



The impact of examining VEGF-C expression, D2-40-based detection of LVI, and LVD on the prediction of lymph node metastasis in endometrial carcinoma

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ABSTRACT

Background: Lymph node metastasis (LNM) is a main route of endometrial carcinoma (EC) spread and it plays a chief prognostic role. Our objective is to find LMN predictors through investigating lymph vessel invasion (LVI) and lymphatic vessel density (LVD), in addition to vascular endothelial growth factor -C (VEGF-C) expression in the primary tumor site of the EC.

Material and Methods: A retrospective immunohistochemical study applying VEGF-C and D2-40 antibodies on 40 EC cases, in addition to 20 cases of proliferative endometrium, and 20 cases with atypical endometrial hyperplasia as control groups. The studied cases were screened for expression of VEGF-C, D2-40-LVI, and LVD. Statistical analysis with the correlation of the findings to various clinicopathological parameters was achieved.

Results: Expression of VEGF-C was moderate-to-high in 87.5% of EC cases, only mild-to-moderate focal staining in atypical hyperplasia cases, and exclusively negative in proliferative endometrium cases. VEGF-C expression could independently predict LNM with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of 100%, 51.6%, 62.5%, 100%, and 62.5%, respectively. D2-40-LVI detection system is more sensitive in predicting LNM in EC than routine H&E stained section. It was detected in 9/9 of EC with LNM cases (sensitivity 100%) compared with 7/9 cases detected by H&E staining (sensitivity 77.8%). D2-40-LVI detection system could predict LNM with sensitivity, specificity, PPV, NPV, and accuracy of 100%, 67.7%, 47.4%, 100%, and 75%, respectively. D2-40-LVI, peritumoral LVD, and VEGF-C expression showed a significant correlation to LNM ($p < 0.05$) and each one of them can independently predict LNM (AUC = 0.839, 0.703, and 0.758), respectively.

Conclusions: Evaluation of LVI, peritumoral LVD by D2-40, and VEGF-C expression in EC at the primary tumor site can predict LNM and helps the clinicians to select the patient who will need further lymphadenectomy.

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Introduction

Worldwide, corpus uteri cancer is one of the high-ranking malignancies in women. It is the fifth most frequent women's malignancy after mammary cancer, colorectal, uterine cervix, and lung cancers [1,2]. Endometrial carcinoma originates from the glandular epithelium of the uterus, representing about 90% of uterine cancers [1,3]. In the developed countries, it is ranked the top most gynecologic malignancy, while in the developing countries, it is the second after cervical carcinoma [1,4]. In Egypt, it represents 2.6%–3.5% of all cancers

[5]. Universally, the 5-year survival rate of endometrial carcinoma (EC) is about 80% when diagnosed at an early stage without lymph node involvement but drops to 50%, if lymph nodes are positive [3]. Surgical evaluation of lymph node involvement in EC, particularly in clinically early stages, remains an issue of debate between the gynecologic oncologist. Up till now, it remains uncertain if systematic lymph node dissection in such cases has a therapeutic value or not. Thus, patients who will subjected to complete systematic lymphadenectomy should be selected to diminish overtreatment and

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undertreatment. So, searching dependable immunohistochemical markers that may improve the prediction of lymph node metastasis (LNM) are of worth and may help in therapeutic decision making.

Pathogenesis of tumor spread to lymph node is a highly sophisticated process that involves detachment of malignant cell, stromal invasion, intravasation of the tumor cell, then extravasation of the tumor cells in the lymph nodes to settle there and proliferate [6]. The lymph vessel is the main channel of malignant cells to go within from the primary tumor site to the draining lymph nodes as well as distant organs [7]. The existence of lymph vessel invasion (LVI) in a primary cancer site is broadly considered as bad prognostic sign and has been estimated to be an independent prognosticator of worse survival in numerous malignant tumors [7–10]. Intralymphatic tumor spread, along with nodal status, encompasses the N category of the staging system. Thus, LVI denotes a possibly informative clinicopathologic parameter for prognostication as it connects T and N categories of the staging system. vascular endothelial growth factor -C (VEGF-C) is a member of VEGF family which is claimed to be involved in lymphangiogenesis, cell proliferation, differentiation, invasion, and migration [11]. Nearly, all types of malignant cells express VEGF, and correlations were found between VEGF expression and both the vascular density in malignant tumors, and patient outcome [7]. D2-40 is a monoclonal antibody that has been categorized as a specific marker for lymphatic endothelial cells [12]. Our study supposes that the ability of EC cells to induce lymphangiogenesis may regulate the possibility lymphatic metastasis.

The purposes of this study are: (1) estimate the incidence of LVI rate evaluated on D2-40 immunostained slide compared with that noted by conventional histology sections; (2) estimate the relation of VEGF-C expression, LVI, and lymphatic vessel density (LVD), to each other and to the other studied clinicopathologic parameters to gain a better understanding of their potential roles in the tumor pathogenesis, patient selection for lymphadenectomy, and to potentially identify novel targets for therapeutic intervention.

Materials and Methods

This is a retrospective study which was implemented in the Department of Pathology, Faculty of Medicine, Banha University from June 2015 to June 2017 after the approval of the Scientific Ethical Committee. It included 40 consecutive female

patients pathologically proved histologically to be endometrioid endometrial carcinoma of the uterine corpus who underwent primary surgical treatment without prior neoadjuvant chemotherapy or radiation therapy. In addition, the study incorporated also 20 patients with proliferative endometrium and 20 patients with atypical hyperplasia as control groups. All these cases were accessed through searching the histopathology data base in-between (January 2013–December 2015) and encompassed to the study according to the availability of the tissue blocks and a relevant clinical data. Surgical treatment entailed abdominal hysterectomy and bilateral salpingo-oophorectomy with complete lymph node dissection (pelvic and para-aortic lymph nodes).

In our study, the exclusion criteria were tumors poorer than International Federation of Gynecology and Obstetrics (FIGO) stage IIIC and other histopathology subtypes, for example, papillary serous or clear cell carcinoma. Clinical data were collected from patients' files.

Tumor classification and grading

Clinical staging as well as grading of EC cases was performed along with FIGO [13].

The final report as well as the H&E stained slides were revised to confirm the previous histopathological diagnosis in addition to record other tumor characteristics as tumor size, grading, depth of myometrial invasion, presence lymphatic invasion, and nodal deposits.

Immunohistochemical staining

The data set of histopathology is searched for picking up the cases of EC, and atypical endometrial hyperplasia, as well as proliferative endometrium cases. The hematoxylin and eosin slides were revised to choose the best section revealing the histological features of the growth; the selected sections were sliced at 4- μ m thickness, went through deparaffinization, and then carried to the water. After that, the sections were incubated in citrate buffer (6 pH) in the microwave at (600 W) for 30 minutes for antigen retrieval. The next step is the blocking of non-specific antigen followed by incubation of primary antibody against VEGF-C (ABCAM) and D2-40 (DakoCytomation, Carpinteria, CA) at 1:200 and 1:100 dilutions, respectively, for 1 hour then incubated for 30 minutes after the addition of secondary antibody. To visualize the reaction, diaminobenzidine (ab143166, DakoCytomation, Carpinteria, CA) were

applied. Hematoxylin (ab143166, DakoCytomation, Carpinteria, CA) were used for counterstaining of the sections then slides were covered. Positive as well as negative controls were submitted with each immunohistochemical (IHC) run.

Interpretation of the conventionally stained and immunostained sections

Interpretation on the conventional histopathology stained slides

Each tumor slide, stained with H&E, was examined to assess tumor grade, the depth of myometrial invasion, and lymphovascular invasion; the later was defined as the presence of tumor cells in blank spaces separated and encircled by the endothelium. In the case of LVI documentation, the presence of lymphovascular invasion was compared with the finding of D2-40-immunostained slide on the successive sections of the same block.

Interpretation on VEGF-C immunostained sections

Cytoplasmic staining of the VEGF-C in epithelial cells was considered as positive according to the immunoreactive score (IRS) proposed by Remmele and Stegner [14] with a slight modification as follows: staining intensity is graded 0, 1, 2, and 3 these correspond to negative, weak, moderate, and strong expression, respectively. percentage of positive cells (PP) was estimated as (0) no cells stained; (1) equal or less than 50% of the cells showed positive staining; (2) 51% or more of the cells showed positive staining. The IRS is the summation of IS and PP as follows: (0–2) score was considered as negative expression, while (3–5) score was considered as positive expression.

Interpretation of D2-40 immunostained sections

The lymphatic vessels delineated with D2-40 immunohistochemical stain were observed in both intra-tumoral and peritumoral areas to assess LVD which was quantified in accordance with Weidner et al. [15] D2-40 positive single cell or group of cells, forming a lumen or not was counted as a lymph vessel. After scanning of the sections at low power, three hotspots' areas (the highest vascularization) in intra-tumoral and three hotspots' areas in peritumoral regions were selected, where the vessels number were counted at 400× magnification (area of 0.1024 mm²). The LVD was estimated as the mean of the three areas. Presence of LVI were confirmed when tumor clusters were observed encircled by d2-40 positively stained endothelium.

Statistical analysis

All data were analyzed statistically by a software program SPSS (Ver. 16.0; SPSS Inc., Chicago, IL). The associations between immunohistochemical findings for VEGF-C, D2-40 and clinicopathological parameters were assessed by Pearson's coefficient of correlation for estimation of *p* value which was considered statistically significant at *p*-values < 0.05.

Sensitivity, specificity, both positive and negative predictive values and accuracy were calculated based on the following formulas:

	Have the disease	Don't have the disease
Positive	a (true positive)	b (false positive)
Negative	c (false negative)	d (true negative)

Sensitivity = $[a/(a + c)] \times 100$

Specificity = $[d/(b + d)] \times 100$

Positive predictive value (PPV) = $[a/(a + b)] \times 100$

Negative predictive value (NPV) = $[d/(c + d)] \times 100$, and

Accuracy = (true positives + true negatives)/(true positives + false positives + true negatives + false negatives) = $[(a + d)/(a + b + c + d)] \times 100$

The ROC curve was used to analyze the probability of LVI-D2-40, LVI-H&E, LVD, and VEGF-C expression to predict LNM.

Results

The mean ages of the studied cases were (52 ± 14 years) for EC patients, (45.5 ± 11.3 years) for atypical hyperplasia cases, and (41 ± 3 years) for proliferative endometrium cases. The maximum tumor diameter ranged between 2 and 6 cm, and its mean was 3.4 ± 2.2. Considering tumor grad, 7 cases were well differentiated, 25 were moderately differentiated, and 8 were poorly differentiated. the depth of myometrial was merely superficial in 22 cases, while 18 cases penetrated deeper. Nine out of 40 (22.5%) cases showed positive nodal deposits. Most patients (22/40, 55%) were limited to the myometrium (FIGO I), 10 patients (25%) were staged as FIGO II, and 8 (20%) as FIGO III.

VEGF-C expression

Out of 40 EC cases, 24 (60%) showed positive cytoplasmic VEGF-C expression (Fig.1c). The atypical endometrial hyperplasia showed only focal mild to moderate cytoplasmic staining (Fig. 1b), while proliferative endometrium cases were exclusively negative (Fig. 1a). Positive VEGF-C expression significantly correlated with the high pathological stage (*p* = 0.008), deep myometrial invasion (*p* = 0.018),

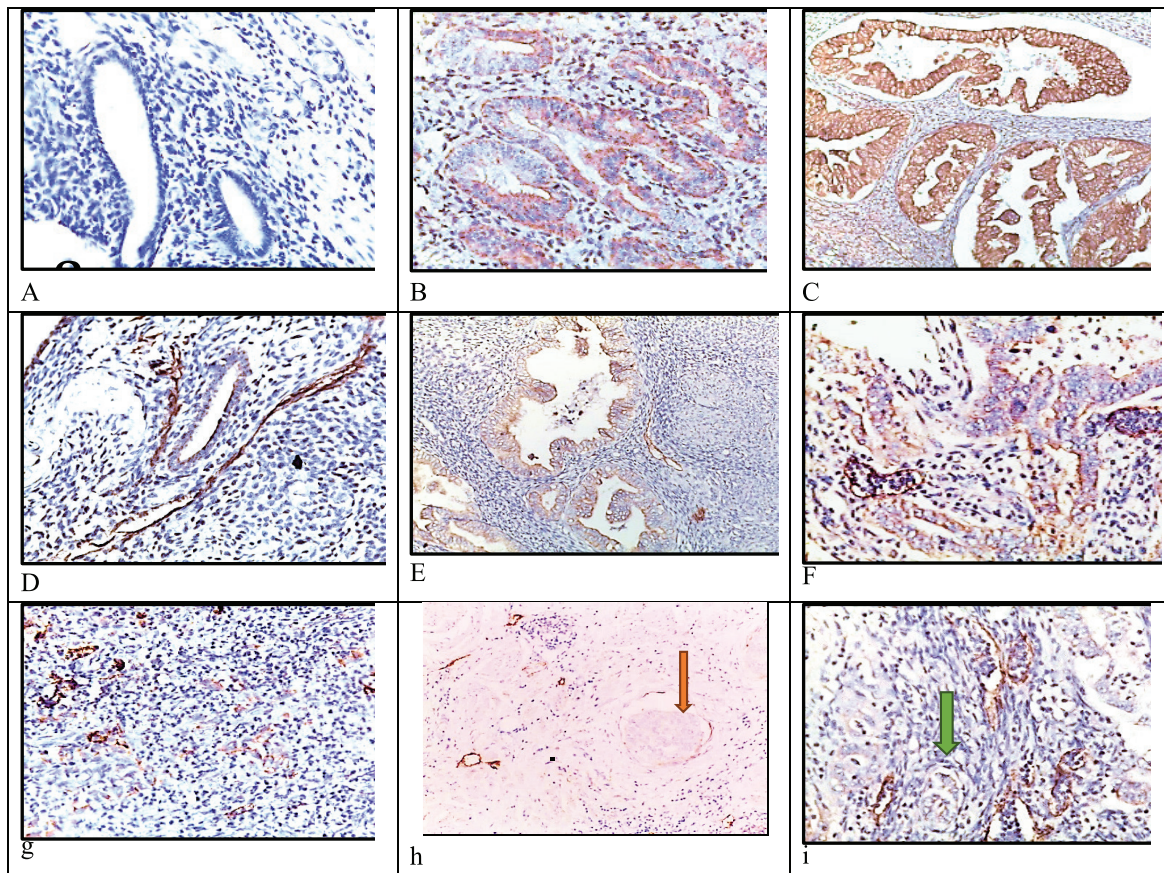


Figure 1. Demonstration of variable immunoexpression of VEGF-C and D2-40 among the studied cases. (a) Proliferative endometrium showing negative expression of VEGF-C (ABC $\times 100$). (b) Atypical endometrial hyperplasia showing focal moderate expression of VEGF-C (ABC $\times 400$). (c) Endometrial carcinoma showing diffuse high expression of VEGF-C (ABC $\times 200$). (d) Proliferative endometrium showing lymph vessels stained with D2-40 (ABC $\times 100$). (e) Atypical endometrium hyperplasia showing lymph vessels stained with D2-40 (ABC $\times 100$). (f) Endometrial carcinoma stained with D2-40 demonstrating intra-tumoral lymphatics distended with lymphocytes (ABC $\times 400$). (g) Endometrial carcinoma stained with D2-40 demonstrating peritumoral lymphatics (ABC $\times 100$). (h) Endometrial carcinoma stained with D2-40 demonstrating lymphatic vessel invasion by tumor cluster, orange arrow (ABC $\times 100$). (i) Endometrial carcinoma stained with D2-40 discriminating false lymphatic invasion due to stromal retraction, green arrow (ABC $\times 100$).

tumor size ($p = 0.13$), high p-LVD ($p = 0.000$), positive LVI-D2-40 ($p = 0.020$), and lymph node metastasis ($p = 0.005$) (Table 1). However, no significant correlation found between VEGF-C expression and differentiation degree ($p = 0.216$). VEGF-C expression could independently predict LNM with sensitivity, specificity, PPV, NPV, and accuracy of 100%, 51.6%, 62.5%, 100%, and 62.5%, respectively, as shown in Table 2, with (AUC = 0.758), shown in ROC curve (Graph 1).

D2-40 expression

D2-40 was used to stain lymphatic endothelium to assess LVD, as well as to detect LVI. Lymphatic vessels in the intra-tumoral area were infrequent, only seen in five cases (12.5%) (Fig. 1f). Though, if they were detected, they were rare; the mean of intra-tumoral (LVD) was 0.6 ± 1.1 . Yet, peritumoral

lymphatics were more common (Fig. 1g), and only two cases revealed no peritumoral lymphatics. The mean of peritumoral LVD (p-LVD) was 5.72 ± 1.41 . Peritumoral LVD were significantly correlated to high tumor stage ($p = 0.004$), deep myometrial invasion ($p = 0.018$), LVI-D240 ($p = 0.05$), lymph node involvement ($p = 0.031$), and positive VEGF-C expression ($p = 0.000$), while no significant association was found between p-LVD and both tumor size ($p = 0.134$), and degree of tumor differentiation ($p = 0.830$). LVD could independently predict LNM (AUC = 0.703), shown on ROC curve (Graph 1). lymph vessel invasion was considered only when tumor clusters were encircled by a D2-40-stained endothelium (Fig. 1h). This was detected in 19 tumor specimens (47.5%), which was more sensitive than detection by H&E stain only (seven cases, 17.5%), this shown by ROC curve as area

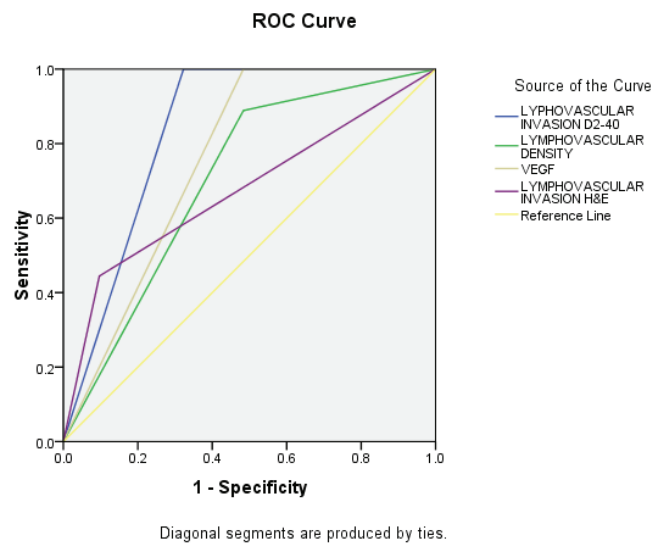
Table 1. Correlation of tumor parameter to lymph vessel invasion based on D2-40.

Tumor parameter	Total no.	D2-40-LVI positive cases		p-value	VEGF-C positive cases		p-value
Tumor grade							
		NO.	%	0.201	NO.	%	0.216
I	7	1	14.3	Non-sig.	2	28.6	Non-sig.
II	25	14	56		17	68.0	
III	8	4	50		5	62.5	
Depth of myometrial invasion							
Superficial	22	5	22.7%	0.000	10	45.5	0.039
Deep	18	14	77.8	Sig.	14	77.8	Sig.
Tumor stage							
I	22	5	22.7	0.000	10	45.5	0.008
II	10	6	60	Sig.	6	60	Sig.
III	8	8	100		8	100	
Nodal status							
N0	31	10	32.3	0.000	15	48.4	0.005
N1	9	9	100	Sig.	9	100	Sig.

Table 2. The sensitivity and specificity of D2-40 and VEGF in perdicting LNM.

Character	D2-40	VEGF
Sensitivity	9/9 (100%)	9/9 (100%)
Specificity	21/31(67.7%)	16/31(51.6%)
positive predictive value	9/19(47.4%)	15/24(62.5%)
Negative predictive value	21/21(100%)	16/16(100%)
Accuracy	30/40(75%)	25/40(62.5%)

under the curve (AUC) was 0.839 on using D2-40 compared with 0.647 on routine H&E staining (Graph 1). Moreover, all cases of D2-40 LVI could be identified H&E slides retrograde after serially sectioning of the paraffine block, only two cases on H&E slide showed LVI (0.05%) but could not be reproduced by D2-40 immunostaining, which was considered a false positive because of retraction artifacts and was simply recognized by D2-40 immunostaining. There were significant correlations between D2-40 LVI and tumor size ($p = 0.006$), progressive tumor stages ($p = 0.000$), high LVD ($p = 0.050$), deep myometrial invasion ($p = 0.000$), lymph node deposits ($p = 0.000$), VEGF-C expression ($p = 0.020$) (Table 1). Five out of 22 (22.7%) patients in FIGO I stage (tumors confined to the mucosa) showed LVI—D2-40 compared with (6/10, 60%) in FIGO tumor stage II. Yet, LVI was detected in all cases histologically diagnosed as FIGO stage III. All cases showed positive lymph node deposits (FIGO IIIC) were positive for D2-40 LVI. Lymphovascular invasion detected by D2-40 immunostaining could predict LNM with sensitivity, specificity, PPV, NPV,



Area Under the Curve

Test Result Variable(s)	Area
LYPHOVASCULAR INVASION D2-40	.839
LYPHOVASCULAR DENSITY	.703
VEGF	.758
LYPHOVASCULAR INVASION H&E	.674

Graph 1. ROC curve that shows the probability of LVI-D2-40, LVI-H&E, and LVD to predict LNM.

and accuracy of 100%, 67.7%, 47.4%, 100%, and 75%, respectively (Table 2).

Discussion

Endometrial cancer patients commonly had serious comorbidities, and the full lymphadenectomy raise the risks for surgical complications. Finding out new predictors for LNM in these cases is a growing clinical demand to discriminate patients who will benefit systematic lymphadenectomy from other patients in whom such procedure will be without clinical advantage and may maximize the risk for sever surgical outcomes. Several studies have established the VEGF role in promoting development and progression of majority of solid tumors, notably malignant types, and is directly related to tumor invasion, and metastasis [15–20]. Data from literature have confirmed that VEGF-C can stimulate angiogenesis and lymphangiogenesis in tumors and can promote cancer cell metastasis through binding to its receptor VEGFR-3 on lymphatic

endothelium that facilitates entry of tumor cells into the lymphatic channels, and allowing lymphatic spread [16,18]. According to Leslie work on EC cases, VEGF is related to the angiogenesis and patients' outcomes [17]. Our study found that the frequency rate of positive VEGF-C immunorepression in EC was 60% (24/40), while few cases of atypical hyperplasia showed focal mild to moderate intensity immunostaining. Statistical analysis showed a positive correlation of VEGF-C positivity to the studied tumor characteristics such as tumor size, depth of myometrial invasion, tumor stage, and lymph node metastasis ($p < 0.05$). Concerning LNM predictive potentiality of VEGF, our work showed a sensitivity and NPV of (100%) for both of them, and to some extent a lower specificity and PPV (51.6% and 62.5%), respectively. These results agreed with the existing studies, which also prove the association of VEGF-C with involvement of the lymph node by tumor deposits of EC [18–20].

We confirmed earlier studies [21–28] findings in that D2-40 (podoplanin) is the most dependable immunohistochemical antibody to identify lymph vessels, which showed a reliable specificity and sensitivity in detecting LVI that was more sensitive than routine H&E-screening as detection rate of D2-40 LVI was detected in 47.5% of the studied cases versus H&E detection rate (17.5%). Moreover, LVI-D2-40 could be found by H&E on looking the slides back. This explore the pathologists' restricted ability to notice lymphatic invasion on screening conventional H&E stained sections. D2-40 immunostaining clearly differentiate between lymphatic and blood vessels, especially in case that the tumor cell aggregates completely fill the vascular lumen, discriminating vascular from lymphatic vessels represented a substantial difficulty on H&E-stained slides. It can also rule out retraction artifacts. Results of previous studies revealed a wide array of LVI rate (14%–52%) in EC [21–27], our result was within this range (47.5%). These diverse results may be overstated by using varying LVI assessment markers and the analysis of different patient assemblies with varied tumor characteristics.

Many authors confirmed that spread of EC cells through lymph vessel is picture of tumor progression, which is associated with deep myometrial invasion and/or high tumor grade according to a number of previous works [21–23]. These also demonstrated throughout our findings as deep myometrial involvement and high stage tumors showed a statistically significant correlation with LVI ($p = 0.000$) each. Despite of statistically non-significant

correlation was found between LVI and tumor grade, the well-differentiated tumors showed less frequent LVI (14.3%) versus high detection LVI rates (56% and 50%) in moderate and high, respectively, as shown in Table 1.

The previous data concerning the predictive role of LVI in endometrial cancer patients to determine lymph node status were contradictory. The present findings showed a high sensitivity and NPV of (100%) for both of them, and to some extent a lower specificity, and PPV (67.4, and 47.4%), respectively, in addition to a statistically significant correlation was detected between LVI and LNM ($p = 0.000$). This go hand in hand with most of literature [21–27]. This in contrary to Vandenput et al. [28] studied 62 EC cases, in which 52% of the tumors showed D2-40 LVI, but no statistically significant correlation between D2-40 LVI and lymph node involvement was found. This contradictory might be due to the difference in patients grouping or because of different LVI evaluation methods used. Most of the literatures found a dependent association between LN metastasis and FIGO grade [27,30–32]. On the other hand, our study supported with other trials failed to prove a direct correlation between tumor differentiation and LN metastasis as an independent predictor [33–35]. Yet caution of interpretation these results, as many factors such as quite small sample size and single institutional nature might subjected our results to sampling errors and bias.

The present work showed that high p-LVD were significantly correlated to high tumor stage ($p = 0.004$), deep myometrial invasion ($p = 0.018$), LVI-D240 ($p = 0.05$), lymph node involvement ($p = 0.031$), and positive VEGF-C expression ($p = 0.000$), this went hand in hand with previous studies which showed that P-LVD was significantly correlated with LVI, LNM, and tumor stage [36,37]; thus, the proliferated peritumoral lymph vessels might participate in the process of lymphatic metastasis could be a appreciated feature denoting increased risk of metastatic disease.

Work limitation and recommendation for future research

As our study had a limitation in the number of participant cases and also that all cases were sampled from a single insitute, then we recommended further researches to be applied on a large number of cases, from different centers and to be followed by meta-analysis studies to enforce the results of the current work.

Furthermore, our study designated as retrospective one that were carried on the whole transmural cut section obtained from those caeses underwent hysterectomy with bilateral salpingo-oophorectomy and complete lymph node dissection (pelvic and para-aortic lymph nodes) that was removed at the same setting. Hence, we advocate our colleagues to designate a retrospective studies to include endometrial carcinoma samples obtained by curettage to be stained by VEGF marker to evaluate its accuracy to predict LNM in theses patients and to compare these results with that after application of D2-40 and VEGF markers on the whole transmural cut section in hysterectomy samples in the same patients (as D2-40 detection system for LVI cannot be judged on curttage specimen) in relation to their sesnsetivity, specificity, positive predictive value and negative predictive value in predicting LNM as a single marker in endometrial curettage samples and as a single marker and incompination with D2-40 in hysterectomy samples. A further verification for the accuracy of VEGF in the early diagnosis of EC and whether it can become a significant target for novel therapeutic intervention is also recommended.

Conclusions

An abnormal high expression of VEGF-C is closely related to the occurrence of endometrial cancer, which may become a useful biomarker for early diagnosis. D2-40 improves the assessment of LVD and LVI in EC that we may advocate that immunohistochemical staining of numerous cancers with D2-40 could facilitate the detection of lymph vessels in more accurate way and more frequent compared with routine H&E-based LVI screening. D2-40 detection system of LVI and LVD can predicts lymph node involvements by malignant deposits; thus, the results of our study support LVI and LVD evaluation by D2-40 to be an important items of endometrial cancer workup to achieve a comprehensive clinicopathological assessment and it might also contribute to the therapeutic decision to perform further systematic lymphadenectomy. VEGF-C and D2-40 could be considered as a new predictors for LNM in EC cases and could be used to distinguish patients who will benefit systematic lymphadenectomy from whom this procedure will give them no advantage to avoid maximizing the hazard of sever surgical outcomes in the later group.

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