Enhancer of zeste homologue 2 (EZH2) expression in synovial sarcomas as a promising indicator of prognosis

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ABSTRACT

Synovial sarcoma (SS) is a type of soft-tissue sarcoma, often linked to poor survival. Although overexpression of enhancer of zeste homologue 2 (EZH2) has been associated with poor prognosis in different tumors, a few studies investigated this link in SS. Here, we analyzed the relationship between EZH2 expression and prognostic factors in SS. We included 29 patients with SS. Immunostaining of EZH2 was performed with (D2C9) XP™ Rabbit mAb antibody, and the results were classified as low EZH2 expression (negative or weak expression) and high EZH2 expression category (moderate or strong expression). Analysis of survival in relation to prognostic factors was performed with Kaplan-Meier survival curves and Cox proportional hazard regression analysis. Our sample included 19/29 female and 10/29 male patients, with age range 16-63 years. The tumor diameter ranged from 2 to 15 cm. Necrosis was observed in 15/29 cases. Sixteen cases had >10 mitoses per 50 high-power fields (HPFs). Out of 29 cases, 14 showed low and 15 had high EZH2 expression. Statistically significant results were obtained for the association between the presence of metastasis and necrosis (p = 0.042), high EZH2 expression and distant metastasis (p = 0.018), high EZH2 expression and necrosis (p = 0.016), and high EZH2 expression and the tumor size >5 cm versus tumor size ≤5 cm (p = 0.014). Patients with all of the following: the tumor size ≤5 cm, low EZH2 expression, and without necrosis and distant metastasis had significantly longer survival time. Our results are consistent with previous studies, suggesting that EZH2 overexpression is an indicator of poor prognosis in SS.

KEY WORDS: Enhancer of zeste homologue 2 (EZH2); immunohistochemistry; prognostic factors; synovial sarcoma; survival analysis
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INTRODUCTION

Synovial sarcoma (SS) is an aggressive soft-tissue tumor with a tendency to recur locally and high potential for distant metastasis. Morphologically, these tumors consist of spindle-like cells positive by immunohistochemical staining for epithelial markers. SS constitutes 5-10% of all soft-tissue sarcomas and typically affects adolescents and young adults [1-4]. This sarcoma is most often found in the lower extremities (especially the knee), upper extremities, and the head and neck (mostly in the parapharyngeal region). SS can be classified into three histological types: monophasic (pure epithelioid or fibroblastic cells), biphasic, and poorly differentiated [1-5], and is diagnosed upon histopathological findings of epithelial differentiation or immunostaining for cytokeratin or epithelial membrane antigen (EMA). While some investigators have reported transducer-like enhancer of split 1 (TLE1) to be an extremely sensitive biomarker for SS [1,2,6], others showed a low specificity of TLE1 in diagnosing SS [7]. Recently, the t(X;18) (SYT-SSX) translocation has been recommended as the most reliable diagnostic tool [1,2,8].

The prognosis for SS is usually poor, with 5- and 10-year survival rates between 36-76% and 20-63%, respectively [3,9-11]. Factors associated with a better prognosis include age <20 years, tumor size smaller than 5 cm, more distal location along the extremities, lower tumor stage, and appropriate excision. Predictors of poor prognosis include less-differentiated tumor areas, presence of necrosis, and high mitotic activity, i.e., >10 mitoses/10 high power fields (HPFs) [3,5,9,12].

Enhancer of zeste homologue 2 (EZH2) belongs to the polycomb group (PcG) proteins of cell cycle regulators that suppress transcription. An excessive expression of EZH2 is found in various carcinomas, lymphomas, and soft tissue

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sarcomas, and growing evidence suggests that this overexpression correlates with the aggressiveness and poor clinical outcome of such tumors [13-22]. However, few studies have investigated the expression of EZH2 in SS [23-25]. The aim of this study was to investigate the relationship between the EZH2 expression as evaluated by immunohistochemistry and known prognostic indicators in SS.

MATERIALS AND METHODS

We retrospectively analyzed the data for 29 patients diagnosed with SS between 2002 and 2014 at our pathology department. All slides were reviewed and re-evaluated. The following information was collected from the medical records: age, gender, tumor site, tumor size, tumor grade, follow-up duration, recurrence, metastasis, and survival time. We used the World Health Organization (WHO) guidelines for the histological type and Fédération Nationale des Centers de Lutte Contre le Cancer (FNCLCC) criteria to determine the tumor grade (differentiation, mitotic index, and necrosis). Tumors were classified histologically into three groups: biphasic, monophasic fibrous, or poorly differentiated. The study was conducted in accordance with the Institutional Ethical Guidelines.

Immunohistochemical study

Four-micron-thick serial sections were obtained from formalin-fixed, paraffin-embedded (FFPE) blocks. Immunohistochemical staining was performed on a Leica BOND-MAX Autostainer (Leica Microsystems, Berlin, Germany), and peroxidase/DAB Bond Polymer Refine Detection System (Leica Microsystems) was used for visualization. EZH2 immunohistostaining was done with D2C9XP™ Rabbit mAb (1:100 dilution) primary antibodies, while other sections were reserved for hematoxylin and eosin (H&E) staining.

Scoring system

To assess the immunohistochemical labeling of EZH2, the immunostained slides were evaluated using a 10× magnification. The nuclear staining was scored as follows: a) negative, no visible staining, b) weak, 1-25% of nuclei were positive, c) moderate, 25-75%, and d) strong, over 75% [19]. The EZH2 staining was classified into two categories according to the nuclear staining: a "low EZH2 expression category" with negative or weak expression, and a "high EZH2 expression category" with moderate or strong expression.

Statistical analysis

The association between EZH2 expression and the presence of distant metastasis was tested by the Fisher's exact test and Fisher-Freeman-Halton test where appropriate. The log-rank test was used to determine the difference between the Kaplan-Meier curves for survival time. Median survival time was reported. To determine the prognostic factors that affect the overall survival time, we performed the Cox proportional hazard regression analysis with backward selection procedure following the Kaplan-Meier analysis. The significant predictors obtained in the Kaplan-Meier analysis were entered into the Cox proportional hazard regression, and the results of the final step were reported. The results were reported as hazard ratios with 95% confidence intervals (CIs) and the related *p*-values. All analyses were performed using IBM SPSS Statistics for Windows, Version 21.0. (IBM Corp., Armonk, NY). A value of $p < 0.05$ was considered as statistically significant.

RESULTS

Clinical data

The study included 19/29 female (65.5%), and 10/29 male (34.5%) patients with age range 16-63 (mean age: 35). The mean diameter of the tumors was 8 cm, ranging from 2 to 15 cm. Eight tumors measured ≤5 cm, 21 tumors were >5 cm. The tumors occurred in the periphery in 22/29 cases (76%) and centrally in 7/29 cases (24%).

The specimens consisted of 15 marginal excisions and 14 wide resections. In 4 cases, recurrence was treated by amputation, including two hemipelvectomies and two finger amputations. Twenty-two patients were treated with both chemo and radiation therapy following the surgery.

The follow-up information for a minimum of 13 months was available for all 29 cases (13-147 months range). Out of the 29 cases, 15 (52%) patients died of the tumor, 9 (31%) had local recurrence, and 5 (17.2%) had distant metastasis.

Pathological features

Histopathologically, 14/29 cases were monophasic (48.3%), 11/29 biphasic (37.9%), and 4/29 poorly differentiated (13.8%) (Figure 1 and 2). In 1 case, there were large areas of epithelial differentiation, and 3 cases localized on the periphery had large areas of calcification.

Out of the 29 cases, 14 (48.3%) were evaluated as Grade II and 15 (51.7%) as Grade III. We observed necrosis in 15 (51.7%) cases, the mitotic rate per 50 HPFs ranged from 4 to 39. In addition, 16 cases had >10 mitoses per 50 HPFs.

We examined the effects of clinicopathological parameters on distant metastasis, and found that there was a statistically significant association between metastases and the presence of necrosis ($p = 0.042$). Other clinicopathological parameters of the tumor (i.e., age, gender, tumor location, tumor size,

TABLE 1. Clinicopathological features of patients with distant metastasis compared with patients without distant metastasis

Clinicopathological features	Distant metastasis		*p* value
	With *n*=5 (%)	Without *n* =24 (%)	
Age			
<19	0	5 (20.80)	0.659
20-34	2 (40)	5 (20.80)	
>35	3 (60)	14 (58.30)	
Sex (male/female)	3/2	7/17	0.306
Tumor location			
Periphery	4 (80)	18 (75)	1.00
Centrally	1 (20)	6 (25)	
Tumor size			
≤5 cm	0	8 (33.30)	0.283
>5 cm	5 (100)	16 (66.70)	
Histological type			
Monophasic	2 (40)	12 (50)	1.00
Biphasic	2 (40)	9 (37.50)	
Poorly differentiated	1 (20)	3 (12.50)	
Grade			
Grade 2	3 (60)	11 (45.80)	0.651
Grade 3	2 (40)	13 (54.20)	
Mitosis			
≤10	1 (20)	12 (50)	0.343
>10	4 (80)	12 (50)	
Necrosis (presence)	5 (100)	10 (41.70)	0.042
Chemo and radiation therapy	5 (100)	17 (70.80)	0.296

histological type, grade, and mitoses) did not show significant association with distant metastasis (Table 1).

Immunohistochemical findings and the relationship with clinicopathological features

Out of the 29 patients, 14 (48.3%) cases were classified as low EZH2 expression and 15 (51.7%) were classified as high EZH2 expression. The relationship between the EZH2 expression and clinicopathological features in SS patients is summarized in Table 2 and Figure 1 and 2.

There were statistically significant correlations between high EZH2 expression and distant metastasis (p = 0.018) and the presence of necrosis (p = 0.016).

In addition, high EZH2 expression was significantly more frequently detected in patients with the tumor size >5 cm compared with the tumor size ≤5 cm (p = 0.014). Patients with low EZH2 expression were predominantly female (p = 0.050).

On the other hand, no statistically significant relationship was observed between EZH2 expression and other clinicopathological factors, including age, tumor location, size, local recurrence, histological type, grade, and mitoses.

Survival analysis

Using the Kaplan-Meier analysis, the overall median follow-up for the entire study population was 68 months (95%, CI: 15.1-34.9 months). The survival was not affected by the age,

TABLE 2. Demographic and clinicopathological characteristics of patients with respect to EZH2 expression

Demographic and clinicopathological characteristics	EZH2 expression		*p* value
	Low n=14 (%)	High n=15 (%)	
Age			
<19	2 (14.30)	3 (20)	0.462
20-34	2 (14.30)	5 (33.30)	
>35	10 (71.40)	7 (46.70)	
Sex (male/female)	2/12	8/7	0.050
Tumor location			
Periphery	9 (64.30)	13 (86.70)	0.215
Centrally	5 (35.70)	2 (13.30)	
Tumor size			
≤5 cm	7 (50)	1 (6.70)	0.014
>5 cm	7 (50)	14 (93.30)	
Histological type			
Monophasic	8 (57.10)	6 (40)	0.612
Biphasic	5 (35.70)	6 (40)	
Poorly differentiated	1 (7.10)	3 (20)	
Grade			
Grade 2	9 (64.30)	5 (33.30)	0.096
Grade 3	5 (35.70)	10 (66.70)	
Mitosis			
≤10	9 (64.30)	4 (26.70)	0.066
>10	5 (35.70)	11 (73.30)	
Necrosis (presence)	4 (28.60)	11 (73.30)	0.016
Local recurrence (presence)	3 (21.40)	6 (40)	0.427
Distant metastasis (presence)	0	5 (33.30)	0.018

EZH2: Enhancer of zeste homologue 2

gender, tumor location, local recurrence, histological type, grade, mitoses, chemo and radiation therapy, and type of surgery (Table 3). However, patients with the tumor size ≤5 cm, without necrosis, without distant metastasis and low EZH2 expression had a significantly longer survival time (p = 0.022, p = 0.006, p = 0.001, and p = 0.017, respectively; Table 3). Moreover, the analysis of independent risk factors affecting survival showed that patients with distant metastasis had a 3.59 risk factor for the total survival time.

DISCUSSION

EZH2 is a PcG protein. This family consists of transcriptional suppressor regulators responsible for the repair of DNA damage, cellular differentiation, cellular aging, and apoptosis. PcG proteins are involved in the maintenance of stem cell character and in the tumor development. Specifically, EZH2 acts as a histone methyltransferase targeting the N-terminal tail of histone H3 to produce a characteristic H3-Lys27 trimethylation (H3K27me3) pattern. EZH2 is expressed at high levels in cells exhibiting an embryonic gene expression pattern, and its expression declines with tissue maturation and differentiation. Recent studies have suggested that EZH2 overexpression might be related to aggressive behavior and poor

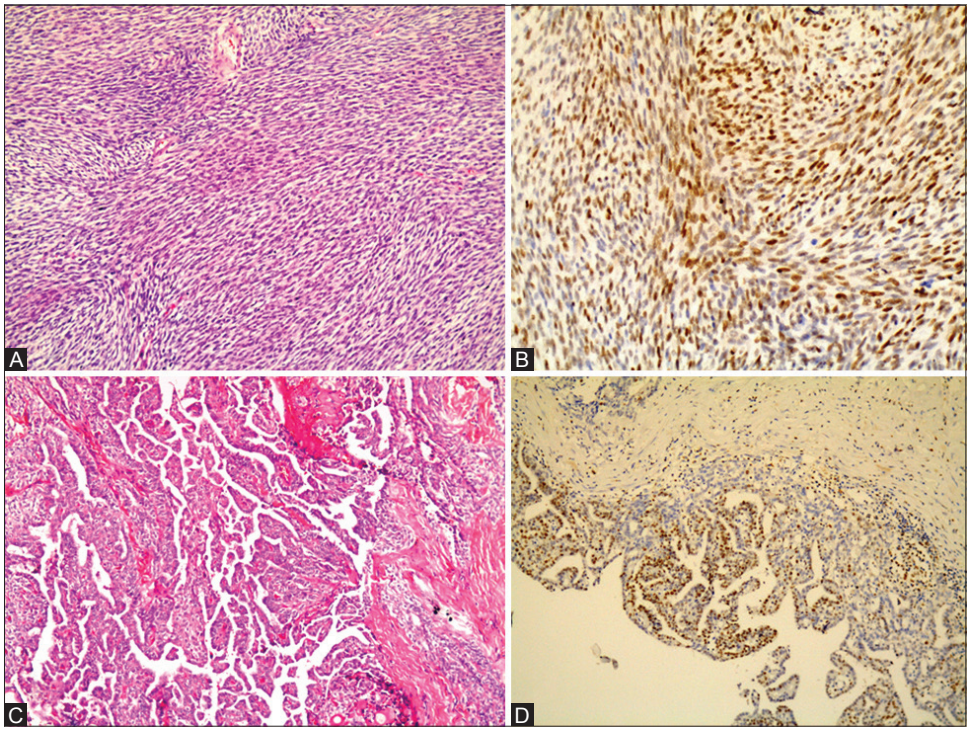

FIGURE 1. Enhancer of zeste homologue 2 (EZH2) expression in two histological types of synovial sarcoma (SS). (A) Monophasic fibrous SS consisting of relatively homogeneous spindle cells arranged in a fascicular pattern (H&E, ×100), (B) EZH2 immunostaining is strong in the same tumor (×200), (C) biphasic SS, (H&E, ×100), and (D) EZH2 immunostaining shows a moderate expression of EZH2 in the glandular epithelium (×100).

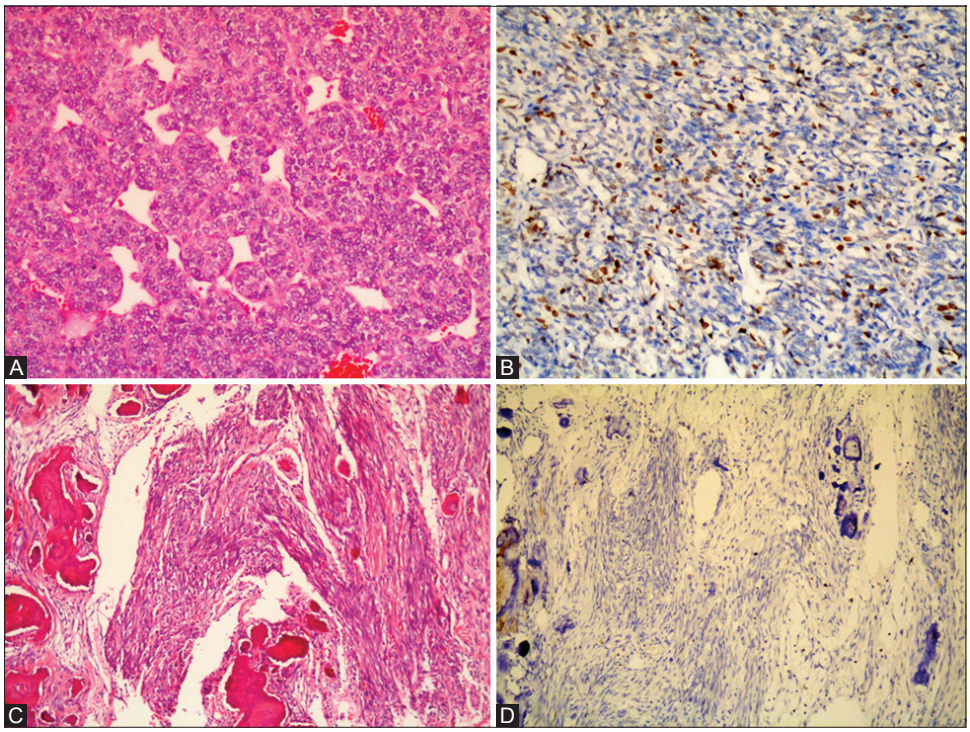

FIGURE 2. (A) Poorly differentiated synovial sarcoma (SS) with a prominent hemangiopericytoma-like vascular pattern (H&E, ×200), (B) in the same tumor, enhancer of zeste homologue 2 (EZH2) immunostaining is weak (×200), (C) monophasic fibrous SS with extensive tumor calcification (H&E, ×100), and (D) in the same tumor, EZH2 immunostaining is negative (×100).

prognosis in various carcinomas, lymphomas, and soft tissue sarcomas [13-25].

Different clinical and pathological factors have been suggested as indicators of SS prognosis. Recent studies including a large number of cases with long follow-up periods have shown that the advanced stage, high grade, male gender, age >40 years, tumor >5 cm in diameter, poor differentiation, as well as occurrence at the proximal sites along the extremities

TABLE 3. Median survival time in months according to the Kaplan-Meier analysis in relation to demographic and clinicopathological characteristics

Risk factor	Number of patients at risk (%)	Number of events (%)	Duration of survival in months	*p* value
Age				
<19	5 (17.20)	3 (60)	37	0.982
20-34	7 (24.10)	4 (57.10)	40	
>35	17 (58.60)	7 (41.20)	68	
Sex				
Male	10 (34.50)	7 (70)	60	0.192
Female	19 (65.50)	7 (36.80)	147	
Tumor location				
Periphery	22 (75.90)	11 (50)	68	0.386
Centrally	7 (24.10)	3 (42.90)	24	
Tumor size				
≤5 cm	8 (27.60)	1 (12.50)	81.17	0.022
>5 cm	21 (72.40)	13 (61.90)	66.04	
Histological type				
Monophasic	14 (48.30)	7 (50)	40	0.998
Biphasic	11 (37.90)	5 (45.50)	21.33	
Poorly differentiated	4 (13.80)	2 (50)	68	
Grade				
Grade 2	14 (48.30)	6 (42.90)	147	0.135
Grade 3	15 (51.70)	8 (53.30)	60	
Mitosis				
≤10	13 (44.80)	6 (46.20)	130	0.183
>10	16 (55.20)	8 (50)	60	
Necrosis				
Presence (n=15)	15 (51.70)	10 (66.70)	26	0.006
Absence (n=14)	14 (48.30)	4 (28.60)	130	
Local recurrence				
Presence	9 (31)	6 (66.70)	60	0.174
Absence	20 (69)	8 (40)	130	
Distant metastasis				
Presence	5 (17.20)	5 (100)	20	0.001
Absence	24 (82.80)	9 (37.50)	130	
EZH2 expression				
Low	14 (48.30)	2 (14.30)	82.75	0.017
High	15 (51.70)	12 (80)	60.01	
Chemo and radiation therapy				
Yes	22 (75.90)	10 (45.50)	147	0.511
No	7 (24.10)	4 (57.10)	60	

EZH2: Enhancer of zeste homologue 2

are associated with poor prognosis [4,5,9,26-28]. On the other hand, younger age (adolescence), small size, distal location, calcification, and thorough resection are indicators of good prognosis [3,9,29].

SS is aggressive, with a 50-70% tendency to recur locally or to metastasize to the lungs, bones, and sometimes to the lymph nodes [3,4,9]. In this study, we found that patients with the tumor size ≤5 cm (p = 0.022) and without distant metastasis (p = 0.001) had better survival. We also demonstrated that the patients with distant metastasis had a 3.59 risk factor for the total survival time. Although the t(X;18) translocation involving the *SSX2* gene has been correlated with better outcomes, no studies have confirmed that it is an independent prognostic factor for SS [3,27,28]. Moreover, necrosis, vascular invasion, high mitotic activity, and high Ki-67 proliferative index are accepted histopathological predictors of the shortened survival [9,13,27]. Our results showed that the patients without necrosis had a significantly higher survival time (p = 0.006).

In the last decade, several new immunohistochemical and molecular markers were investigated as indicators of SS prognosis and potential therapeutic targets [9]. To this end, we aimed to evaluate whether immunohistochemical EZH2 expression correlates with known prognostic indicators of SS.

A histological type may serve as a prognostic indicator for SS. Tumors with >20% of poorly differentiated histology show more aggressive behavior [5,8,9]. Similarly, Paulino [30] reported that biphasic histology is associated with a better clinical outcome more frequently than the monophasic histology; in addition, Krieg et al. [3] found that a histological

subtype can also serve as an independent prognostic factor for SS [3,30]. Changchien et al. [23] observed higher EZH2 expression in poorly differentiated SS and suggested that EZH2 expression might correlate with aggressive clinical behavior [23]. We found high EZH2 expression in 3 out of 4 cases of poorly differentiated SS, but there was no statistically significant relationship between EZH2 expression and the histological subtype.

Although some studies suggest that gender does not correlate with prognosis in SS, other authors report that male gender is associated with a worse prognosis [3,31,32]. In this study, patients with low EZH2 expression were predominantly female (p = 0.050), which is consistent with the previous studies.

Similar to other soft tissue sarcomas, SS tends to have a better prognosis in younger patients [12,26,30,31]. A study from a single institution on 271 cases of SS found a significant correlation between advanced age and poor prognosis [26]. Similarly, another study on 121 SS cases reported that the age over 25 years was correlated with the lower disease-free survival [9]. Furthermore, SS located in the extremities is reported to have a better prognosis than the tumor in the head-neck region [3,30,33,34]. For example, in children and adolescents SS in the extremities had a better prognosis than the tumors in other locations, and it was suggested that the treatment should be planned accordingly [34,35]. In our study, no significant relationship was observed between the age, tumor location, and EZH2 expression.

The standard treatment for SS is wide resection of the tumor and surrounding tissue. SS recurs in 70-83% of cases with inadequate marginal resection. Recurrence may increase the risk of metastasis, and thus negatively influence the prognosis. Aggressive surgery accompanied by chemo or radiation therapy increases disease-free survival [3,4,26,33-35]. We found no statistically significant relationship between EZH2 expression and treatment modality and local recurrence, and we demonstrated a significant correlation between high EZH2 expression and distant metastasis (p = 0.018).

In most studies of carcinoma, lymphoma, and soft-tissue sarcomas, EZH2 overexpression was an indicator of poor prognosis [13-15,19-25]. Similarly, we observed that low EZH2 expression was associated with prolonged survival (p = 0.017).

Generally accepted indicators of prognosis in SS include tumor stage at presentation, tumor size, and FNCLCC tumor grade [5,27,28]. The tumor size >5 cm is strongly associated with poor prognosis [3,5,9,28,33], as well as the presence of distant metastasis at diagnosis and FNCLCC grade 3 [3,27,28]. Changchien et al. [23] reported that tumors overexpressing EZH2 were also >5 cm in diameter and had distant metastases, suggesting that EZH2 overexpression is associated with greater tumor size, presence of distant metastasis, and poor prognosis [23]. We also found a significant correlation between high EZH2 expression and tumor size >5 cm (p = 0.014), and the presence of distant metastasis (p = 0.018).

Tumor necrosis and high mitotic counts (>10 mitosis/10 HPFs) have been reported as indicators of a poor prognosis in SS [5,9,27]. We found no significant relationship between EZH2 expression and mitosis, but there was a significant correlation between high EZH2 expression and the presence of necrosis (p = 0.016).

CONCLUSION

Our findings suggest that patients with the tumor size ≤5 cm, without necrosis, without distant metastasis, and with low EZH2 expression had a significantly longer survival time. In addition, patients with distant metastasis had a 3.59 risk factor for the total survival time. Furthermore, we demonstrated a significant correlation between high EZH2 expression and tumor size >5 cm, necrosis, and distant metastasis. This is consistent with the previous studies suggesting that EZH2 overexpression is an indicator of poor prognosis in SS. Our findings warrant the confirmation by future studies with larger cohorts to determine whether EZH2 expression is an indicator of prognosis in SS.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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