Clinical Significance of Lymphatic Infiltration Detected by Immunohistochemical Double Staining in Patients with Endometrial Cancer

Clinical Medicine Insights: Oncology
Volume 17: 1–7

DOI: 10.1177/11795549231152308

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ABSTRACT

BACKGROUND: The presence of lymph-vascular space invasion is a powerful predictor of lymph node metastasis. However, most studies do not distinguish lymph vessel invasion (LVI) and blood vessel invasion (BVI). The aim of this study was to distinguish the role of LVI and BVI in lymphatic metastasis and recurrence in patients with endometrial cancer.

METHODS: We examined 171 patients with endometrial cancer. Immunohistochemical double staining was used to distinguish lymphatic invasion and vascular invasion. First, the relationship between lymphatic/vascular invasion and clinicopathological features and lymphatic metastasis was studied. Then, the expression of D2-40/LVI and CD31/BVI in patients with recurrence was analyzed.

RESULTS: Pathological grading (G3) and D2-40/LVI were independent high-risk factors for lymph node metastasis of endometrial cancer. The area under the receiver operating characteristic curve values for predicting lymphatic metastasis using pathological grading (G3) or D2-40/LVI alone were .642 and .680, respectively, and the area under the curve value for the combined detection of pathological grading (G3) and D2-40/LVI was .726, which was greater than the values obtained for the abovementioned independent variables. Among the 15 recurrent patients, 5 (33.3%) were D2-40/LVI positive, 2 (13.3%) were CD31/BVI positive, and 8 (53.3%) were both D2-40/LVI and CD31/BVI positive.

CONCLUSION: D2-40/LVI combined with G3 can effectively predict lymph node metastasis of endometrial carcinoma.

KEYWORDS: blood vessel invasion, CD31, D2-40, endometrial carcinoma, lymphatic metastasis, lymphatic vessel invasion

RECEIVED: May 16, 2022. **ACCEPTED:** January 5, 2023.

TYPE: Original Research Article

FUNDING: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Science and Technology Project of Tianjin Health Committee (project no: ZC20144).

DECLARATION OF CONFLICTING INTEREST: The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: All authors have completed the ICMJE uniform disclosure form. The authors declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence their work. There is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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Introduction

Endometrial cancer (EC) is one of the most common gynecological cancers, which accounts for approximately 20% mortality rate worldwide. Recently, the incidence of EC has shown an upward trend with each passing year.[1] In 2020, there were 81 964 new cases of EC and 16 607 deaths in China.[2] Most of ECs were diagnosed early (80% in stage I), and the 5-year survival rate was about 90%. However, if there is lymph node metastasis, the 5-year survival rate is much lower (60%).[3]

The main treatment of EC is surgery and chemoradiotherapy. In recent years, with the development of clinical research, targeted therapy and immunotherapy have also shown good efficacy in advanced, recurrent, and metastatic EC. According to the National Cancer Comprehensive Network (NCCN), the conventional surgical methods are total hysterectomy and bilateral salpingo-oophorectomy, with or without pelvic and para-aortic lymphadenectomy. However, lymphadenectomy for Type I EC patients still remains controversial, both in the early stage and at later stages. Some studies believe that patients in the early stages of EC should undergo surgical staging.[4] However, other studies disagree with the effect of lymphadenectomy in patients with EC because it does not prolong disease-free survival and overall survival.[5,6] Therefore, it is necessary to predict which patients will benefit from lymphadenectomy and avoid overtreatment that leads to many complications.[7] Evidence of serious adverse events suggests that women undergoing lymphadenectomy are more prone to lymphedema/lymphocyst formation and surgery-related systemic complications.[8-10] One of the challenges of EC is to identify specific factors for predicting lymph node metastasis, so as to optimally identify the patient cohort who should undergo lymphadenectomy.

In addition, the choice of chemotherapy and/or radiotherapy and the optimal treatment sequence are also another controversial topic for patients with high-risk factors who need supplementary treatment after surgery. Pelvic radiotherapy has

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always been the standard adjuvant treatment to reduce the risk of pelvic recurrence, while systemic chemotherapy can reduce the risk of distant metastasis and improve the survival rate.

Lymphangiogenesis and angiogenesis are essential for tumor growth, invasion, metastasis, and diffusion, which can be evaluated based on lymph vessel invasion (LVI) and blood vessel invasion (BVI) detected using specific markers. Previous studies on EC have shown that the presence of lymph-vascular space invasion (LVSI) is a powerful predictor of lymph node metastasis, recurrence, and cancer-specific death. However, most tissue-based studies do not distinguish between vascular and lymphatic systems. Traditional hematoxylin and eosin (H&E) staining cannot accurately distinguish the gap around cancer cells from the real tumor thrombus in the process of tissue treatment,[11] and there may be over diagnosis or missed diagnosis. In recent years, immunohistochemical CD31/D2-40 is often used to label vascular and lymphatic endothelial cells. However, a single immunohistochemical marker of CD31 or D2-40 can only prove the presence of neovascularization or lymphatic vessels but cannot determine whether there is cancer cell infiltration in the lumen. Immunohistochemical cocktail double staining is a method that can label 2 different antigens in tissues at the same time. It can double stain vascular endothelial cells and cancer cells at the same time, which can significantly improve the accuracy of pathological diagnosis.

The purpose of this study is to differentiate lymphatic invasion and vascular invasion using immunohistochemical cocktail double staining and investigate the relationship between lymphatic and vascular infiltration and clinicopathological features and lymphatic metastasis in patients with EC.

Materials and Methods

Patients

A total of 435 patients with EC were diagnosed by diagnostic curettage pathology in Tianjin Central obstetrics and gynecology hospital, Tianjin, China, from January 2018 to December 2019. A total of 181 patients underwent total hysterectomy and bilateral salpingo-oophorectomy with complete systematic lymph node dissection. And 171 patients met the inclusion criteria and were included in the study (Figure 1).

Inclusion criteria were as follows: (1) patients in whom EC was confirmed by pathological analysis after diagnostic curettage or hysteroscopic-assisted diagnostic curettage; (2) the patients who were subjected to laparotomy or laparoscopic hysterectomy and bilateral salpingo-oophorectomy with complete systematic lymph node dissection (pelvic and/or abdominal para-aortic lymphadenectomy); (3) patients in whom EC was confirmed by postoperative pathology; (4) patients who did not receive radiotherapy and chemotherapy, high-dose hormone therapy, or any other treatments before surgery; (5) patients with complete clinical and pathological data; (6) patients with no history of other malignant tumors.

Contrastingly, the following were the exclusion criteria: (1) patients who were initially treated with total hysterectomy, for whom pathological indication of EC was suggested after surgery, and the second surgery was performed in stages; (2) patients having a history of other malignant tumors; (3) patients who had incomplete clinical and pathological data; (4) patients who received radiotherapy and chemotherapy, high-dose hormone therapy, or other treatment methods before surgery; and (5) patients who had a combination of other serious diseases, which made it difficult for them to tolerate the full-stage operation.

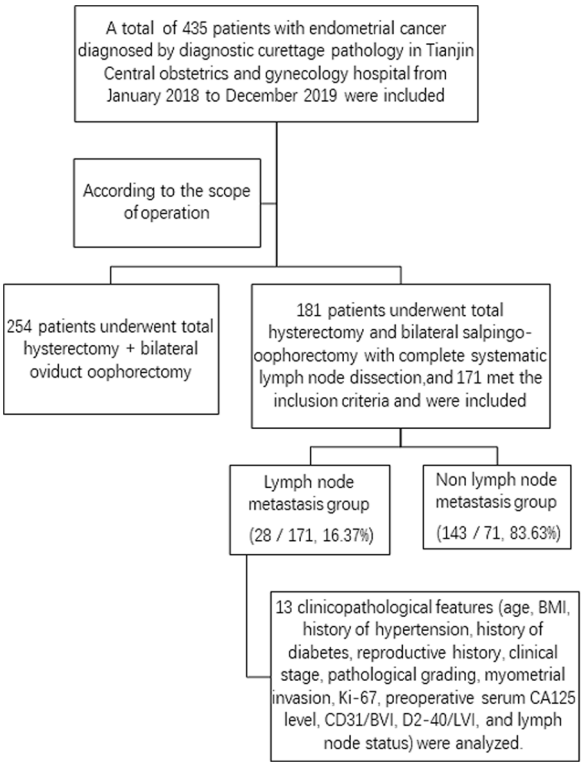

Figure 1. Flow chart of patient recruitment.

Tissue samples

Paraffin-embedded tissue samples were collected from the pathology department. All immunohistochemically stained slides were re-reviewed by a gynecological pathologist to confirm the diagnosis, surgical stage, histological grade, histological type, CD31/BVI, and D2-40/LVI. Clinical staging was determined according to International Federation of Gynecology and Obstetrics (FIGO) criteria.[12] Histological grade was performed according to the World Health Organization (WHO) system in well-differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3) carcinomas.

Ethics approval and consent

This study was performed according to the international ethical guidelines for biomedical research involving human beings (CIOMS) and was approved by the Institutional Medical Ethics Committee of Tianjin Central Hospital of Obstetrics

and Gynecology (ethics no: 2020KY008); the clinical study informed consent forms were signed by the patients.

Immunohistochemistry

CD31/D2-40 and CKpan (AE1/AE3) mouse antihuman monoclonal antibodies were purchased from Fuzhou Maixin company in China, and cocktail double staining kit was purchased from Roche company in the United States. For each patient, 3 paraffin-embedded tissue samples from different sections of the tumor were examined (after avoiding necrotic tissue).

Immunohistochemistry was performed by elivision TM plus method. The primary antibody was D2-40/CK low antibody mixed with 1:1. The antigen was repaired by high temperature and high pressure. The DAB (Diaminobenzidine) color was developed. The neutral resin was sealed and observed under the microscope.

Criteria for result reading

Criteria for LVI/BVI diagnosis: LVI was considered positive when the presence of at least one tumor cell were detected in D2-40-positive lymphatic vessels, and BVI was considered positive when the presence of at least one tumor cell were detected in CD31 positively but D2-40-negative stained vessels. The artifact is defined as tumor emboli detected in the clear space without D2-40 and CD31 stained. To understand the differences in reading results among different readers, 30 patients were randomly selected, and their immunohistochemically stained slides were read, measured, and scored by 2 researchers. The resulting LVI and BVI data agreement rate were both 96.7%.

Statistics

The data were analyzed with SPSS 20.0(SPSS Inc., Chicago, III., USA). Pearson's chi-square test or Fisher's exact probability method was used for univariate analysis, and logistic regression method was used for multivariate analysis. The receiver operating characteristic (ROC) curve combined with clinicopathological parameters was plotted to predict lymph node metastasis, and the predictive value was compared by calculating the area under the curve. SPSS 22.0 software was used for statistical analysis of the above data, and a P value of $<.05$ indicated a statistically significant difference.

Results

General data

The mean age of patients in this study was 56.13 ± 7.30 (range, 33–70) years. The tumors were classified as endometrioid (136 cases, 79.53%) and nonendometrioid (35 cases, 20.47%), including serous (16 cases, 9.36%), clear cell (6 cases, 3.51%), undifferentiated (5 cases, 2.92%), and mixed (8 case, 4.68%); carcinosarcoma was excluded. According to the status of lymph node metastasis, they were divided into 2 groups: (1) positive group: patients with pelvic/abdominal para-aortic lymph node metastasis (28, 16.37%) and (2) negative group: patients without pelvic/abdominal para-aortic lymph node metastasis (143, 83.63%). There was no significant difference in age, body mass index (BMI), history of hypertension, history of diabetes and reproductive history between the 2 groups.

Staining of lymphatic vessels in EC tissue using D2-40/CD31 marker

D2-40/CD31-positive staining is localized in the cell membrane or cytosol. D2-40/CD31-positive lymphatic/blood vessels are composed of flat tubes or cavities formed by monolayer endothelial cells. The lymphatic lumen is irregular in size and shape and occasionally contain lymphocytes but do not contain platelets, red blood cells, and other vascular content. In the immunohistochemical cocktail double-staining section, the lymphatic vessels and blood vessels were stained into continuous and smooth brown tubules. The cell membrane and cytoplasm of cancer cells in the tubes were evenly red, and the brown tubules surrounded the red cell clusters (Figure 2).

Univariate and multivariate analysis for factors affecting lymphatic metastasis of endometrial carcinoma

Univariate analysis showed that pathological grade ($P = .0075$), depth of myometrial invasion ($P < .001$), CD31/BVI ($<.001$), and D2-40/LVI ($P < .001$) were the risk factors of lymphatic metastasis in EC (Table 1).

The results of collinearity diagnosis for the above variables indicate that all variance expansion factors (VIF values) are less than 5, so it is considered that there is no multiple collinearity problem for the above variables. The statistically significant indicators were input to the multivariate logistic regression model, and the screening results were as follows: pathological grading (G3) ($P = .016$) and D2-40/LVI ($P = .013$) are independent high-risk factors for lymph node metastasis of EC (Table 1).

Predictive value of G3 and D2-40/LVI in lymph node metastasis of endometrial carcinoma

We plotted the ROC curve of pathological grading (G3) and D2-40/LVI to predict lymph node metastasis. The areas under the curve value obtained from the individual ROC plot of pathological grading (G3) and D2-40/LVI was 0.642 and 0.680, respectively. The areas under the curve value obtained from the combined ROC plot of pathological grading (G3) and D2-40/LVI was 0.726, which was greater than the independent predictor value (Table 2, Figure 3).

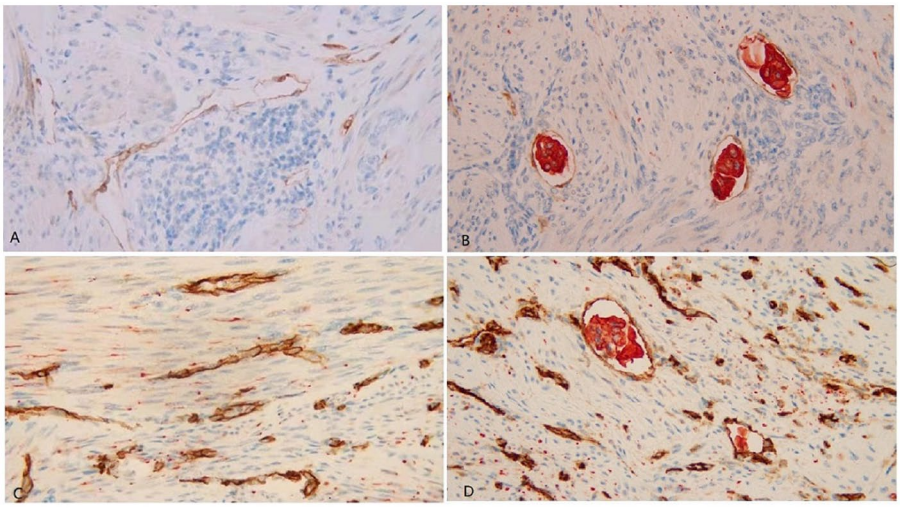

Figure 2. Immunohistochemical double staining results (×400): (A) CD31/ckpan, CD31 positive in vascular endothelium was brown, and ckpan negative in intravascular cancer cells; (B) CD31/ckpan, vascular endothelial CD31 positive was brown, and intravascular cancer cell ckpan was red; (C) D2-40/ckpan, lymphatic endothelium D2-40 positive was brown, and intraductal cancer cells ckpan negative; and (D) D2-40/ckpan, lymphatic endothelium D2-40 positive was brown, and intraductal cancer cells ckpan positive was red.

Expression status of D2-40/LVI and CD31/BVI in recurrent patients

The median follow-up for the cohort of 171 patients was 41 (range, 27-51) months. The median follow-up for the recurrent cohort was 22 (range, 9–32) months. Among 137 patients with stage I-II EC, 5 recurred, all of which were stage II and pelvic recurrences, and there were no dead cases. Among the 34 patients with stage III-IV, 10 had recurrence, 2 had pelvic recurrence, 7 had multiple pelvic and abdominal metastases, and 1 had distant metastasis (including multiple metastasis of supraclavicular lymph nodes), 3 cases died (2 cases with multiple metastasis in pelvic and abdominal cavity and 1 case with distant metastasis).

Among the 15 recurrent patients, 5 (33.3%) were D2-40/LVI positive, 2 (13.3%) were CD31/BVI positive, and 8 (53.3%) were both D2-40/LVI and CD31/BVI positive.

Among 156 patients without recurrence, 135 patients were negative for D2-40. Among 15 patients with recurrence, only 2 patients were negative for D2-40. The sensitivity is 86.67%, the specificity is 86.54%, and the Youden index is 0.732, the positive predictive value is 38.23%, and the negative predictive value was 98.54%.

The expression status of D2-40/LVI and CD31/BVI in recurrent patients is shown in Table 3.

Discussion

Lymphatic metastasis is the most common way of metastasis of EC and is one of the main risk factors for poor prognosis of EC. At present, the commonly used pathological diagnostic methods, such as H&E staining and single immunohistochemical markers, cannot distinguish lymphatic vessels from blood vessels; moreover, it is impossible to determine whether there are cancer cells in the cell mass in the vessel, and there may be over diagnosis or missed diagnosis. The immunohistochemical cocktail double-staining method is a method that can label 2 different specific antigens in tissues at the same time, and can double dye vascular endothelial cells and cancer cells at the same time.

In our study, we used the immunohistochemical cocktail double-staining method to stain the lymphatic/blood vessels of EC tissues using specific anti-body D2-40/CD31, and at least one cancer cell should be present in the columnar structure formed by the linear arrangement of D2-40-positive or CD31-positive endothelial cells. Univariate and multivariate logistic regression models were used, and the result revealed that pathological grading (G3) and D2-40/LVI are independent high-risk factors for lymph node metastasis of EC. This is inconsistent with other research results. According to Federation International of Gynecologic and Obstetrics (FIGO) and Gynecologic Oncology Group (GOG), the most important risk factors for lymph node metastasis in patients with EC were the grade of tumor and the depth of myometrial invasion.[13] Some studies believe that nonendometrioid histology, age greater than 60 years, pathological grading (G3), deep myometrial invasion, lymphatic vascular space infiltration, primary tumor size, and cervical stromal invasion are high-risk factors for lymphatic metastasis of EC.[14-16]

The relationship between myometrial invasion and lymphatic metastasis has been confirmed by many studies,[17] and myometrial invasion has been included in the staging of EC, but the results of this study have not reached a consistent conclusion. Maybe because the early symptoms of EC are typical, most of them are in the early stage of the disease, and the incidence of lymphatic metastasis is low. Furthermore, the number of positive cases of lymphatic metastasis in this study is relatively small, which may have a certain impact on the experimental results.

Table 1. Univariate and multivariate analysis of risk factors for lymph node metastasis of endometrial carcinoma.

VARIABLE	CATEGORIES	NUMBER OF PATIENTS	UNIVARIATE ANALYSIS			MULTIVARIATE ANALYSIS	
			LYMPH NODES NEGATIVE N (%)	LYMPH NODES POSITIVE N (%)	*P*	OR	*P*
Age (years)	<60	112	96 (85.71%)	16 (14.29%)	.3849		
	⩾60	59	47 (79.66%)	12 (20.34%)			
Histological type	Endometrioid	136	117 (86.03%)	19 (13.97%)	.1226		
	Nonendometrioid	35	26 (74.29%)	9 (25.71%)			
Grade[a]	I-II	114	102 (89.47%)	12 (10.53%)	.0075	3.030 (1.232-7.455)	.016
	III	57	41 (71.93%)	16 (28.07%)			
Myometrial infiltration[b]	<1/2	131	121 (92.37%)	10 (7.63%)	<.001	1.488 (0.488-4.541)	.485
	⩾1/2	40	22 (55.00%)	18 (45.00%)			
Ki-67	<40%	79	68 (86.08%)	11 (13.92%)	.5349		
	⩾40%	92	75 (81.52%)	17 (18.48%)			
CD31/BVI	Positive	42	19 (45.24%)	23 (54.76%)	<.001	2.830 (0.883-8.901)	.080
	Negative	129	124 (96.12%)	5 (3.88%)			
D2-40/LVI	Positive	34	16 (47.06%)	18 (52.94%)	<.001	3.880 (1.377-11.259)	.013
	Negative	137	127 (92.70%)	10 (7.30%)			
CA125	<35	92	82 (89.13%)	10 (10.87%)	.0585		
	⩾35	79	61 (77.22%)	18 (22.78%)			
Cervical stromal invasion	Yes	6	3 (50.0%)	3 (50.0%)	.0562		
	No	165	140 (84.85%)	25 (15.15%)			
Lesion size (cm)	<2	71	64 (90.14%)	7 (9.86%)	.0836		
	⩾2	100	79 (79.0%)	21 (21.0%)			

Abbreviation: OR, odds ratio.
[a]Pathological grade III includes poorly differentiated endometrioid adenocarcinoma and all nonendometrioid adenocarcinoma.
[b]Myometrial infiltration: ⩾1/2 refers to the external 1/2 (including 1/2) of the whole muscle layer infiltrated by cancer tissue observed under the microscope; <1/2 refers to the inner 1/2 of the whole muscle layer invaded by cancer tissue under the microscope.

Table 2. Area under the curve.

TEST RESULT VARIABLE	STANDARD			95% CONFIDENCE INTERVAL	
	AUC	ERROR	*P* VALUE	LOWER LIMIT	UPPER LIMIT
G3	0.642	0.059	0.017	0.527	0.758
D2-40/LVI	0.680	0.061	0.003	0.560	0.800
G3 + D2-40/LVI	0.726	0.059	0.000	0.609	0.842

There are different views on the relationship between lesion size and lymph node metastasis of EC.[18-20] The reason may be related to the different ways of recording the initial lesion size in different studies. The common methods are hysteroscopy, ultrasonic prompt before subsection diagnosis and curettage and pathological examination after operation.

Based on the above results, we plotted the area under the curve of pathological grade (G3) and D2-40/LVI, and concluded that D2-40/LVI can effectively predict lymph node metastasis of EC, especially combined with G3. There were also other consistent findings that lymphatic infiltration was significantly correlated with regional lymph node metastasis and poor prognosis.[21] BVI is an important factor associated with the hematogenous spread of tumor cells.[22] In addition, this study found that 80% of the relapsed patients with positive D2-40/LVI were pelvic metastases, and 20% were distant metastases outside the pelvic cavity. However, distant metastases outside the pelvic cavity accounted for 75% of relapsed patients with positive D2-40/LVI and CD31/BVI. This conclusion has important clinical significance. It provides a hypothesis for postoperative adjuvant therapy of patients with EC, that is, patients with LVI positive have a high risk of pelvic

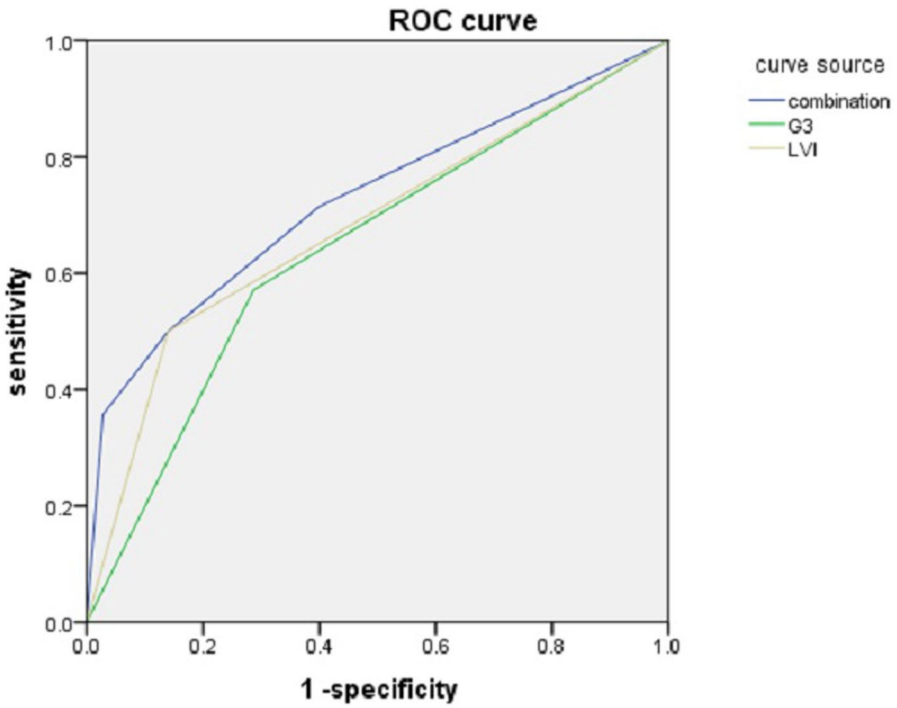

Figure 3. ROC curve of single and joint index.

Table 3. Expression status of D2-40/LVI and CD31/BVI in recurrent patients.

	NUMBER OF PATIENTS	PELVIC METASTASIS	PERITONEAL/DISTANT METASTASIS
D2-40/LVI (+)	5	4 (80%)	1 (20%)
CD31/BVI (+)	2	1 (50%)	1 (50%)
D2-40/LVI (+) + CD31/BVI (+)	8	2 (25%)	6 (75%)

local recurrence, and these patients will benefit from radiotherapy. When D2-40/LVI and CD31/BVI are both positive, the proportion of distant metastasis outside the pelvic cavity is high (75%). Therefore, we proposed to assume that patients with D2-40/LVI and CD31/BVI positivity may benefit from chemotherapy. However, due to the limited sample size, this conclusion needs further study.

Endometrial carcinoma can be divided into 4 subtypes according to its molecular characteristics: POLE ultra-mutated, microsatellite instable hypermutated (MSI-H), copy-number-low (CN-L), and copy-number-high (CN-H). This classification method has higher prognostic value than the traditional classification based on histopathological characteristics. For example, POLE mut endometrial carcinoma has a good prognosis and only rarely relapses.[23] The prognosis of patients with CN-H is poor. MSI-H EC also elicits a strong immuno-genic response and has an intermediate prognosis.[24] The increased expression of progesterone receptor of CN-L type suggests that patients with CN-L type EC may benefit from endocrine therapy.[1] CN-H type has the worst prognosis, including some high-grade endometrioid carcinoma and most serous carcinoma patients. Therefore, chemotherapy rather than adjuvant radiotherapy should be carefully considered clinically.[25] Since this study is a retrospective study, the initial treatment time of the selected cases was about 4 years ago. At that time, there were few molecular classification tests for EC in China. Therefore, this is one of the shortcomings of this study.

This study has several limitations. First, because the clinical symptoms of EC are typical and most of them are in the early stage at the time of initial treatment, the number of EC cases with lymph node metastasis among the cases included in this study is small, which may have a certain impact on the reliability of the research results, to more effectively explain the predictive value of detection of D2-40/LVI and CD31/BVI for lymphatic metastasis, it is necessary to further expand the sample size. In addition, this experiment only focuses on tumor thrombus from epithelial tissue, but it is not suitable for cocktail double staining CD31/D2-40/CKpan, such as sarcoma.

Conclusion

D2-40/LVI combined with G3 can effectively predict lymph node metastasis of EC. In addition, it may have certain clinical value in the adjuvant treatment of EC after surgery

Acknowledgements

The authors wish to acknowledge the assistance of Yan Shen, and of other doctors from Tianjin Central Hospital of Gynecology Obstetrics.

Author Contributions

(I) Conception and design: JX; (II) Administrative support: PQ and CL; (III) Provision of study materials or patients: XW and QD; (IV) Collection and assembly of data: JX and XW; (V) Data analysis and interpretation: JX; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors; (VIII) Manuscript arrangement and revision: JX and CL.

Ethical Approval and Consent to Participate

The study was approved by the Institutional Ethical Committee of Tianjin Central Hospital of Obstetrics and Gynecology (approval no: 2020KY008), and written informed consent was obtained from all patients.

Consent for Publication

Written informed consent for publication was obtained from all participants.

Availability of Data and Materials

The data sets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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