic HeLa cells from the experiments represented in panel B. Data are presented as mean ± SD. *** $p < 0.001$ indicates a statistically highly significant difference compared to the designated control group. Groups: 1, HeLa cells + PBS; 2, HeLa cells + cisplatin; 3, NC; 4, KD; 5, KD + cisplatin; 6, NC + cisplatin. D: Western blot analysis of Bcl-2 and Bax protein expression in three groups: 1, HeLa cells + PBS; 2, HeLa cells + control lentivirus (NC); 3, HeLa cells + HDAC6 knockdown lentivirus (KD). E. Western blot analysis of p21 protein expression in three groups: 1, HeLa cells + PBS; 2, HeLa cells + control lentivirus (NC); 3, HeLa cells + HDAC6 knockdown lentivirus (KD).

tients compared with that in non-recurrent patients ($p < 0.05$).

Similarly, p21 expression was predominantly localized in the cytoplasm, as displayed in Figure 2E - H. In patients with a promising prognosis, p21 expression level was elevated (Figure 2E & F), while it was reduced in patients with a poor prognosis (Figure 2G & H). Table 4 provides a statistical summary of p21 protein expression level in recurrent and non-recurrent patients. The data indicated that p21 expression level was significantly lower in recurrent patients compared with that in non-recurrent patients ($p < 0.05$). These results indicate that HDAC6 is more commonly expressed in patients with poor outcomes, while p21 is linked to better prognosis, suggesting their potential roles in cervical cancer prognosis.

Effects of HDAC6 knockdown on HeLa cell proliferation and apoptosis

In HeLa cells, lentiviral transfection for HDAC6 knockdown yielded an infection efficiency of over 70% at 72 hours (Figure 3A - D). RT-qPCR showed a 67.3% reduction in HDAC6 mRNA level (Figure 3E), and western blotting confirmed a significant decrease in HDAC6 protein expression in the knockdown group ($p < 0.05$, Figure 3F).

As illustrated in Figure 4A, HDAC6 knockdown and cisplatin treatment inhibited the proliferation of HeLa cells. The HeLa + cisplatin group significantly reduced cell proliferation compared with the HeLa + PBS group. In the HDAC6 knockdown group (KD), a significant inhibition of HeLa cell proliferation was found on day 5 compared with the control group (NC). Moreover, the KD + cisplatin group exhibited a statistically significant reduction in cell proliferation on day 5 compared with the NC + cisplatin group.

The induction of apoptosis was assessed by flow cytometry (Figure 4B and C). HDAC6 knockdown alone significantly increased the apoptosis rate compared with the negative control group. Expectedly, cisplatin treatment alone also induced apoptosis. Notably, the combination of HDAC6 knockdown with cisplatin resulted in a markedly enhanced apoptotic effect, which was significantly greater than that of cisplatin treatment alone in the control background. These results clearly demonstrated that HDAC6 knockdown could promote apoptosis and synergize with cisplatin-induced cell death.

As depicted in Figure 4D & E, HDAC6 knockdown modulated the expression levels of key proteins involved in cell survival and apoptosis. Specifically, in HeLa cells transfected with HDAC6 knockdown lentivirus, the expression level of the anti-apoptotic protein Bcl-2 was reduced, while the expression level of the pro-apoptotic protein Bax was elevated, compared with the NC group. Additionally, HDAC6 knockdown led to an upregulation of p21 protein expression level, further supporting the role of HDAC6 in regulating cell cycle progression and apoptosis.

DISCUSSION

This study highlighted the critical role of HDAC6 in cervical cancer progression, particularly its overexpression in advanced stages and its association with poorer prognosis [21]. The findings of the present study, along with existing literature, underscore the multifaceted role of HDAC6 in oncogenesis, extending beyond its classical function as a histone deacetylase. While HDAC6 can modulate gene expression by altering chromatin architecture (its canonical role) [22], a growing body of evidence highlighted its significance in regulating a suite of non-histone substrates. The deacetylation of proteins, such as α-tubulin, cortactin, and HSP90 by HDAC6 influences critical cancer phenotypes, including cell migration, metastasis, and stress response [23, 24]. It is noteworthy that HDAC6's role is highly context-dependent, as it also mediates protective functions in neuronal regeneration [25]. Furthermore, the interaction between different epigenetic modifications, such as the synergistic regulation of gene expression by H3K79 methylation and H3K36 trimethylation [26], highlights a complex regulatory network where HDAC6-mediated deacetylation may participate in cross-talk with other epigenetic marks. This functional versatility makes HDAC6 a potent regulator of cellular homeostasis and a compelling therapeutic target. The emerging concept that epigenetic regulators often exhibit functions beyond their canonical enzymatic activities is further exemplified by lysine-specific demethylase 1 (LSD1), which can modulate enhancer activity and drive cell fate transitions through demethylase-independent mechanisms [27]. This parallel suggests that the full scope of HDAC6's role in carcinogenesis, potentially including functions independent of its deacetylase activity, warrants further investigation. The significantly enhanced pro-apoptotic effect observed with the combination of HDAC6 knockdown and cisplatin treatment is consistent with the increasing recognition of HDAC6 as a pivotal regulator of protective cellular pathways, including autophagy. Targeting HDAC6 has been proposed as a strategic approach to disrupt autophagy-mediated survival mechanisms, thereby sensitizing cancer cells to conventional chemotherapeutic agents and overcoming drug resistance [20]. It is therefore plausible that the synergistic effect may, at least in part, be mediated through the inhibition of HDAC6-driven pro-survival autophagy.

The significant overexpression of HDAC6 in cervical cancer tissues reflects its importance in tumorigenesis, particularly in driving aggressive phenotypes. Moreover, its strong correlation with clinicopathological features, such as tumor size, grade, and lymph node metastasis suggest that HDAC6 may serve as a biomarker for assessing disease severity and progression.

HDAC6's involvement in cancer extends beyond its regulatory role in protein degradation [28]. Previous research has demonstrated that HDAC6 can mediate the deacetylation of heat shock protein 90 (HSP90), which

is crucial for the stability and function of several oncogenic proteins [29]. By promoting the activation of oncogenes through its effects on HSP90, HDAC6 may support tumor cell survival and resistance to stress, including chemotherapeutic agents. This study's findings reinforce the notion that HDAC6 is not merely a passive marker of disease severity, but also an active contributor to the malignant phenotype. The correlation between high HDAC6 expression level and advanced FIGO stages, as well as poorer survival outcomes, highlights the potential of HDAC6 as both a prognostic marker and a therapeutic target. Specifically, targeting HDAC6 in patients with advanced cervical cancer could inhibit key pathways that promote tumor growth and resistance to therapy, providing a new direction for treatment of cervical cancer.

In contrast, p21 (gene name: CDKN1A), a well-known cyclin-dependent kinase inhibitor, plays a central role in cell cycle regulation and acts as a tumor suppressor by inducing cell cycle arrest in response to various stress signals, including DNA damage [30,31]. In this study, p21 expression level was inversely correlated with HDAC6 level, and patients with higher p21 expression level had better clinical outcomes. This inverse relationship suggests a compensatory mechanism, where the downregulation of p21 in more aggressive tumors may be compensated by the overexpression of HDAC6, helping to sustain uncontrolled proliferation. Notably, p21 exerts its effects primarily by inhibiting cyclin-CDK complexes, thereby preventing the transition from the G1 phase to the S phase of the cell cycle, which is crucial for DNA replication [32,33]. In the present study, the reduced expression level of p21 in advanced-stage tumors indicates that tumor cells may evade cell cycle arrest, enabling unchecked proliferation.

The relationship between p21 expression level and cervical cancer prognosis has been explored in other studies, where its expression level has exhibited to predict patient outcomes [34-36]. However, the precise mechanisms by which p21 interacts with other molecular pathways, including those regulated by HDAC6, require further investigation. In this context, it is important to consider the broader implications of the HDAC6/p21 axis in cancer biology. HDAC6 may indirectly suppress p21 by influencing upstream regulators, such as p53, a tumor suppressor protein that controls p21 expression. Previous research has demonstrated that HDAC6 can deacetylate and inactivate p53, thereby diminishing its ability to induce p21 and other downstream effectors [37]. This could explain why tumors with a higher HDAC6 expression level tend to exhibit a lower p21 expression level, contributing to a more aggressive clinical phenotype. In this study, patients with a higher p21 expression level had better responses to therapy and improved prognosis, suggesting that p21 may serve as a predictive biomarker for chemotherapy efficacy.

The experimental results presented in this study further underscore the therapeutic potential of targeting HDAC6 in cervical cancer. The knockdown of HDAC6 in HeLa cells led to a significant reduction in cell proliferation and an increase in apoptosis, confirming the critical role of HDAC6 in maintaining cervical cancer cell survival. These findings are consistent with those of previous studies in other cancer types, where HDAC6 inhibition has exhibited to disrupt key oncogenic pathways, leading to the reduced tumor growth and increased sensitivity to chemotherapeutic agents [38,39]. In this study, the knockdown of HDAC6 not only impaired cell proliferation, but also induced apoptosis, suggesting that HDAC6 could regulate both the cell cycle and cell survival pathways. The upregulation of pro-apoptotic proteins, such as Bax, and the downregulation of anti-apoptotic proteins (e.g., Bcl-2) found in this study support the hypothesis that HDAC6 inhibition could trigger apoptosis by disrupting the balance of pro- and anti-apoptotic signals.

One of the most significant findings of this study is the synergistic effect of HDAC6 knockdown when combined with cisplatin treatment. Cisplatin, a platinum-based chemotherapeutic agent commonly utilized in the treatment of cervical cancer, mainly has its clinical efficacy limited by the development of resistance [40]. The enhanced anti-proliferative and pro-apoptotic effects observed in HeLa cells when HDAC6 knockdown was combined with cisplatin suggest that HDAC6 may contribute to chemoresistance by promoting cell survival pathways. By inhibiting HDAC6, it was revealed that cervical cancer cells became more susceptible to cisplatin-induced DNA damage, leading to the increased apoptosis. This synergistic effect could potentially overcome one of the most challenging aspects of cervical cancer treatment, including resistance to chemotherapy. Future research should concentrate on elucidating the molecular mechanisms behind this synergism, particularly the role of HDAC6 in modulating DNA repair pathways and stress responses in cancer cells.

While this study provided valuable insights into the role of HDAC6 in cervical cancer, several limitations must be acknowledged. Firstly, the study was conducted *in vitro*, and the findings need to be validated in vivo using animal models and clinical samples. Additionally, while the potential of HDAC6 inhibition was demonstrated as a therapeutic strategy, the molecular mechanisms underlying the interaction between HDAC6 and cisplatin require further investigation. Future studies should also explore the potential side effects of HDAC6 inhibitors and their efficacy in combination with other chemotherapeutic agents or targeted therapies. Clinical trials will be necessary to determine the safety and efficacy of HDAC6 inhibitors in cervical cancer patients.

CONCLUSION

In conclusion, this study demonstrated that the elevated HDAC6 expression level could be a strong indicator of poor prognosis and disease recurrence in cervical cancer patients, while higher p21 expression level was correlat-

ed with more favorable clinical outcomes. Although the association between HDAC6 and FIGO stage was noted as a statistical trend in the cohort, the strong correlation with recurrence highlights its potential as a clinically relevant biomarker. Most importantly, the functional experiments confirmed that HDAC6 knockdown significantly inhibited HeLa cell proliferation and induced apoptosis, and its combination with cisplatin yielded a synergistic anti-tumor effect. We postulate that these observed effects may be mediated through a combination of HDAC6's histone with non-histone deacetylase activities, ultimately impacting the p21 signaling pathway. While the data provided a strong phenotypic and correlative link, the precise contribution of specific acetylation mechanisms warrants further investigation. Collectively, these findings strongly confirm that targeting HDAC6, particularly in combination with standard chemotherapeutic agents, such as cisplatin, represents a promising therapeutic strategy for cervical cancer.

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Ethical Approval:
This study was approved by the ethics committee of the Second Hospital of Jilin University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Availability of Data and Materials:
The data included in this study could be provided upon a reasonable request.

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Declaration of Interest:
The authors declare that there is no conflict of interest.

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