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ORIGINAL RESEARCH

Upregulated Histone Deacetylase 6 Associates with Malignant Progression of Melanoma and Predicts the Prognosis of Patients

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Background: Melanoma is the most malignant tumor among skin tumors, and its morbidity and mortality are increasing year by year. Although melanoma biology has been increasingly studied, no prognostic biomarkers have yet been incorporated into clinical protocols. Histone deacetylase 6 (*HDAC6*) has been shown to act as a prognostic biomarker in several cancers. Here, we aimed to investigate the predictive value of *HDAC6* for the prognosis of cutaneous melanoma patients.

Methods: Eighty cutaneous melanoma patients were enrolled in this study. The protein and mRNA expression levels of *HDAC6* were detected, and the clinical features and survival time of cutaneous melanoma patients with *HDAC6* expression were analyzed.

Results: The results suggested that high *HDAC6* expression was significantly associated with unfavorable clinicopathological features. High *HDAC6* expression was related to melanoma metastasis and was also associated with a reduced survival time in melanoma patients, and this association remained significant in multivariate analysis adjusted for all other factors.

Conclusion: These findings validate the utility of *HDAC6* expression as an independent biomarker for the prognostication of patients with cutaneous melanoma.

Keywords: *HDAC6*, cutaneous melanoma, prognostic biomarker, survival, metastasis

Introduction

Melanomas are malignant tumors arising from melanocytes, which are derived from neural crest cells.[1] Cutaneous melanoma represents 3% of all skin cancer diagnoses each year but accounts for 65% of the deaths.[2] Cutaneous melanoma has been described as one of the most immunogenic cancers with heterogeneous histological and clinical features and a significant number of mutations, which may explain the low rate of tumor regression, multidrug resistance to targeted therapies, and reduced survival rate.[3] Analysis of biomarkers may predict disease prognosis or sensitivity to treatment and is a widely accepted approach that is very important for precision medicine. Previous studies have reported that various biomarkers are closely related to cutaneous melanoma, including diagnostic molecular biomarkers, such as *S100*, glycoprotein 100,[4] and chondroitin sulfate proteoglycan 4 (*CSPG4*), and prognostic biomarkers, such as S100 family, *Ki-67*, metallothionein (*MT*) I and II and melanoma cell adhesion molecule (*MCAM*, also known as *CD146/MUC18*).[5] However, there are no biomarkers confirmed to be related to the prognosis of melanoma that are used in clinical practice.

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Histone deacetylases (*HDACs*) are a family of epigenetic regulatory enzymes that can condense chromatin structure and suppress gene expression by removing acetyl groups from the lysine residues of histone tails. *HDAC6* has duplicated deacetylase domains and a C-terminal binder of the ubiquitin zinc finger domain.[6] The known substrates of *HDAC6* include α-tubulin, heat shock protein 90 and cortactin.[7] As a key modulator, HDAC6 is involved in many cellular processes, including proliferation, apoptosis, autophagy and DNA repair.[8] Furthermore, *HDAC6* inhibitors have shown strong potential therapeutic effects in many diseases, such as neurodegenerative diseases,[9] kidney disease,[10] lymphoproliferative disease,[11] ovarian cancers[12] and glioma.[13] *HDAC6* was found to promote melanoma cell proliferation and migration and inhibit cell apoptosis.[14,15] *HDAC6* inhibitors were discovered to augment the T-cell immune response in melanoma patients,[16] impair tumor growth and reduce *PD-L1* production.[17]

In our study, we collected pathological specimens from 80 melanoma patients and analyzed *HDAC6* expression to examine the associations of *HDAC6* expression with clinicopathological factors and survival.

Methods

Patients

A total of 80 melanoma tissue samples were collected from patients who underwent radical surgery at the First Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China). Patients with melanoma who were younger than 18 years old, suffered from other types of cancer, had a poor general health, were unable to describe the symptoms, had consciousness disorders, had severe infections or had respiratory insufficiency were excluded. The tumor grade was detected by pathological sectioning. Data on location, tumor size, and depth of invasion were obtained from the clinical and pathology records. Until the end of follow-up on 31 December 2019, overall survival was defined as the period from the date of surgery to the date of death (or the last follow-up). This study was approved by the ethics committee of First Affiliated Hospital of Sun Yat-sen University, and written informed consents were obtained, for their medical records to be used in this study and that this study complied with the Declaration of Helsinki.

Immunohistochemistry and Evaluation of HDAC6 Staining

Paraffin-embedded tumor specimens were collected from the archives of the pathology departments of the First Affiliated Hospital at Sun Yat-Sen University. The paraffin-embedded tissue sections were baked at 60°C for 2 h, deparaffinized with xylene and rehydrated with decreasing concentrations of alcohol. The endogenous peroxidase activity was blocked with 3% H_2O_2 at room temperature for 15 min. Antigen retrieval was conducted at a high temperature in saline sodium citrate (pH 6.0) for 15 min. After slow cooling, the tissue sections were stained with an anti-*HDAC6* antibody (1:200, ab133541; Abcam, USA) at 4°C overnight. After washing with phosphate-buffered saline (PBS), the sections were then incubated with HRP-labeled goat anti-rabbit IgG for 15 min at room temperature (Beyotime, Shanghai, China), stained with the DAB+ Staining Kit (Beyotime, Shanghai, China) and counterstained with hematoxylin. The score of the positive staining intensity was defined using ImageJ software (National Institutes of Health, USA). The cutoff value used was 3. Accordingly, tissue with an immunohistochemistry staining score ≤3 was defined as having low *HDAC6* expression, while a score >3 referred to high *HDAC6* expression.

Cell Culture

Melanoma cell lines (A375, A875, MeWo, WM35, SK-Mel-2, and SK-Mel-28) and the melanocyte PIG1 cell line were purchased from the Chinese Academy of Sciences (CAS, Shanghai, China). PIG1, A375, MeWo and WM35 cells were maintained in DMEM supplemented with 10% FBS (Gibco), 100 U/mL penicillin and streptomycin. A875, SK-Mel-2 and SK-Mel-28 cells were maintained in MEM supplemented with 10% FBS, 100 U/mL penicillin and streptomycin. All cell lines were cultured at 37°C in a humidified incubator with 5% CO_2.

RNA Extraction and Quantitative Real-Time Reverse Transcription PCR (qRT-PCR) Analysis

Total RNA was isolated from the tumor samples by TRIzol reagent (Invitrogen, CA, USA) and transcribed into cDNA using the High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific, USA) following the manufacturer's protocol. The qRT-PCRs were performed with qRT-PCR Brilliant II SYBR ROX (Agilent Technologies, USA) and detected by the Mx3000P real-time PCR system

Table 1 Baseline Characteristics of Melanoma Patients (n=80)

Characteristics	Category	Number of Cases	(%)
Age (years)	<60	51	63.7
	≥60	29	36.3
Sex	Male	44	55
	Female	36	45
TNM stage	Stage 0	2	2.5
	Stage I	23	28.7
	Stage II	31	38.8
	Stage III	18	22.5
	Stage IV	6	7.5
Radiotherapy	Yes	20	25
	No	60	75
Systemic therapy	Yes	21	26.3
	No	59	73.8
Metastasis	Yes	6	7.5
	No	74	92.5

(Agilent Technologies, USA). PCR conditions were as follows: 95° C for 300s, followed by 40 cycles of 95°C for 20 s, 55°C for 20s and 72°C for 20s. Melting curves were generated to verify the specificity of each qRT-PCR. Relative gene expression levels were calculated using the ΔΔCt method, and the *GAPDH* gene was used as a reference gene for mRNA quantification. Each independent experiment was repeated three times for accuracy. The primers were as follows: forward primer of *HDAC6*: 5′-AAGAAGACCTAATCGTGGGACT-3′, reverse primer of *HDAC6*: 5′-GCTGTGAACCAACATCAGCTC-3′; forward primer of *GAPDH*: 5′-TGACTTCAACAG CGACACCCA-3′, reverse primer of *GAPDH*: 5′-GATGCCTGCTTCACCACCTTCT-3′.

Protein Extraction and Western Blot Analysis

Total proteins of tumor samples were extracted with the ProteoPrep® Total Protein Extraction Kit (Sigma, USA), and protein concentration was determined by the bicinchoninic acid assay kit (Beyotime, Shanghai, China). Equal amounts of protein extract (40 μg) were subjected to electrophoresis in 5–10% SDS-PAGE gels and then transferred to PVDF membranes. After blocking with 5% nonfat milk in a mixture of 5% skim in Tris-buffered saline solution at room temperature for 1 h, the membranes were incubated with primary antibodies against *HDAC6* (1:2000, ab133541; Abcam, USA) and *GAPDH* (1:6000, G8795; Sigma-Aldrich), which served as an internal control, on an orbital shaker at 4°C overnight, after which secondary antibodies (HRP-labeled goat-anti-mouse (1:1000, 14709S; Cell Signaling Technology, USA) and HRP-labeled goat-anti-rabbit (1:1000, 14708S; Cell Signaling Technology, USA)) were added and incubated for 1 h at room temperature. The protein-antibody

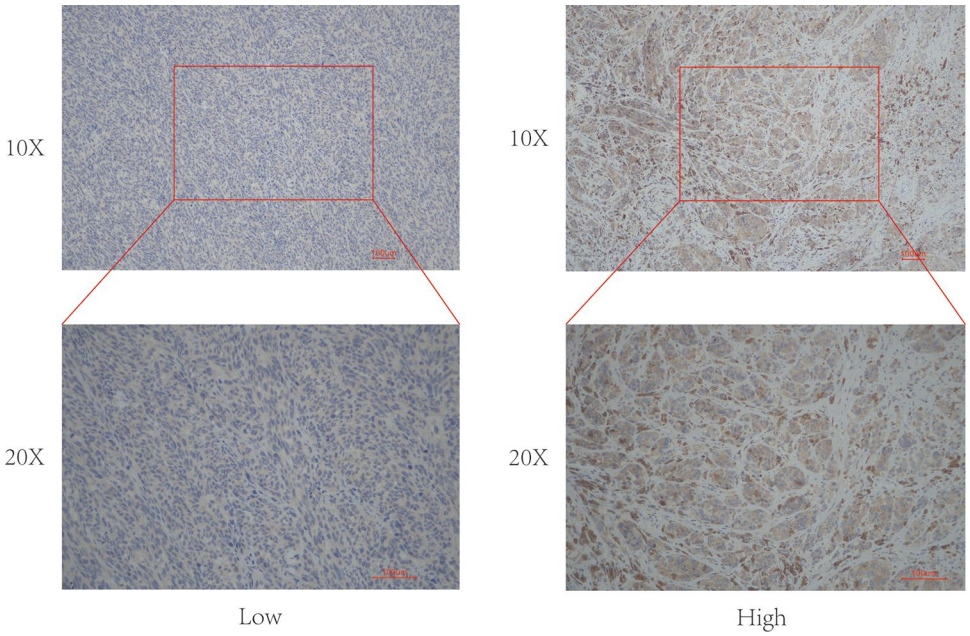

Figure 1 Representative examples of high and low *HDAC6* expression in melanoma tissues. ×20 and ×40 magnification.

complexes were then detected by chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Scientific Pierce).

Statistical Analysis

Chi-square tests were used for correlation analyses of *HDAC6* expression with clinicopathological characteristics. Survival analysis was performed by Kaplan–Meier analysis and Log rank test. Multivariate Cox regression was performed to further analyze the factors that were judged to have significant influence by univariate analysis. All calculations were performed using IBM SPSS Statistics Version 25.0 (SPSS Inc, Chicago, IL). Values are expressed as the mean and 95% confidence interval (CI). A P value <0.05 was considered statistically significant.

Results

The demographic characteristics of the patients, including age, gender, TNM stage, metastasis status, and whether they received radiotherapy or systemic therapy, were similar between the two groups (Table 1).

Association Between HDAC6 Expression and the Clinicopathological Characteristics of Patients with Melanoma

To determine the role of *HDAC6* protein expression in melanoma development, the relationship between the clinicopathological characteristics of melanoma patients and *HDAC6* protein expression was analyzed. Among the 80 patients with melanoma, 29 showed strong *HDAC6* expression, and 51 showed weak *HDAC6* expression. Representative examples of *HDAC6* expression in melanoma tissues are shown in Figure 1. The data presented in Table 2 show that high *HDAC6* expression was significantly associated with unfavorable clinicopathological features. The data demonstrate that a high expression level of *HDAC6* was associated with age (P=0.002), TNM stage (P=0.0001) and metastasis (P=0.02). There were no obvious associations between *HDAC6* protein expression and sex (P=0.657), radiotherapy (P=0.893) or systemic therapy (P=0.838).

The Expression of HDAC6 Increased in Melanoma

Protein and RNA were extracted from 3 pairs of cutaneous melanoma tissues and adjacent tissue clinical samples, and the mRNA and protein expression levels of *HDAC6* were detected by qRT-PCR and Western blot. The results showed that the mRNA expression of *HDAC6* in melanoma tissue was significantly higher than that in adjacent tissue ($p<0.001$) (Figure 2A), and the protein expression of *HDAC6* was consistent with the mRNA expression (Figure 2B). Moreover, protein and RNA were extracted from the melanocyte cell line PIG1 and 6 different melanoma cell lines to determine the mRNA and protein expression of *HDAC6*. The results showed that the mRNA expression of *HDAC6* in melanoma cell lines was significantly higher than that in the melanocyte cell line ($p<0.005$) (Figure 3A), and the protein expression of *HDAC6* was consistent with the mRNA expression (Figure 3B).

The Expression of HDAC6 Increased from Stage 0 to Stage IV

To demonstrate the relationship between melanoma staging and the expression of *HDAC6*, we analyzed the expression of *HDAC6* in patients of different stages. The protein expression levels of *HDAC6* are shown in Figure 4. It can

Table 2 The Correlation Between *HDAC6* Expression and Clinical Characteristics in Patients with Melanoma (n=80)

Characteristics	HDAC6 Expression		*P*-value
	Low Expression	High Expression	
Age			
≥60	12	17	0.002
<60	39	12	
Sex			
Male	29	15	0.657
Female	22	14	
TNM stage			
Stage 0	2	0	0.0001
Stage I	23	0	
Stage II	20	11	
Stage III	6	12	
Stage IV	0	6	
Radiotherapy			
Yes	13	7	0.893
No	38	22	
Systemic therapy			
Yes	13	8	0.838
No	38	21	
Metastasis			
Yes	0	6	0.002
No	51	23	

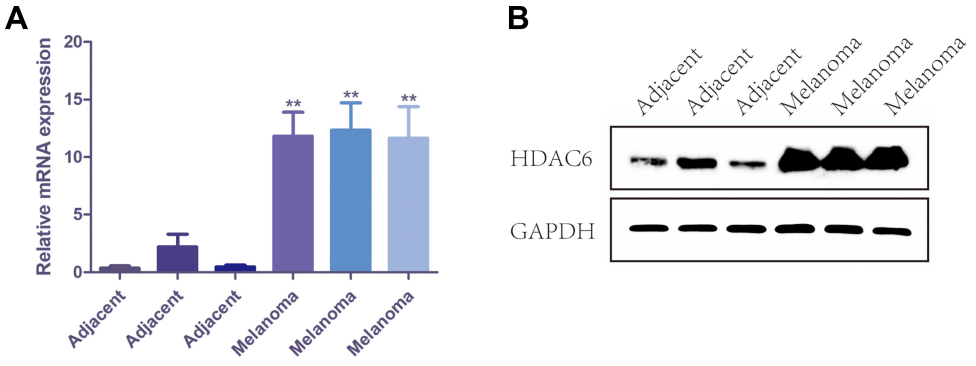

Figure 2 The expression of *HDAC6* in melanoma tissue and adjacent tissue. (**A**) The mRNA level of *HDAC6* in melanoma tissue and adjacent tissue. (**B**) The protein expression of *HDAC6* in melanoma tissue and adjacent tissue. Data were expressed as mean ± SD, **p<0.001.

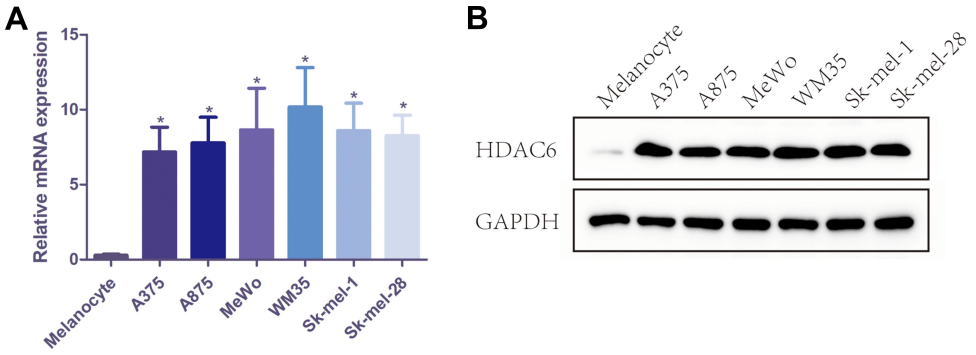

Figure 3 The mRNA and protein expression of *HDAC6* in melanocyte cell line PIG1 and 6 different melanoma cell lines. (**A**) The mRNA level of *HDAC6* in melanocyte cell line PIG1 and 6 different melanoma cell lines. (**B**) The protein expression of *HDAC6* in melanocyte cell line PIG1 and 6 different melanoma cell lines. Data were expressed as mean ± SD, *p<0.005.

be seen that the expression level of *HDAC6* increased from stage 0 to stage IV and showed significant differences between stage 0 and stage I (P<0.05), stage II and stage III (P<0.05), and stage III and stage IV (P<0.01). To further confirm the relationship between melanoma stage and the expression of *HDAC6*, protein and RNA were extracted from cutaneous melanoma tissues of different stages. The results showed that the mRNA expression of *HDAC6* in stage I–IV patients was higher than that in stage 0 patients and became higher from stage 0 to stage IV (Figure 5A), and the protein expression of *HDAC6* was consistent with the mRNA expression (Figure 5B).

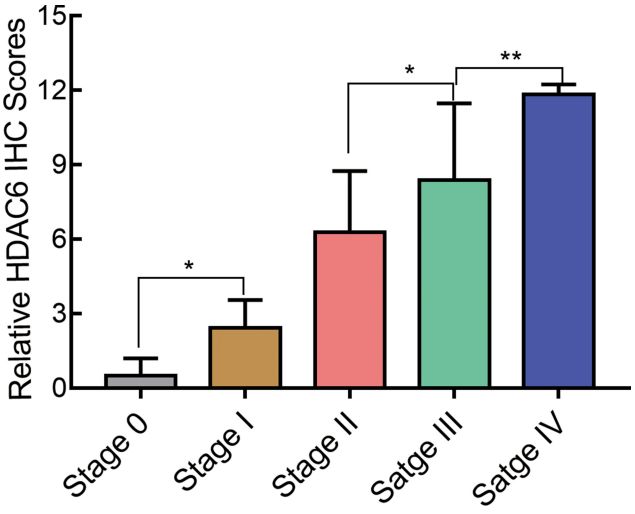

Figure 4 The immunohistochemistry staining score of *HDAC6* in melanoma tissues of different stages. Data were expressed as mean ± SD, *p<0.005, **p<0.001.

The Expression of HDAC6 Increased in Metastatic Melanoma Tissues

In this study, 6 patients had metastatic melanoma. The expression level of *HDAC6* in metastatic tissue was higher than that at the original site and showed a significant difference between in situ and metastatic tissues (p=0.002) (Figure 6). To further confirm the relationship between melanoma metastasis and the expression of *HDAC6*, protein and RNA were extracted from 3 pairs of in situ melanoma tissues and metastatic melanoma tissue clinical samples. The results showed that the mRNA expression of *HDAC6* in metastatic melanoma patients

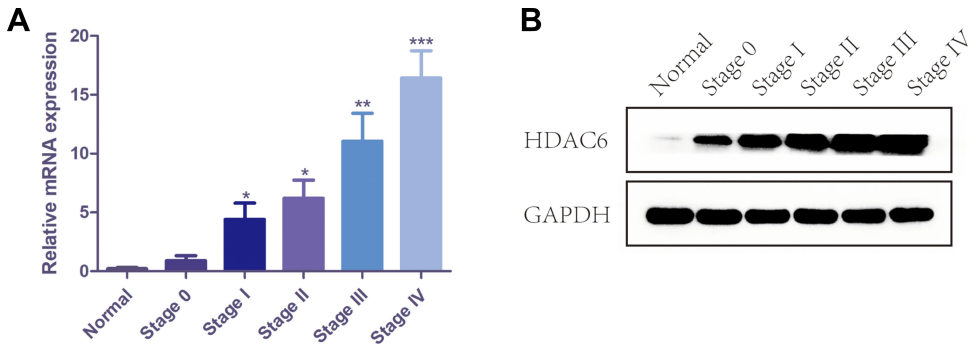

Figure 5 The mRNA and protein expression of *HDAC6* in melanoma tissues of different stages. (**A**) The mRNA level of *HDAC6* in melanoma tissues of different stages. (**B**) The protein expression of *HDAC6* in melanoma tissues of different stages. Data were expressed as mean ± SD, *p<0.005, **p<0.001, ***p<0.0001.

was higher than that in patients without metastasis (Figure 7A), and the protein expression of *HDAC6* was consistent with the expression of mRNA (Figure 7B).

Prognostic Value of HDAC6 Expression

Next, we examined the prognostic value of *HDAC6* expression in melanoma tissues and a dichotomized variable of low (staining score≤3) and high (staining score>3) *HDAC6* expression. As demonstrated in Figure 8, Kaplan–Meier analysis revealed a significant association between high *HDAC6* expression and a reduced overall survival time (p<0.0001). To further demonstrate the prognostic value of *HDAC6* expression, patients were further stratified by melanoma stage, and survival within each subgroup was analyzed using the Kaplan–Meier method. In low-stage patients (stage 0/I/II, n=56), the results showed that patients with low expression of *HDAC6* had longer survival times (p<0.0001) (Figure 9). In advanced patients (stage III/IV, n=24), the high expression of *HDAC6* correlated with worse overall survival (p=0.0016) (Figure 10). According to the *HDAC6* expression level of the melanoma patients, a receiver operating characteristic (ROC) curve of *HDAC6* and melanoma 10-year survival rate was generated. The results showed that the area under the curve (AUC) of *HDAC6* expression level predicting the 10-year survival rate of melanoma was 0.847 (95% CI=0.762~0.931, P<0.0001) (Figure 11). The prognostic value of *HDAC6* was confirmed in univariate Cox regression analysis (HR=1.57, 95% CI=1.41–1.74, P=0.0001) and remained significant in a multivariate model (HR=1.39, 95% CI=1.21–1.58, P=0.0001) adjusted for clinicopathological factors (Table 3).

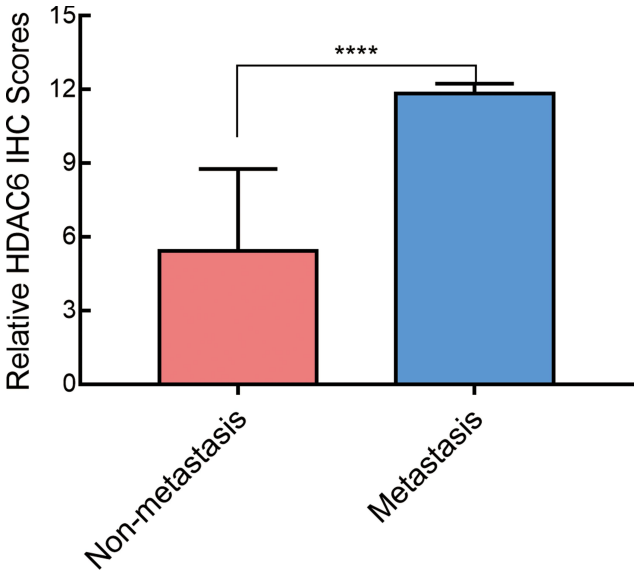

Figure 6 The immunohistochemistry staining score of *HDAC6* in melanoma tissues with and without metastasis. Data were expressed as mean ± SD, ****p<0.00001.

Discussion

The incidence of cutaneous melanoma continues to increase each year, and its aggressiveness and high mortality rate make it the deadliest type of skin cancer.[18–20] Therefore, it is necessary to find new biomarkers to evaluate the prognosis of melanoma patients.

HDAC6 is an important type II histone deacetylase family member that promotes cell proliferation and migration in different cancers.[21] *HDAC6* was discovered to be negatively related to the prognosis of patients suffering from lung adenocarcinoma and can promote lung adenocarcinoma cell proliferation through the activation of the *EGFR* signaling pathway.[22] For ovarian clear cell carcinoma patients, high nuclear *HDAC6* expression was confirmed to be related to patient death. In a multivariate analysis of overall survival, *HDAC6* nuclear expression was one of

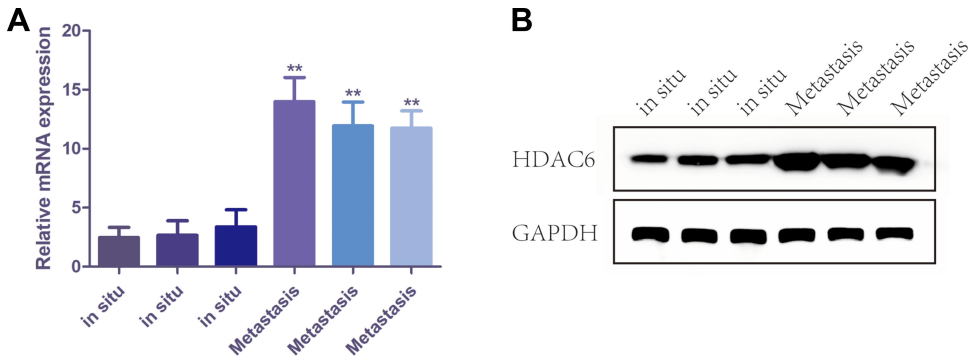

Figure 7 The mRNA and protein expression of *HDAC6* in melanoma tissues with and without metastasis. (**A**) The mRNA level of *HDAC6* in melanoma tissues with and without metastasis. (**B**) The protein expression of *HDAC6* in melanoma tissues with and without metastasis. Data were expressed as mean ± SD, **p<0.001.

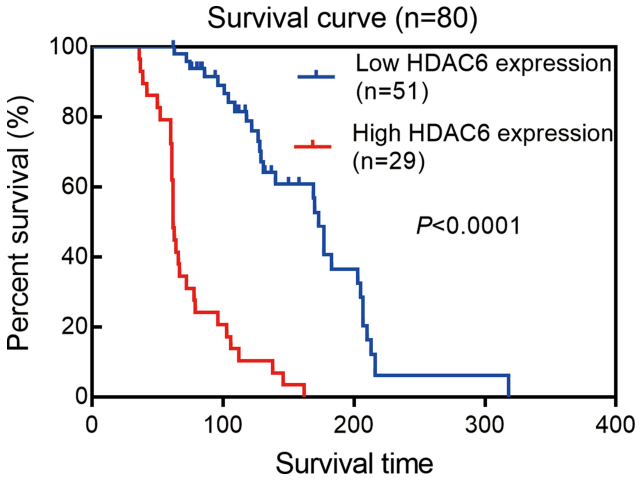

Figure 8 Kaplan–Meier estimates of the impact of *HDAC6* expression on melanoma survival, logrank p<0.0001.

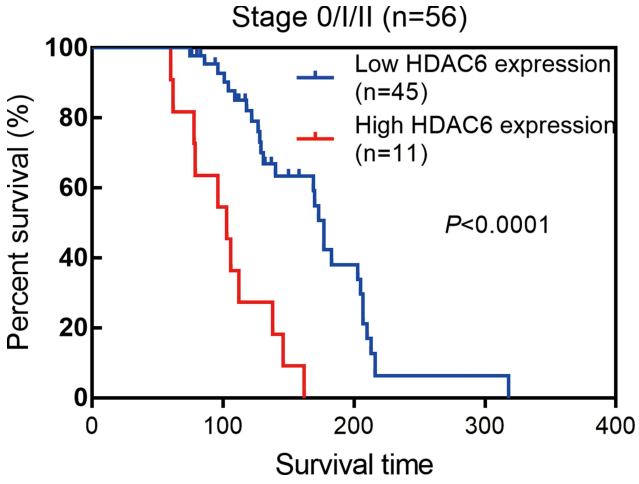

Figure 9 Kaplan–Meier estimates of the impact of *HDAC6* expression on the survival of melanoma patients of Stage 0/I/II, logrank p<0.0001.

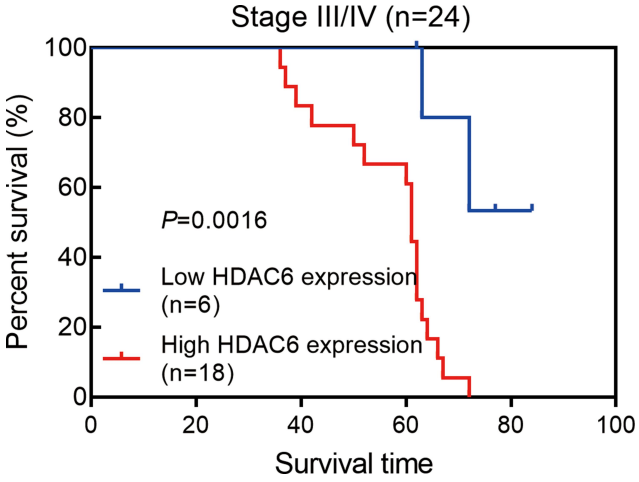

Figure 10 Kaplan–Meier estimates of the impact of *HDAC6* expression on the survival of melanoma patients of Stage III/IV, logrank p=0.0016.

the independent prognostic factors.[23] A recent study discovered that higher expression of *HDAC6* indicated a worse clinical prognosis in breast cancer patients.[24] *HDAC6* was found to be related to poor prognosis of patients with prostatic foamy gland carcinoma, and multivariate Cox regression analysis showed that *HDAC6* level was a significant prognostic factor for survival of patients suffering from prostatic foamy gland carcinoma.[25] That is, it has been found that *HDAC6* might play a predictive role in the prognosis of certain tumors.

Some recent studies found that *HDAC6* can promote melanoma cell proliferation and metastasis through various pathways, and knockdown of *HDAC6* can inhibit tumor growth in vivo. Moreover, compared with normal

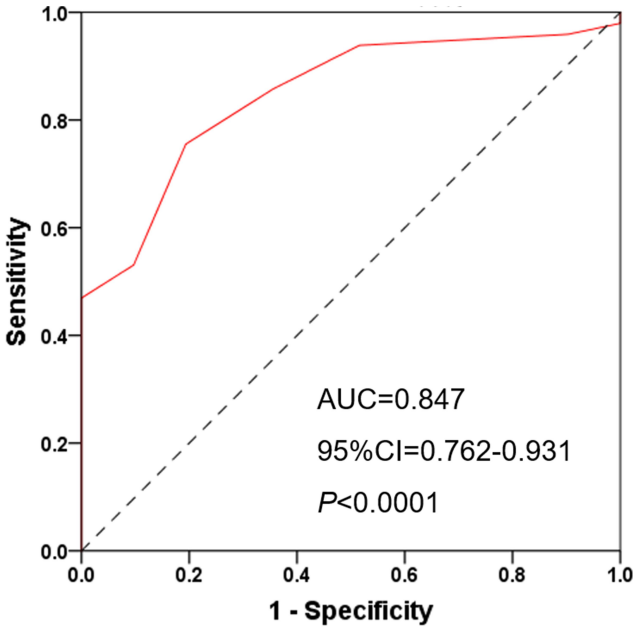

Figure 11 ROC curve of *HDAC6* and melanoma 10-year survival rate, AUC=0.847, 95% CI=0.762~0.931, P<0.0001.

skin tissues, the expression levels of *HDAC6* were higher in melanoma tissues.[14,15,26] Furthermore, some research discovered that *HDAC6* inhibitors can act as the anti-melanoma agents by promoting T-cell immune properties, or impairing the *STAT3* activation in melanoma cells.[16,17] In this study, we investigated the correlation between the expression level of *HDAC6* and the clinical features of melanoma patients. We also studied the impact of *HDAC6* expression and clinical features on the survival time of melanoma patients. We found that the protein and mRNA expression levels of *HDAC6* in the cancer tissue were significantly higher than those in the adjacent nontumor tissue, which was consistent with Liu's[14] and Bai's[15] results. Moreover, *HDAC6* expression in melanoma cells was also higher than that in normal melanocytes. The expression level of *HDAC6* was correlated with the pathological stages of the patients, which increased from stage I to stage IV. In the metastatic tumor site, the expression of *HDAC6* was also significantly higher than that in tumors in situ. Kaplan–Meier survival analysis showed that *HDAC6* positivity was significantly associated with a poorer prognosis of melanoma patients, and multivariate Cox analysis indicated that *HDAC6* was an independent predictor of the survival time of melanoma patients. In our study, we demonstrated that high expression of *HDAC6* was an independent predictor of an increased risk of metastasis and death from melanoma in a retrospective clinical study. However, this study had certain limitations, such as some information (the type and anatomical site of the metastases) cannot be collected, and no information on *HDAC6* protein expression in TCGA dataset was available, thus limiting a more detailed comparison.

Conclusion

In conclusion, our study found that the expression level of *HDAC6* was related to the prognosis of melanoma patients, and *HDAC6* was an independent predictor of the survival time of melanoma patients. Moreover, we demonstrated that higher *HDAC6* expression was associated with melanoma metastasis. The mechanistic basis for the above observations is worthy of in-depth exploration in future studies. Based on the results we have so far, we propose that *HDAC6* may be a prognostic biomarker of melanoma, and the mechanism of *HDAC6* inhibitors, which are widely used in clinical investigations as promising drug targets, should be further studied.

Acknowledgments

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Table 3 Univariate Analysis and Multivariate Analysis in Patients with Melanoma (n=80)

Characteristic	Univariate Analysis			Multivariate Analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age	1.05	1.02–1.07	0.0001	1.03	1.01–1.05	0.011
Sex	0.82	0.48–1.39	0.458			
TNM stage	8.15	4.88–13.61	0.0001	4.15	1.90–9.09	0.0001
Radiotherapy	0.75	0.39–1.45	0.395			
Systemic therapy	2.11	1.16–3.82	0.014	1.60	0.84–3.04	0.149
Metastasis	0.04	0.01–0.11	0.0001	1.18	0.28–5.00	0.823
HDAC6 expression	1.57	1.41–1.74	0.0001	1.39	1.21–1.58	0.0001

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Disclosure

The authors have no conflicts of interest to declare.

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