Immunohistochemical Characterization of Histone Deacetylase as a Potential Prognostic Marker and Therapeutic Target in Endometrial Stromal Sarcoma

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Abstract. *Aim: Endometrial stromal sarcoma (ESS) is a rare tumor with limited treatment options. Histone deacetylase (HDAC) is a potential therapeutic target in ESS showing a good rate of response in laboratory studies. In this study we investigated the expression of HDAC enzymes in 41 ESS patients. Materials and Methods: Immunohistochemical expression of HDACs was analyzed by tissue microarrays. Results: Strong positive immunoreactivity was observed in 32 (78.0%), 23 (56.1%), 8 (19.5%), 36 (87.8%), 7 (17.1%), 30 (73.2%), 31 (75.6%), and 33 (80.5%) for HDACs 1-8, respectively. Although not statistically significant, HDAC 1, 4, 6, 7, and 8 exhibited a high frequency of strong immunoreactivity linked to a lower 10-year DFS (100.0% vs. 81.3%, p=0.202; 100.0% vs. 83.3%, p=0.393; 90.9% vs. 83.3%, p=0.579; 90.0% vs. 83.9%; and 100.0% vs. 81.8%, p=0.207; respectively). Conclusion: HDACs 1, 4, 6, 7, and 8, that showed an especially high frequency of strong immunoreactivity, may represent potential therapeutic targets for ESS.*

Uterine sarcoma is a rare disease that accounts for 3-5% of uterine malignancies (1). Among these malignancies, endometrial stromal sarcoma (ESS) is a rare malignant stromal disease that represents approximately 10% of uterine sarcomas with an annual incidence of 0.19 per 100,000 women (2). Previously, ESS was sub-grouped into high- and low-grade. High-grade ESS has been reclassified as undifferentiated endometrial sarcoma (UES) because of its poor prognosis and different clinical outcomes compared with low-grade ESS (3). Although, ESS shows relatively indolent clinical characteristics compared to UES, it also shows a poor survival outcome with a 30~40% rate of recurrence, even when diagnosed at an early stage (4, 5). Because of its rarity, specific treatment guidelines have not yet been established (6), and there exists a need to investigate new therapeutic modalities to improve its prognosis.

Although hormonal therapy may be considered for adjuvant therapy and has shown clinical benefits in some progressive and metastatic diseases (7, 8), it is only effective when a tumor expresses the respective steroid receptor (9). More than one-third of previously studied ESS cohorts received hormonal therapy with or without chemo- and radio-therapy, and eventually 64% and 22% of patients recurred and died of disease (7, 10, 11), respectively. New therapeutic modalities, thus, need to be investigated that can compensate or substitute for traditional treatments.

Targeted therapeutic agents that inhibit certain receptors in tumors that are associated with tumorigenesis, progression, and metastasis represent a promising treatment modality. Histone deacetylases (HDACs) play an important role in the covalent modification of histone proteins, which results in changes in chromatin architecture and gene expression. HDACs are involved in the pathogenesis of various hematological malignancies and solid tumors, and some HDAC inhibitors are emerging as novel promising therapeutic agents with few side-effects and high specificity (12).

Studies of the expression of specific receptor targets and their clinical impact is important and a prerequisite for the use of a therapeutic agent in the treatment of rare types of

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cancer. Our current study aimed to investigate the expression of HDACs and the clinical impact of these enzymes in ESS.

Materials and Methods

Patients. Patient electronic medical charts from the Asan Medical Center from 1990 to 2014 were reviewed under approval of the institutional review board (IRB). A total of 72 ESS patients were identified who were diagnosed, treated, and underwent regular follow-up at our hospital. Overall, paraffin blocks had been preserved for 41 of these ESS cases. Clinico-pathological characteristics, such as age, menopause, parity, body mass index (BMI), recurrence, death, and the International Federation of Obstetrics and Gynecology (FIGO) stage, were reviewed. Treatment methods, including hysterectomy, ovarian preservation, lymph node dissection, and the type of adjuvant treatment, were also evaluated.

Methods. Paraffin blocks of tumor tissues that were obtained at the time of surgery and pathologically confirmed as ESS were sectioned and mounted onto a slide. Tumors in paraffin blocks were punctured with a 2-mm diameter needle and samples were sent to the Bio-Resource Center (BRC) at our Institution to generate a tissue microarray (TMA) under approval of the IRB (following the ethical standards of the responsible committee on human experimentation (Institutional or regional) and with the Helsinki Declaration of 1975, as revised in 1983). Three different parts of each tumor were obtained and three samples were included per case to minimize bias and maintain the quality of the immunohistochemistry results. The samples that showed the strongest immunoreactivity were considered to represent the intensity of positivity for each case.

According to the instructions of the manufacturer, the custom-generated TMA sections were utilized for immunohistochemical analysis of the HDAC series using a BenchMark ULTRA automatic immunostaining device (Ventana Medical Systems, Tucson, AZ, USA) with OptiView DAB IHC Detection Kit (Ventana Medical Systems, Tucson, AZ, USA). Four-micrometer-thick sections (obtained with a microtome) were transferred onto silanized charged slides and allowed to dry for 10 min at room temperature, followed by 20 min in an incubator at 65˚C. Sections were performed by heat-induced epitope retrieval method using cell conditioning 1buffer for 32 min and incubated for 16 min with rabbit anti-HDAC1 (1:1,000 dilution; GTX100513, Genetex), anti-HDAC2 (1:400 dilution; NBP2-03980SS, Novus), anti-HDAC3 (1:25 dilution; NB100-1669SS, Novus), anti-HDAC4 (1:100 dilution; GTX110231, Genetex), anti-HDAC5 (1:25 dilution; NBP2-22152SS, Novus), anti-HDAC6 (1:200 dilution; NB100-91805-0.02mg, Novus), anti-HDAC7 (1:100 dilution; GTX114179, Genetex), and anti-HDAC8 (1:50 dilution; GTX113981, Genetex) in the autoimmunostainer. Using Ventana OptiView DAB IHC Detection Kit (Optiview HQ Linker 8min, Optiview HRP Multimer 8min, Optiview H_2O_2/DAB 8min, Optiview Copper 4min), antigen-antibody reactions were visualized. Counterstaining was performed by using Ventana Hematoxylin II for 24 min and Ventana Bluing reagent for 4 min. Finally, all slides are removed from the stainer, dehydrated, and cover-slipped for microscopic examination. For positive controls, adequate immunoreactive tissue samples were used. Incubation with the primary antibody was omitted in the negative controls.

Diagnoses were performed by one pathologist who was blind to clinical data and patient characteristics, and one physician with a sub-specialization in gynecological oncology. A semi-quantitative scoring system was used to determine the immunostaining intensity (categorized as 0, 1, 2, or 3). The percentage of the total width that was stained was then multiplied to yield the total score; 0-20 points was considered to be negative, 21-80 was weakly positive (1+), 81-180 was moderately positive (2+), and 181-300 was strongly positive (3+).

Statistical analysis. The Student's *t*-test and the Mann–Whitney *U*-test were used to analyze mean and median values for each variable. The frequency of each variable was analyzed using the χ^2 test. Disease-free survival (DFS) was defined as the period from the day of surgery to the day of recurrence or last follow-up, and overall survival (OS) was defined as the period from the day of surgery to the day of death or the last follow-up. Survival curves and their analysis were performed using the Kaplan–Meier method and statistical significance was analyzed using the log-rank test. SPSS software version 22.0 (SPSS Inc., Chicago, IL) was used for statistical analyses and $p<0.05$ was used as our threshold for statistically significant differences.

Results

Table I lists the clinicopathological characteristics of the current study patients with ESS. The median patient age was 46 years (range=23-64 years), and 7 (17.1%) patients were older than 50 years. The median tumor size was 5.5 cm (range, 1.0-26.0 cm), and 18 (43.9%) patients had a tumor size ≥5.5 cm. Additionally, the median body mass index (BMI) was 23.65 kg/m^2 (range=17.29-31.13 kg/m^2), and 15 (36.6%) patients had a BMI ≥25 kg/m^2. Finally, 6 (14.6%) patients had parity ≥3, 5 (12.2%) were menopausal, and 9 (22.0%) had FIGO stage III/IV.

Table II presents the types of treatment and adjuvant therapy used for the ESS patients analyzed in this study. Hysterectomy, ovarian preservation, and lymphadenectomy were performed in 38 (92.7%), 20 (48.8%), and 18 (43.9%) patients, respectively. Among these cases, lymph node metastasis was identified in 2 (8.7%) patients. A total of 23 (56.1%) patients underwent adjuvant treatment after surgery. Overall, adjuvant hormone therapy was performed in 23 (56.1%) patients. The median follow-up period was 47.8 months (range=2.5-197.7 months). Recurrence and death were observed in 7 (17.1%) and 2 (4.9%) patients, respectively, during the follow-up period (Table I).

Table III and Figure 1 show the expression of each HDAC in the present ESS series. A strong positive immunoreactivity was observed in 32 (78.0%), 23 (56.1%), 8 (19.5%), 36 (87.8%), 7 (17.1%), 30 (73.2%), 31 (75.6%), and 33 (80.5%) of the study patients for HDACs 1-8, respectively. Table IV lists the survival outcomes according to the type of treatment and adjuvant therapy for the study patients. Hysterectomy, ovarian preservation, lymphadenectomy, lymph node metastasis, and adjuvant treatment had no clinical impact on the 10-year DFS or OS. HDAC expression showed no significant association with survival outcomes. Although the associations were not statistically significant, HDACs 1, 4, 6,

Table I. *Clinico-pathological characteristics of patients (n=41)*

Variable	N (%)
Age	
Median (range)	46.0 (23-64)
≥50	7 (17.1)
<50	34 (82.9)
Parity	
≥2	35 (85.4)
<2	6 (14.6)
Body mass index (kg/m^2)	
Median (range)	23.65 (17.29-31.13)
≥25	15 (36.6)
<25	26 (63.4)
Menopause	
Yes	5 (12.2)
No	36 (87.8)
Tumor size (cm)	
Median (range)	5.5 (1.0-26.0)
≥5.5 cm	18 (43.9)
<5.5 cm	23 (56.1)
FIGO stage	
I	30 (73.2)
II	2 (4.9)
III	6 (14.6)
IV	3 (7.3)
Recurence	
Yes	7 (17.1)
No	34 (82.9)
Death	
Yes	2 (4.9)
No	39 (95.1)

FIGO, International Federation of Gynecology and Obstetrics.

Table II. *Type of treatment and adjuvant therapy in patients (n=41).*

Variable	N (%)
Hysterectomy	
Yes	38 (92.7)
No	3 (7.3)
Ovarian preservation	
No	21 (51.2)
Yes	20 (48.8)
Lymphadenectomy	
Yes	18 (43.9)
No	23 (56.1)
Lymph node metastasis	
Positive	2 (8.7)
Negative	21 (91.3)
Adjuvant treatment	
None	18 (43.9)
Chemotherapy with or without hormone therapy	8 (19.5)
Radiotherapy with or without hormone therapy	5 (12.2)
CCRT with or without hormone therapy	4 (9.8)
Hormone therapy only	6 (14.6)
Hormone therapy with or without other treatment	23 (56.1)

CCRT, Concurrent chemo-radiotherapy.

Table III. *Expression of each biomarkers categorized by semi-quantitative scoring system (n=41).*

Biomarkers	Positive			
	Negative, N (%)	Weak, N (%)	Moderate, N (%)	Strong, N (%)
HDAC 1	0 (0)	2 (4.9)	7 (17.1)	32 (78.0)
HDAC 2	3 (7.3)	3 (7.3)	12 (29.3)	23 (56.1)
HDAC 3	3 (7.3)	29 (70.7)	1 (2.4)	8 (19.5)
HDAC 4	0 (0)	0 (0)	5 (12.2)	36 (87.8)
HDAC 5	8 (19.5)	10 (24.4)	16 (39.0)	7 (17.1)
HDAC 6	2 (4.9)	2 (4.9)	7 (17.1)	30 (73.2)
HDAC 7	0 (0)	1 (2.4)	9 (22.0)	31 (75.6)
HDAC 8	0 (0)	0 (0)	9 (19.5)	33 (80.5)

HDAC, Histone deacetylase.

7, and 8 showed a high frequency of strong immunoreactivity and a lower DFS trend (100.0% *vs*. 81.3%, p=0.202; 100.0% *vs*. 83.3%, p=0.393; 90.9% *vs*. 83.3%, p=0.579; 90.0% *vs*. 83.9%; and 100.0% *vs*. 81.8%, p=0.207, respectively; Figure 2). HDAC expression showed no significant association with pattern of recurrence, lymph node metastasis, tumor size, and FIGO stage (Table V).

Discussion

Recently, many clinical trials have investigated novel and promising therapeutic agents for the treatment of cancer. There may only exist a few ongoing studies of endometrial cancer in gynecological malignancies because of its relatively favorable prognosis compared to other cancers. However, further investigations of rare subtypes of endometrial cancer with poor prognoses, such as uterine sarcoma, are required. Among uterine sarcomas, ESS is a rare type of cancer with a high rate of recurrence, even when diagnosed at an early stage, and there is a need for new treatments that can support classical interventions for this subtype (4, 13). To date, no management consensus has been determined for ESS and, because traditional treatments have not produced any improvement in survival outcomes, there is a need for new and targeted therapeutic agents.

Hysterectomy has served as the mainstay of surgery (14, 15) for ESS, whilst cytoreductive surgery that leaves no residual mass in locally advanced disease has an unproven survival benefit (6) and did not improve the survival outcome in ESS according to a previous report (3). Additionally, the removal of both adnexae does not improve the clinical course of this disease (16, 17). The incidence of reported lymph node metastasis from ESS has generally been low in previous

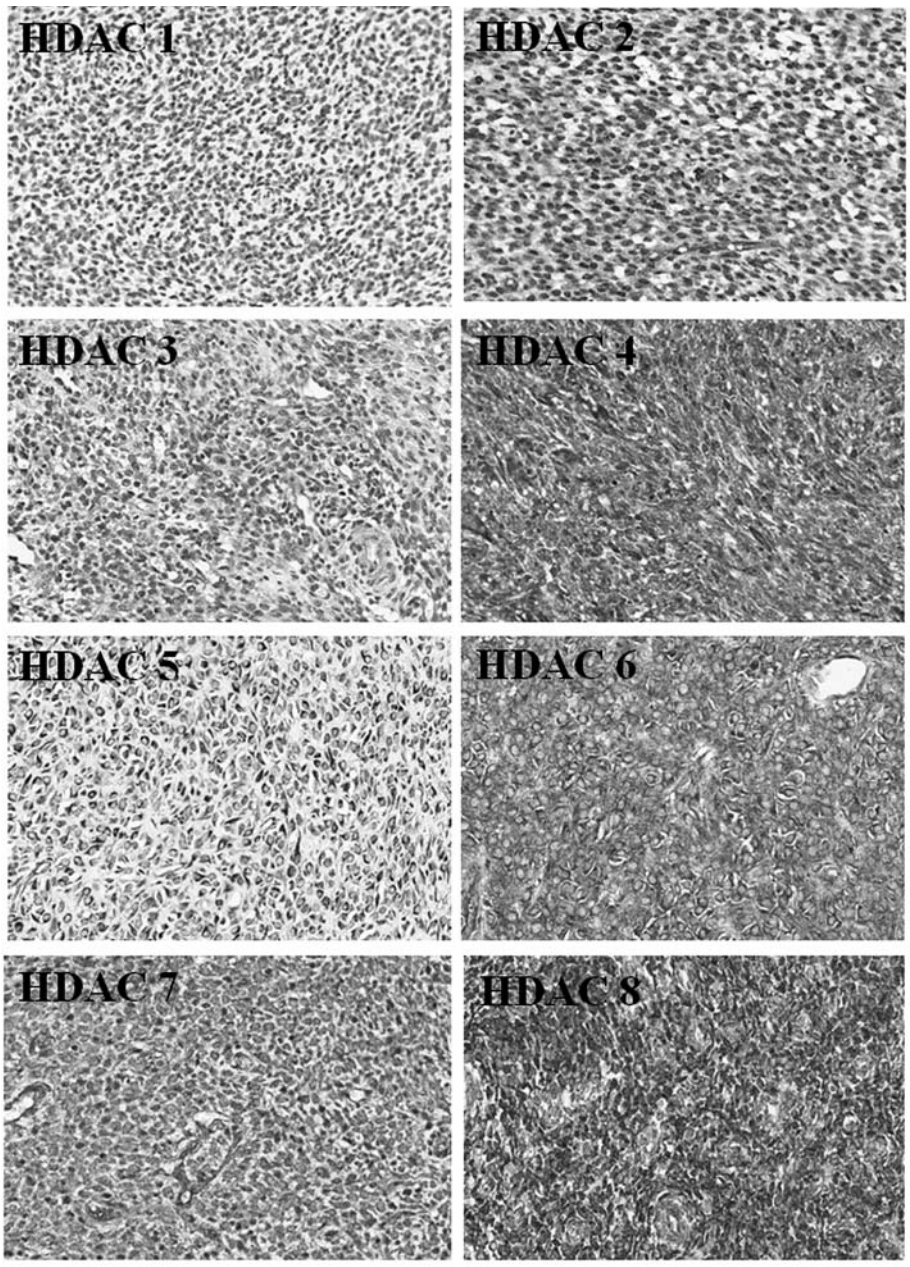

Figure 1. *Strong expression (3+) of histone deacetylase (HDACs) in endometrial stromal sarcoma. Images were provided by the Department of Pathology, Asan Medical Center (magnification ×200).*

studies at 7~9.9%, and lymphadenectomy appears to have no clinical benefit in treating ESS (16, 17). Lymph node metastasis has only been frequently observed when the tumors have been obviously enlarged or related to extrauterine spread (18). Consequently, lymphadenectomy is not indicated for ESS unless the lymph nodes are clearly enlarged on preoperative image findings (6). In our present ESS case series, only 8.7% of patients had lymph node metastases, which was similar to the incidence reported previously.

Radiation therapy has been reported to have a modest role in the locoregional control of ESS, but ultimately did not prolong overall survival (19). This treatment is only considered for palliative therapy in progressive and recurrent cases when systemic therapy or surgery is expected to be ineffective (6). Additionally, it is difficult to apply this treatment in every case of ESS. There have only been a few studies of adjuvant chemotherapy, and it is only considered in cases of recurrent disease after hormonal therapy (6). Furthermore, previous

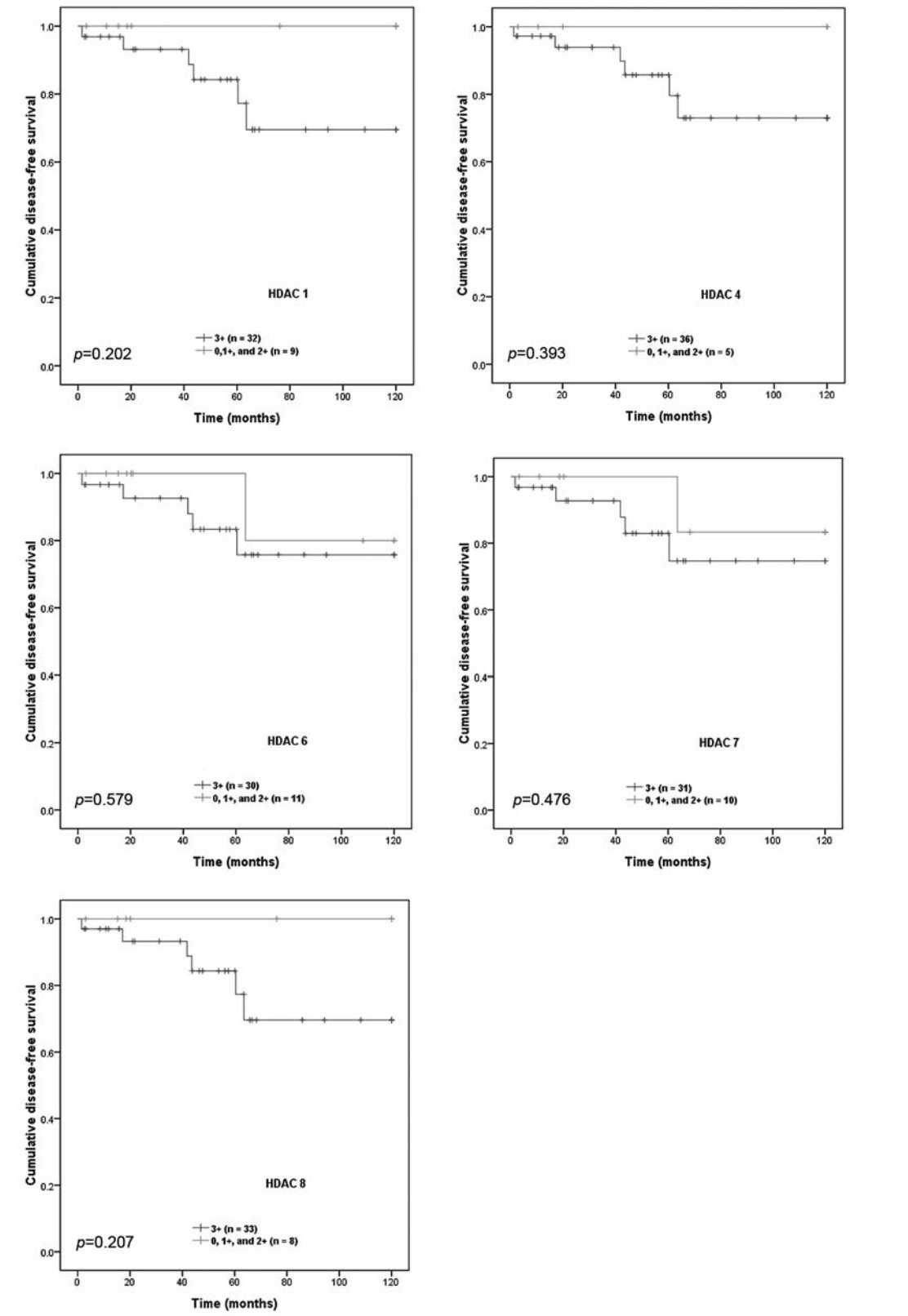

Figure 2. *Disease-free survival based on the intensity of immunoreactivity (Semi-quantitative scoring system) for histone deacetylase (HDAC) 1, 4, 6, 7, and 8 in endometrial stromal sarcoma.*

Table IV. *Differences in 10 year survival outcome by treatment type of patients (n=41).*

Variables	DFS (%)	*p*-Value	OS (%)	*p*-Value
Hysterectomy (No *vs*. Yes)	100 *vs*. 84.2	0.478	100 *vs*. 97.4	0.785
Ovarian preservation (No *vs*. Yes)	81.0 *vs*. 90.0	0.681	95.2 *vs*. 100	0.434
Lymphadenectomy (No *vs*. Yes)	87.0 *vs*. 83.3	0.927	95.7 *vs*. 100	0.301
Adjuvant therapy (No *vs*. Yes)	88.9 *vs*. 82.6	0.996	100 *vs*. 95.7	0.434
HDAC 1 (0, 1+, 2+ *vs*. 3+)	100 *vs*. 81.3	0.202	100 *vs*. 96.9	0.689
HDAC 2 (0, 1+, 2+ *vs*. 3+)	77.8 *vs*. 91.3	0.417	94.4 *vs*. 100	0.301
HDAC 3 (0, 1+, 2+ *vs*. 3+)	82.4 *vs*. 100	0.159	97.1 *vs*. 100	0.573
HDAC 4 (0, 1+, 2+ *vs*. 3+)	100 *vs*. 83.3	0.393	100 *vs*. 97.2	0.785
HDAC 5 (0, 1+, 2+ *vs*. 3+)	82.4 *vs*. 100	0.156	97.1 *vs*. 100	0.573
HDAC 6 (0, 1+, 2+ *vs*. 3+)	90.9 *vs*. 83.3	0.579	100 *vs*. 93.7	0.648
HDAC 7 (0, 1+, 2+ *vs*. 3+)	90.0 *vs*. 83.9	0.476	100 *vs*. 96.8	0.610
HDAC 8 (0, 1+, 2+ *vs*. 3+)	100 *vs*. 81.8	0.207	100 *vs*. 97.0	0.689

DFS, Disease-free survival; HDAC, histone deacetylase; OS, overall survival.

Table V. *Association of expression of therapeutic target with clinico-pathologic characteristics in patients with endometrial stromal sarcoma (n=41)*

Variables	Pattern of recurrence (n=7)			Lymph node metastasis (n=18)			Tumor size (<5.0 cm *vs*. ≥5.0 cm)			FIGO stage (I, II *vs*. III, IV)		
	Loco-region	Distant	*p*-Value	No	Yes	*p*-Value	<5.0 cm	≥5.0 cm	*p*-Value	I, II	III, IV	*p*-Value
HDAC 1												
0, 1+, 2+	1 (100)	0 (0)	0.429	2 (100)	0 (0)	1.000	6 (66.7)	3 (33.3)	0.128	8 (88.9)	1 (11.1)	0.654
3+	2 (33.3)	4 (66.7)		14 (87.5)	2 (12.5)		11 (34.4)	21 (65.6)		24 (75.0)	8 (25.0)	
HDAC 2												
0, 1+, 2+	3 (60.0)	2 (40.0)	0.429	6 (100)	0 (0)	0.529	8 (44.4)	10 (55.6)	0.760	14 (77.8)	4 (22.2)	1.000
3+	0 (0)	2 (100)		10 (83.3)	2 (16.7)		9 (39.1)	14 (60.9)		18 (78.3)	5 (21.7)	
HDAC 3												
0, 1+, 2+	3 (42.9)	4 (57.1)	-	13 (92.9)	1 (7.1)	0.405	15 (44.1)	19 (55.9)	0.679	26 (76.5)	8 (23.5)	1.000
3+	-	-		3 (75.0)	1 (25.0)		2 (28.6)	5 (71.4)		6 (85.7)	1 (14.3)	
HDAC 4												
0, 1+, 2+	1 (100)	0 (0)	0.429	1 (100)	0 (0)	1.000	4 (80.0)	1 (20.0)	0.141	4 (80.0)	1 (20.0)	1.000
3+	2 (33.3)	4 (66.7)		15 (88.2)	2 (11.8)		13 (36.1)	23 (63.9)		28 (77.8)	8 (22.2)	
HDAC 5												
0, 1+, 2+	3 (42.9)	4 (57.1)	-	12 (92.3)	1 (7.7)	0.490	13 (38.2)	21 (61.8)	0.421	26 (76.5)	8 (23.5)	1.000
3+	-	-		4 (80.0)	1 (25.0)		4 (57.1)	3 (42.9)		6 (85.7)	1 (14.3)	
HDAC 6												
0, 1+, 2+	2 (100)	0 (0)	0.143	3 (100)	0 (0)	1.000	7 (63.6)	4 (36.4)	0.151	8 (72.7)	3 (27.3)	0.680
3+	1 (20.0)	4 (80.0)		13 (86.7)	2 (13.3)		10 (33.3)	20 (66.7)		24 (80.0)	6 (20.0)	
HDAC 7												
0, 1+, 2+	2 (100)	0 (0)	0.143	2 (100)	0 (0)	1.000	5 (50.0)	5 (50.0)	0.714	8 (80.0)	2 (20.0)	1.000
3+	1 (20.0)	4 (80.0)		14 (87.5)	2 (12.5)		12 (38.7)	19 (61.3)		24 (77.4)	7 (22.6)	
HDAC 8	-	-	-	-	-	-	-	-	-	-	-	-

FIGO, International Federation of Gynecology and Obstetrics; HDAC, histone deacetylase.

studies report a low response rate and that this approach is only considered when hormone therapy is ineffective (10, 19, 20). In our present series, the type of treatment, such as radical extended surgery, removal of ovaries, and various postoperative adjuvant treatments, did not result in an improved prognosis, which is in accordance with previous findings.

Hormone therapy has been reported to reduce recurrence and prolong overall survival with a high response rate in ESS (3, 21). However, in a study of a large number of patients, Cheng *et al*. reported a 64% rate of recurrence, that more than one-third of patients underwent hormone therapy, but only 27% of the patients showed a response (10). In our present

series, 17.1% of the patients recurred and more than half underwent hormone therapy with or without treatment with another adjuvant. Although hormone therapy represents the cornerstone of adjuvant therapy in ESS, we argue that there is a need for studies of new adjunctive treatment options, such as targeted therapy, to improve survival outcomes.

The identification of certain therapeutic potential biomarkers in rare types of cancer is a prerequisite for clinical trials that incorporate novel targeted agents because it is difficult to perform large-scale randomized clinical trials for such conditions, for example epithelial ovarian cancer (22). Investigations of new promising therapeutic targets that will reduce the time, cost, and effort involved in choosing the optimal target have a high probability of improving the prognosis of rare types of disease. In our present study, HDAC proteins were found to be highly expressed in ESS and we propose that a HDAC inhibitor may represent a promising new therapeutic agent for ESS that reduces recurrence and improves its clinical outcome. Recently, the potential use of HDAC inhibitors for targeted therapy of cancer has received attention because of their high specificity and low side-effects. HDAC inhibitors can trigger various anticancer mechanisms, such as apoptosis, cell-cycle arrest, and terminal differentiation (23). Studies of HDAC expression in gynecological cancer and its clinical impact will aid in the elucidation of new HDAC inhibitors that may be beneficial for treating certain malignancies. Numerous clinical trials are ongoing, and HDAC inhibitors will be administered along with traditional standard-treatment modalities (24). HDACs both control and balance histone acetylation, which is related to cell growth, and altered HDAC activity can lead to tumorigenesis and tumor progression (25).

Suberoylanilide hydroxamic acid (SAHA) is one of the most promising targeted agents that has a well-characterized mechanism and shows favorable bioavailability compared with other types of HDAC inhibitors. Generally, SAHA, which is known to be a pan-HDAC inhibitor, mainly acts on class I and II HDACs, although recent studies have shown that it can also act on HDAC 7 (26, 27). In previous experimental studies, SAHA showed its synergistic effect on mTOR, AKT pathway, and tumor cell growth inhibition when it was combined with phosphoinositid-3-kinase inhibitor and rapamycin in ESS (26, 28). To date, few studies have investigated the clinical efficacy and feasibility of vorinostat (Merck & Co, NJ, USA) in ESS (29).

Previous studies have found that HDAC inhibitors have synergistic effects when combined with other targeted agents, chemotherapies, and radiation (24). Vorinostat with paclitaxel and carboplain in non-small cell lung cancer or advanced solid tumors and valproate with epirubicin in advanced solid tumors have shown a response. Depsipeptide and MGCD 0103 in combination with gemcitabine in ovarian cancer or other advanced or refractory solid tumors has shown minor responses or a delay in disease progression. Belinostat in conjunction with paclitaxel and carboplatin in relapsed epithelial ovarian cancer is associated with a high frequency of a partial response. In our present study, HDACs 1, 4, 6, 7, and 8 showed strong expression and were associated with a high rate of recurrence in ESS patients. Further studies will be required to determine whether new drugs that target these potential biomarkers in combination with traditional therapeutic modalities will improve the prognostic outcomes in ESS.

The strength of our study was its consistency in arriving at pathological diagnoses and measurements of the expression of HDACs in ESS with its large number of patients from a single Institution. We used a semi-quantitative scoring system to diagnose the immunoreactivity of HDACs in ESS, which is the most widely accepted criterion in the field and allows our findings to be compared to those of other studies. The major limitation of our study was its retrospective design and use of paraffin blocks that were made using tissues obtained at the time of surgery. Changes in policies for diagnoses and treatment must be considered as a potential source of bias because patients who were treated more than 10 years ago were also included in our cohort. Also, current series was not able to demonstrate statistical significance between immunoreactivity of HDAC 1, 4, 6, 7, and 8 and lower 10-year DFS because of relatively indolent clinical course of ESS compared to other high grade uterine sarcomas.

A prospective large-scale randomized clinical trial that follows a well-designed protocol and study design is now needed to best assess the expression patterns and clinical impact of promising new therapeutic agents for the treatment of ESS. We believe that our current study will aid the identification of the characteristics of this rare type of cancer and improve the selection of novel targeted agents for inclusion in future trials.

Conflicts of Interest

The Authors declare no conflicts of interest.

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None.

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