Original Article
The prognostic and predictive values of SPARC, E-cadherin, and EZH2 in endometrial carcinoma

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Abstract: Endometrial carcinoma (EC) is one of the most common malignancies among gynecological tumors. Currently, sensitive and effective biomarkers for the clinical diagnosis, treatment, and efficacy evaluation of EC are urgently needed. Among the most promising cancer prognostic biomarkers, SPARC, E-cadherin, and EZH2 play crucial roles in the tumorigenesis and progression of different cancer types. However, the roles of SPARC, E-cadherin, and EZH2 in EC are still unclear. Here, quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC) were performed to measure the SPARC, E-cadherin, and EZH2 expressions in EC tissues and in normal adjacent proliferative phase endometrium (PPE) tissues. The IHC staining results on the tissue microarrays (TMAs) with EC tissues and their clinical data were used to statistically analyze the prognostic roles of SPARC, E-cadherin, and EZH2. We found that the relative mRNA and protein levels of SPARC and E-cadherin were strongly downregulated, but EZH2 was markedly upregulated in EC compared with the PPE tissues. The abnormal SPARC, E-cadherin, and EZH2 expressions were correlated with the FIGO stage, grade, and poor clinical outcomes. In addition, we also found that a decreased expression of SPARC was related to histological type and lymph node metastasis, but decreased expression of E-cadherin was related to lymph node metastasis. Moreover, the SPARC expression was shown to be positively associated with E-cadherin, but EZH2 was negatively associated with SPARC or E-cadherin. Taken together, our findings improve the understanding of the SPARC, E-cadherin, and EZH2 expression levels in EC and suggest novel prognostic biomarkers for EC.

Keywords: SPARC, E-cadherin, EZH2, endometrial carcinoma

Introduction

Endometrial carcinoma (EC) is one of the most prevalent gynecological malignancies among women [1]. The clinical incidence of EC has generally maintained a steady increase worldwide [1, 2]. However, the etiology and nosogenesis of EC are multifactorial and not yet fully understood. At present, the diagnosis and prognosis of EC are mainly based on a histopathological examination of the endometrium, but its application has limitations. Thus, novel biomarkers with prognostic value are urgently needed.

Secreted protein, acidic and rich in cysteine (SPARC) is also known as osteonectin or basement membrane-40 protein (BM-40). It is

expressed at different levels in human chondrocytes, vascular smooth muscle cells, placental trophoblastic cells, and macrophages. SPARC has been shown to be highly expressed in some malignant tumor cells or fibroblasts and endothelial cells associated with tissue repair; meanwhile SPARC exhibits low expressions in other malignant tumors [3, 4]. For example, SPARC is highly expressed in gastric cancer [5], pancreatic cancer [6], cervical cancer [7], but it is lowly or not expressed in T-cell lymphoma [8] and prostate cancer [9]. E-cadherin is an important adhesive molecule that can maintain the stability of intercellular adhesion. Currently, E-cadherin has been confirmed to be a molecule that inhibits tumor adhesion. Studies have shown that E-cadherin is downregulated in gas-

tric cancer [10] and bone and soft tissue sarcomas [11] and the downregulated expression of E-cadherin is closely related to the occurrence, invasion, and metastasis of malignant tumors [12]. Enhancer of zester homolog 2 (EZH2) is considered to be a candidate oncogene. EZH2 expression is high in many human tumors and also highly associated with the occurrence and development of malignant tumors. For instance, EZH2 has been found to be significantly upregulated in glioma [13] and breast cancer [14]. Highly expressed EZH2 was also found to be a significant factor for poor prognosis [15].

So far, there are few studies on the joint expressions of SPARC, E-cadherin, and EZH2 in EC. Thus, we analyzed the expressions and correlations of SPARC, E-cadherin, and EZH2 in EC, as well as their relationship with EC's clinicopathological parameters and prognosis. Our findings indicate that SPARC/E-cadherin/EZH2 can predict biological behavior, providing theoretical and experimental bases for the clinical diagnosis, treatment, and evaluation of EC.

Materials and methods

Clinical tissue samples

All human tissue samples were obtained from randomly selected EC patients during surgery. For the mRNA expression level determinations, the samples were collected from January 2016 to January 2017 and immediately stored in RNA*later* (Ambion) at the Department of Obstetrics and Gynecology, Gansu Province Hospital Rehabilitation Center. The diagnoses of all the EC patients were confirmed through pathological sections.

For the immunohistochemical (IHC) analysis, 130 cases of EC tissue samples were randomly collected during surgery from May 2008 to May 2010 and embedded in formalin fixation. None of the 130 cases of EC patients included in this study received any prior chemotherapy or radiation therapy. All the patients were diagnosed with EC through cytology or pathology. All their clinical data were available and reviewed. The follow-up period was 108 months. The follow-up was updated by a review of the patients' records, telephone calls, and text messages. All the patients were followed up from their admission to their death, loss of fol-

low-up, or follow-up until May 2019 (14 of the 130 EC patients were lost during the follow-up). The date of death or relapse was used to calculate the overall survival (OS) or the disease-free survival (DFS). Informed consents were signed by all the participants upon full notification. Meanwhile, the whole research protocol was approved by the Joint Ethics Committee of the Gansu Province Hospital Rehabilitation Center and carried out in accordance with the ethical standards set in the Helsinki declaration.

All of the EC patients' clinical data, such as age, menopause, histological type, FIGO stage, lymph node metastasis, grade, ER status, vascular invasion, PR status, P53 status, and Ki-67 status were available and reviewed. All the EC patients were grouped according to their clinical features as shown in **Table 1**.

Tissue microarray (TMA) construction

A representative portion of the EC tissue samples used for the TMA preparation were identified by three independent experienced pathologists in our hospital. The TMA block was designed by TMA Designer® software and then constructed at a MiniCore Control Station (Tissue Arrayer MINICORE3, Alphelys, France). The core tissue biopsies (1.0 mm) were used to construct the TMA blocks. The tissue samples were taken from the 130 paraffin-embedded tissue blocks to construct a new recipient block. The new recipient block was used to make tissue slides which were then stained with hematoxylin and eosin [16].

IHC analysis and scoring system

We used 90% methanol and 3% H_2O_2 solution to block the endogenous peroxidase of the tissue slides (10 min, T = room temperature) after the deparaffinization and rehydration. Then, a sodium citrate buffer was used to retrieve the antigen. After blocking using BSA, an antibody against SPARC/E-cadherin/EZH2 (overnight, T = 4°C, CST, USA), a biotinylated secondary antibody (10 min, T = room temperature, CST, USA), and HRP-Streptavidin (10 min) were used to incubate the slides.

After the DAB staining, the protein expression results of SPARC/E-cadherin/EZH2 were graded by their intensities. The final score (0-3) for

Table 1. The correlations between the SPARC/E-cadherin/EZH2 expressions and the clinicopathologic features in 130 patients with EC

Characteristics	Total (n = 130)	SPARC High/Low (67/63)	P value	E-cadherin High/Low (70/60)	P value	EZH2 High/Low (83/47)	P value
Age (years)			0.232		0.223		0.093
< 45	12	4/8		4/8		5/7	
≥ 45	118	63/55		66/52		78/40	
Menopause			0.754		0.120		0.084
Yes	87	44/43		51/36		60/27	
No	43	23/20		19/24		23/20	
Histological type			0.046*		0.760		0.377
Endometrioid	116	56/60		63/53		72/44	
Non-endometrioid	14	11/3		7/7		11/3	
FIGO stage			0.027*		0.003*		0.002*
I-II	119	65/54		69/50		81/38	
III-IV	11	2/9		1/10		2/9	
Grade			0.030*		0.018*		0.001*
G1-G2	112	62/50		54/58		78/34	
G3	18	5/13		16/2		5/13	
Vascular invasion			1.000		0.725		0.710
Yes	8	4/4		5/3		6/2	
No	122	63/59		65/57		77/45	
LNM			0.025*		0.019*		0.351
Yes	5	0/5		0/5		2/3	
No	125	67/58		70/55		81/44	
ER status			0.175		0.889		0.076
Positive	81	38/43		44/37		47/34	
Negative	49	29/20		26/23		36/13	
PR status			0.736		0.177		0.161
Positive	20	11/9		8/12		10/10	
Negative	110	56/54		62/48		73/37	
Ki-67 status			0.319		0.501		0.100
Positive	48	22/26		24/24		35/13	
Negative	82	45/37		46/36		48/34	
P53 status			0.681		0.055		0.580
Positive	95	50/45		56/39		62/33	
Negative	35	17/18		14/21		21/14	

*means statistically significant ($P < 0.05$), LNM: lymph node metastasis.

the SPARC/E-cadherin/EZH2 expression was equal to the score of intensity (0-3) multiplied by the positive cells' percentage, which were assessed by three independent pathologists. The SPARC expression was recognized as high expression when the final score was not less than 1.33 (median score), and a case was recognized as low expression when the final score was between 0 and 1.33. The E-cadherin and EZH2 were considered to be high expression when the final scores were not less than 1.42 or 1.59, respectively.

RNA isolation from EC and PPE tissues

We isolated total RNAs from the lysed EC and PPE tissue samples with TRIzol reagent (Invitrogen, USA). Then, the mRNA was separated from the total RNAs (Invitrogen). We stored all the tissue samples at -80°C. The

operation steps were consistent with the manufacturer's instructions.

Quantitative real-time PCR analysis (qRT-PCR)

The relative SPARC, E-cadherin, and EZH2 mRNA expression levels were determined using 35 pairs of EC and PPE tissue samples from the EC patients. Total RNAs were extracted and used for the qRT-PCR reactions (SYBR Green-containing PCR kit, Invitrogen, USA) in triplicate. The relative expression ratios of the target genes were generated based on the comparative CT method ($2^{-\Delta\Delta Ct}$ method).

Statistical analysis

SPSS 19.0 software (IBM, USA) was used for all the statistical calculations. All the data are presented as the mean ± SE. Student's t-tests and χ^2 tests were used to evaluate the differences between groups. The Kaplan-Meier method and log-rank tests were carried out to plot the overall survival (OS) and disease-free survival (DFS) curves. The results were considered statistically significant when P < 0.05.

Results

The SPARC/E-cadherin/EZH2 expressions were altered in endometrial carcinoma

The SPARC, E-cadherin, and EZH2 expressions in the 35 pairs of EC tissues and adjacent PPE tissues were determined using qRT-PCR. We found that the relative SPARC and E-cadherin mRNA expressions in the EC tissues were markedly lower than they were in the PPE tissues, but the relative EZH2 mRNA expressions in the EC tissues were much higher than they were in the PPE tissues (**Figure 1A**).

Subsequently, we examined the SPARC, E-cadherin, and EZH2 expressions using IHC. The cytoplasmic staining of SPARC was more frequently observed in the PPE cells than it was in the EC cells. Both the plasma membrane and cytoplasmic staining of E-cadherin was also more frequently observed in the PPE cells than in the EC cells. In addition, the nuclear staining of EZH2 was more frequently observed in the EC cells than in the PPE cells as well. Representative IHC images of SPARC, E-cadherin, and EZH2 under a microscope are shown (**Figure 1B**, 200×). Among the 35 EC tissues, approximately 28.57% (P < 0.01, 10 of 35),

25.71% (P < 0.01, 9 of 35), and 74.29% (P < 0.01, 26 of 35) of the EC samples showed significantly positive expressions of SPARC, E-cadherin, and EZH2, respectively (**Figure 1B**). Our data indicated that the downregulation of SPARC/E-cadherin and the upregulation of EZH2 were frequent events in the EC tissues. This could be related to EC carcinogenesis.

The SPARC/E-cadherin/EZH2 expressions and the FIGO stage/grade were related

The TMAs of the other 130 EC samples were used for the IHC analysis. All 130 cases of the EC samples were classified into low or high expression groups according to the SPARC/E-cadherin/EZH2 expressions cutoff of the median score. Chi-square tests were performed to analyze the correlations between the altered SPARC/E-cadherin/EZH2 protein expressions and the clinicopathologic parameters. The clinicopathologic parameters included age, menopause, histological type, FIGO stage, lymph node metastasis, vascular invasion, ER status, grade, PR status, P53 status, and Ki-67 status. The results indicated that all the SPARC/E-cadherin/EZH2 expressions in the EC tissues were closely correlated with FIGO stage (P = 0.027, 0.003 and 0.002, respectively) and grade (P = 0.030, 0.018 and 0.001, respectively). Additionally, the SPARC expressions in the EC tissues were closely correlated with histological type and lymph node metastasis (P = 0.046 and P = 0.025, respectively). The E-cadherin expressions in the EC tissues were also closely associated with lymph node metastasis (P = 0.019). There was no significant correlation between the SPARC/E-cadherin/EZH2 expressions and age, menopause, ER status, vascular invasion, PR status, P53 status, or Ki-67 status (**Table 1**, all P > 0.05).

Next, we determined the correlations between the SPARC, E-cadherin, and EZH2 expression using a Pearson correlation analysis. The correlation coefficient was 0.465 (linear R^2 = 0.258, P < 0.001) between SPARC and E-cadherin, -0.544 (linear R^2 = 0.219, P < 0.001) between E-cadherin and EZH2, and -0.519 (linear R^2 = 0.239, P < 0.001) between SPARC and EZH2, respectively (**Figure 2**). Our data indicated that the SPARC expression was positively associated with E-cadherin, but EZH2 was negatively associated with SPARC or E-cadherin, respectively (2-tailed).
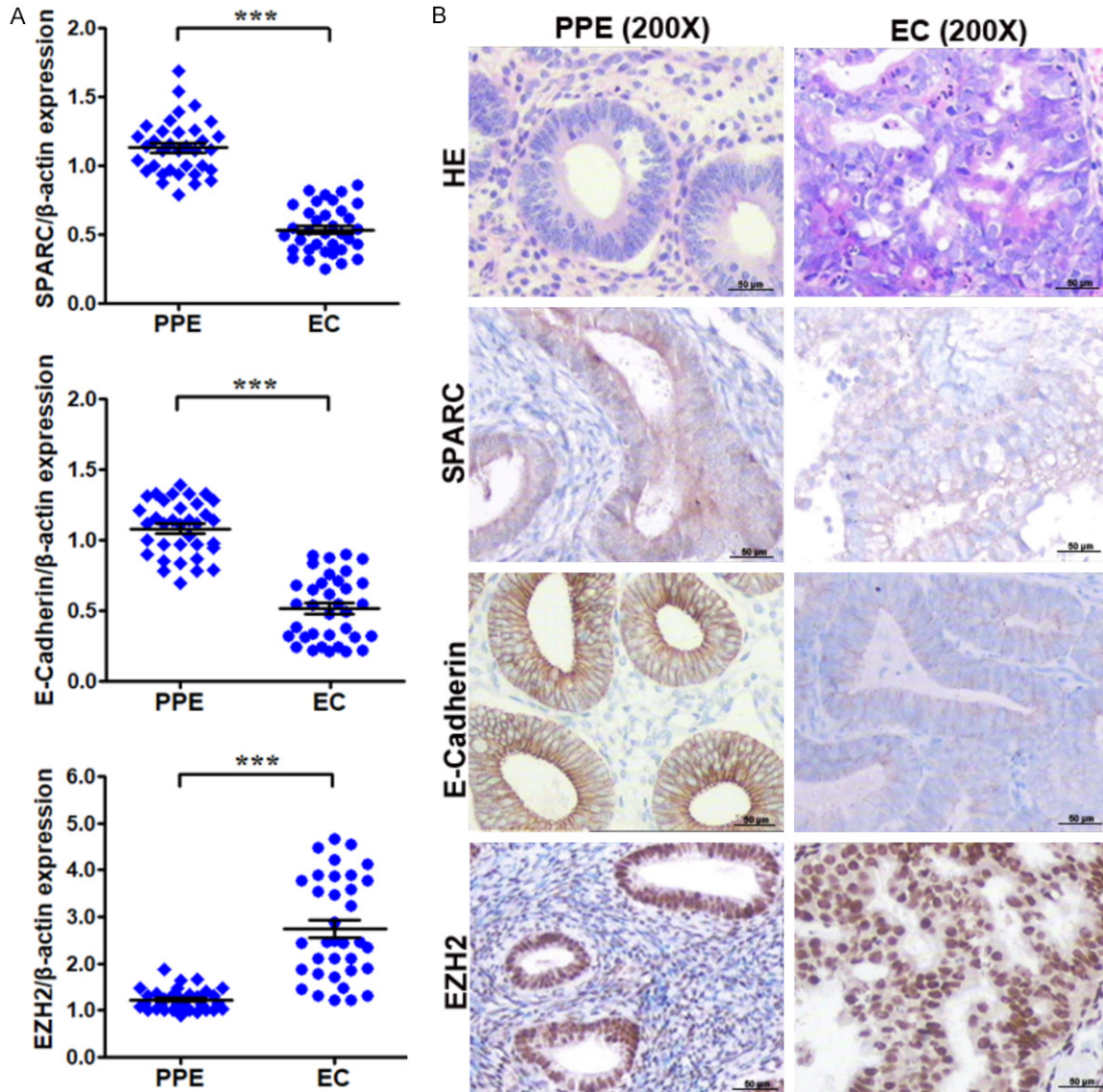
Figure 1. The SPARC/E-cadherin/EZH2 expression was altered in endometrial carcinoma. A: The expression levels of SPARC/E-cadherin/EZH2 in the 35 pairs of freshly frozen EC tissues and adjacent PPE tissues were determined using qRT-PCR analysis. The results are shown as the mean ± SE. *** P < 0.01. B: IHC microscopy (200×) reveals the cellular location of the SPARC/E-cadherin/EZH2 protein. SPARC is primarily observed in the cytoplasms (brown). E-cadherin is primarily observed in the plasma membranes and cytoplasms (brown). EZH2 is primarily observed in the nuclei (brown), respectively.

These results demonstrated that alternative SPARC/E-cadherin/EZH2 expressions contribute to the occurrence and progression of EC.

SPARC/E-cadherin/EZH2 expression and poor clinical outcomes were related

The results above indicated that SPARC/E-cadherin/EZH2 contributes to the pathogenesis of EC. Thus, we subsequently explored the prognostic function of SPARC/E-cadherin/EZH2. A

Kaplan-Meier survival analysis was carried out to generate the overall survival (OS) and disease-free survival (DFS) curves of the EC patients. As shown in **Figure 3A** and **3B**, the EC patients had a shorter mean OS (P < 0.05) and DFS (P < 0.001) with lower SPARC or E-cadherin expressions than higher expressions. But the EC patients had a shorter mean OS (P < 0.001) and DFS (P < 0.001) with a higher EZH2 expression level than lower expression.
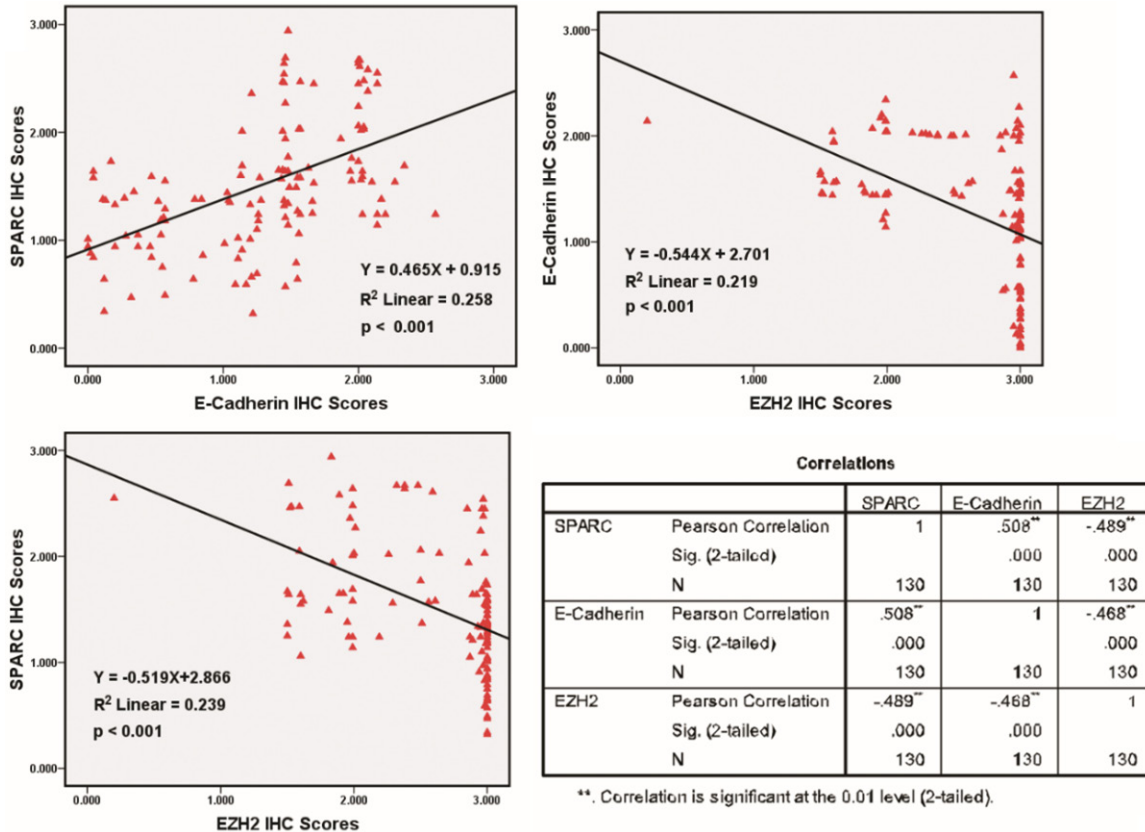
Figure 2. The SPARC/E-cadherin/EZH2 expressions and FIGO stages/grades were related. The correlations between the SPARC, E-cadherin, and EZH2 expression levels were analyzed using a Pearson correlation analysis. The linear equations were Y = 0.456X + 0.915 (linear R^2 = 0.258, P < 0.001) between SPARC and E-cadherin, Y = -0.544X + 2.701 (linear R^2 = 0.219, P < 0.001) between E-cadherin and EZH2, and Y = -0.519X + 2.866 (linear R^2 = 0.239, P < 0.001) between SPARC and EZH2, respectively.

These results demonstrated that the SPARC/E-cadherin/EZH2 expressions were strongly associated with patient OS and DFS. SPARC/E-cadherin/EZH2 may be a potential prognostic biomarker for EC.

Discussion

EC is one of the most prevalent types of malignant gynecological tumors. Its incidence increases yearly, but its pathogenesis has not been well defined [17]. Due to the advancements in genetics and molecular biology, much evidence has shown that the activation of protooncogenes or the inactivation of tumor suppressor genes are closely related to the occurrence and progression of tumors, during which many molecular biomarkers indicating the prognosis of malignant tumors have been found [18]. These molecular biomarkers have led to further breakthroughs in the early diagnosis

and gene-targeted therapy of cancer. Researchers expect a reliable assessment of the risk factors for EC to determine whether patients should be treated surgically or conservatively and to guide the early and accurate diagnosis and prognostic assessment of EC patients.

In this study, SPARC was found to be downregulated in EC tissues. SPARC frequently exhibits a low expression or a deletion in a variety of malignant tumors, including T-cell lymphoma, prostate cancer, and EC [8, 9, 19]. This phenomenon suggests that SPARC may function as an inhibitor in the formation of tumors. SPARC is a common target for aberrant methylation in EC. This may be the main reason why SPARC is downregulated in EC [20]. In our study, a lower SPARC expression was found to be closely related to histological type, lymph node metastasis, FIGO stage, shorter OS and DFS, and grade in EC tissues. It has been reported
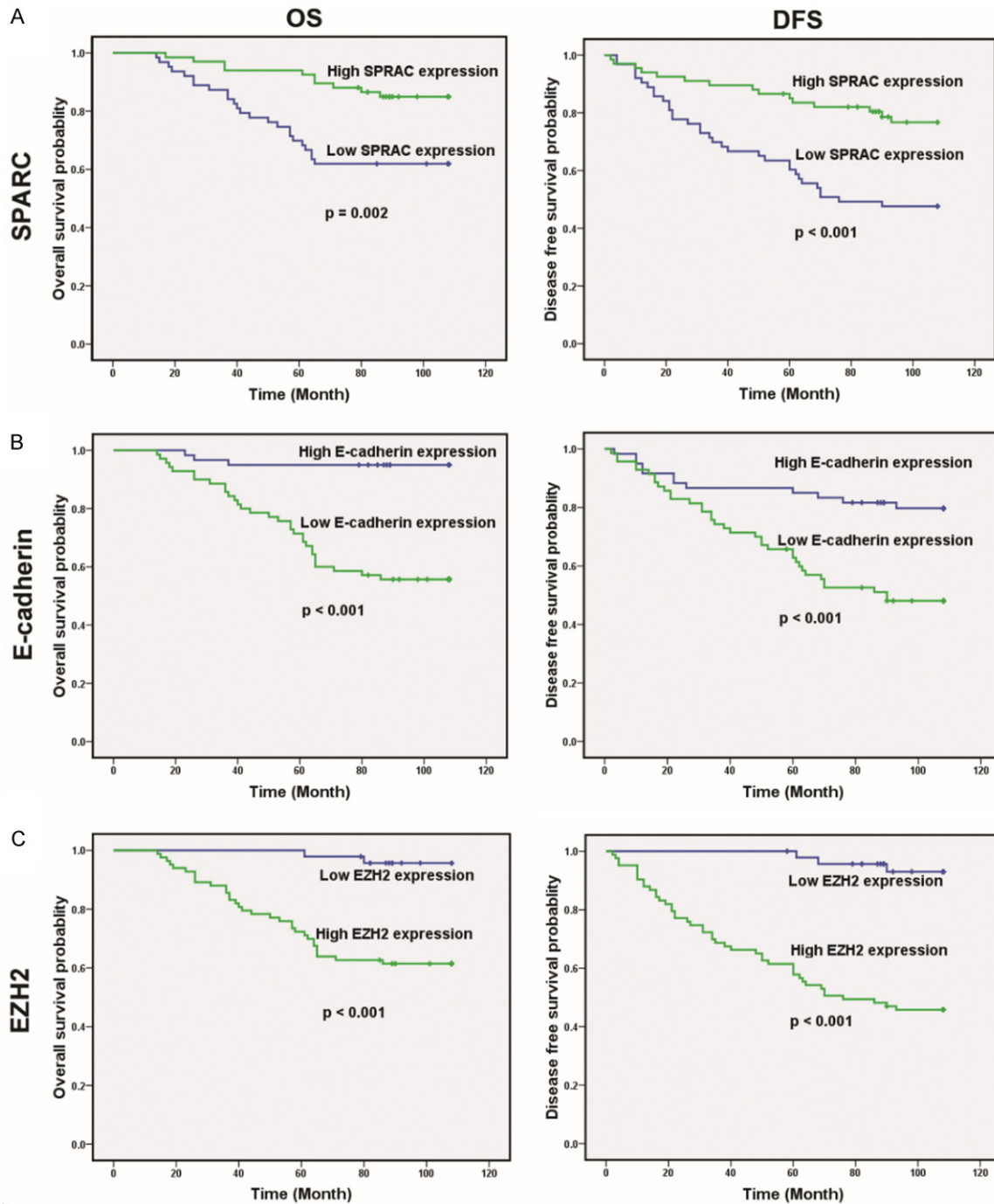
Figure 3. The SPARC/E-cadherin/EZH2 expressions and poor clinical outcomes were related. A: OS and DFS curves for the 130 EC patients with low or high SPARC expressions. The prognostic significance of the SPARC expression was analyzed for the EC patients using Kaplan-Meier and log-rank analyses. A lower level of SPARC expression and shorter OS and DFS were markedly related. B: Lower levels of E-cadherin expression and shorter OS and DFS were strongly related. C: Higher levels of EZH2 expressions and shorter OS and DFS were markedly related. The results are shown as the mean ± SE, P < 0.05.

that the regulation of cell adhesion, the cell cycle, angiogenesis, tissue differentiation, and embryonic development are the main physio-

logical functions of SPARC [21, 22]. SPARC shows aberrant expression, which is related to the clinical staging and histological grading of

endometrial cancer [19]. Although SPARC expression varies from cancer to cancer, SPARC is actually correlated with the occurrence, development, and prognosis of endometrial cancer [19].

Similarly, E-cadherin was also downregulated and closely correlated with FIGO stage, lymph node metastasis, grade, and prognosis in EC tissues in this study. It is known that E-cadherin is one of the important molecules that mediates cell adhesion and is the major intercellular adhesion protein in epithelial cells [23]. By inhibiting the production of matrix metalloproteinases by tumor cells and host cells, E-cadherin inhibits the degradation of various protein components in the matrix and basement membrane surrounding tumor cells [24]. E-cadherin maintains the stability and polarity of cell morphology by promoting intercellular adhesion. Therefore, when E-cadherin is downregulated in malignant tumors, it will cause adhesion between tumor cells, resulting in tumor progression. The stability between cells is affected and becomes more susceptible to invasion and metastasis [25]. The E-cadherin gene has hypermethylation in the promoter region in EC tissues, and it was found that the deepening of myometrial invasion and lymph node metastasis of tumor cells show an upward trend, but its expression shows a tendency to decline or even disappear. This suggests that E-cadherin is downregulated in EC, and its gene promoter hypermethylation is highly associated with tumor invasion, metastasis and prognosis in EC [26, 27]. Our results are consistent with the existing research.

In addition, we also found that EZH2 is significantly upregulated and closely related to FIGO stage, grade, and prognosis in EC tissues. EZH2 expression has been found to be upregulated in many EC cells and tissues [28], and EZH2 is also positively correlated with the histological grade, TNM stage, lymph node metastasis, and depth of tumor invasion, factors which could be used as new indicators to assess the poor prognosis of tumors in EC [29]. These results show that EZH2 expression is markedly different in EC tissues from the corresponding expression in the adjacent tissues. EZH2 is now considered to be a new protooncogene since it is highly expressed in EC. EZH2 is also believed to promote EC development and is closely related to its prognosis [30, 31].

This study also showed a strong association between the SPARC, E-cadherin, and EZH2 protein expressions in EC. The SPARC expression was positively related to E-cadherin, but EZH2 was negatively related with SPARC or E-cadherin. As reported, SPARC is mainly involved in tumor invasion and metastasis, affecting cell adhesion, regulating cell proliferation, and regulating tumor angiogenesis [32, 33]. E-cadherin is considered to be an intercellular adhesion molecule whose main function is to promote intercellular adhesion and maintain the stability of epithelial cell morphology in EC [34-37]. The EZH2 protein has a role in regulating cell proliferation, invasion, and abrogated cancer stem cell-like properties in EC [30]. EZH2 can inhibit the progression of the cell cycle from the G1 to the S phase and cell proliferation by downregulating E2F1 and MMP9 in EC cells [38]. Therefore, a low expression of SPARC in EC may down-regulate E-cadherin, and low expressions of SPARC and E-cadherin in EC may up-regulate EZH2. Thus, it is speculated that SPARC, E-cadherin, and EZH2 expressions and the biological behavior of EC malignancy are related, and there is a correlation between their expression levels. SPARC, E-cadherin, and EZH2 have their independent roles in the occurrence, development, and prognosis of EC. They may exert synergistic inhibition effects on each other.

Tumor formation is subject to the synergistic influence of multiple oncogenes and suppressor genes. It is necessary to jointly quantify multiple genes to assess the occurrence, development, and prognosis of cancer [39]. Therefore, the early detection of the SPARC, E-cadherin, and EZH2 expressions in EC patients plays an important role in the disease's diagnosis, treatment, and prognosis. It could improve the survival rate of patients with malignant EC.

Disclosure of conflict of interest

None.

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