Tumor budding index and microvessel density assessment in patients with endometrial cancer: A pilot study

TOMASZ KLUZ[1*], TOMASZ ŁOZIŃSKI[2*], SYLWIA CZEKIERDOWSKA[3], NORBERT STACHOWICZ[3], GRZEGORZ GURYNOWICZ[4], MIECZYSŁAW CHRÓŚCIEL[5] and ARTUR CZEKIERDOWSKI[3]

[1]Department of Obstetrics and Gynecology, Fryderyk Chopin University Hospital No. 1, Faculty of Medicine, Rzeszow University, 35-055 Rzeszow; [2]Department of Obstetrics and Gynecology, Pro-Familia Hospital, 35-302 Rzeszow; [3]Department of Gynecological Oncology and Gynecology; [4]Division of Perinatology and Women's Diseases, Poznan University of Medical Sciences, 60-535 Poznan; [5]Chair and Department of Epidemiology and Clinical Research Methodology, Medical University of Lublin, 20-081 Lublin, Poland

Received December 8, 2019; Accepted May 27, 2020

DOI: 10.3892/ol.2020.11811

Abstract. The present study aimed to analyze the association between tumor budding index (TBI) and microvessel density (MVD) and selected clinicopathological features in female patients with endometrial cancer (EC). The present study included 137 patients, of whom 117 had endometrial endometrioid cancer and 3 had non-endometrioid EC (NEEC). Additionally, 8 cases of simple endometrial hyperplasia and 9 cases of atypical endometrial hyperplasia were included in the present study. Patient age, menopausal status, tumor histological type, grade and International Federation of Gynecologists and Obstetricians (FIGO) clinical stage were investigated. Immunohistochemistry was utilized to detect MVD using a CD34 antibody, and a laminin-5γ2 antibody was used for TBI assessment. In nonmalignant endometrial lesions, the TBI was significantly lower than that in patients with EC and NEEC (P=0.002). Significant differences in median TBI (MD-TBI) were also observed between patients with low-grade EC (MD-TBI, 4.5) and high-grade EC (MD-TBI, 16.2; P=0.01). Age, body mass index and tumor FIGO stage were not indicated to be associated with the MD-TBI. Premenopausal patients with EC had lower MD-TBI values than postmenopausal patients (0.3 vs. 11.1; P<0.005). The median MVD-CD34 in the study group was 19 (range, 13-29). Significant differences in MVD-CD34 were observed between malignant and nonmalignant endometrial lesions (P=0.01). Histological grade was markedly associated with tumor MVD-CD34 (P=0.001). The MVD was higher in high-grade cancer (G3; MVD-CD34, 24.9) than in grade G1 and G2 lesions (MVD-CD34, 14 and 18.6, respectively; P=0.01). FIGO clinical stage was not associated with MVD-CD34 in low and high stage lesions (MD, 18.4 for FIGO stage I/II; MD, 17.6 for FIGO stage III/IV; P=0.2). High MVD was markedly associated with high MD-TBI (P=0.0002). In conclusion, TBI could be a valuable indicator of tumor aggressiveness in patients with EC. The presence of the tumor budding phenomenon with increased MVD may have the potential to further refine clinical management decisions when endometrial malignancy is detected.

Correspondence to: Professor Tomasz Kluz, Department of Obstetrics and Gynecology, Fryderyk Chopin University Hospital No. 1, Faculty of Medicine, Rzeszow University, Szopena 2, 35-055 Rzeszów, Poland
E-mail: jtkluz@interia.pl

*Contributed equally

Abbreviations: AEH, atypical endometrial hyperplasia; BMI, body mass index; CD34, cluster differentiation 34 (microvessels marker used in immunohistochemistry); EC, endometrial cancer; EEC, endometrioid endometrial cancer; EH, endometrial hyperplasia; EMT, epithelial-mesenchymal transition; ESCC, early-stage cervical cancer; FIGO, International Federation of Gynecologists and Obstetricians; G1, G2, G3, histological grades of malignant tumors; H&E, histological hematoxylin and eosin staining; HPF, high power field of a microscope; IHC, immunohistochemistry; ITB, intratumoral budding; L5γ2, laminin subchain 5γ2; MD, median value; MVD-CD34, microvessel density assessed with CD34 expression; MVD, microvessel density; NEEC, non-endometrioid endometrial cancer; OSCC, oral squamous cell carcinoma; PTB, peritumoral budding; TB, tumor budding; TBI, tumor budding index

Key words: endometrial cancer, tumor budding index, laminin-5γ2, microvessel density

Introduction

Endometrial cancer (EC) is the most common gynecological malignancy in high-income countries, and it generally is associated with a good prognosis due to early-stage diagnosis (1). In patients with EC, the survival rates are associated with tumor stage, the depth of myometrial invasion and the histological type of cancer, including the cancer cell grade of differentiation. Following surgery and tumor removal, the key factors associated with prognosis are histological grade and clinical cancer stage, and these are important for determining further treatment decisions. EC has been traditionally divided into type 1 and 2 based on microscopic findings, and these are associated with different clinical outcomes. Furthermore, ~85% of newly-diagnosed cases are histologically endometrioid type endometrial cancer (EEC) and belong to the type 1 group, whereas type 2 cancers, such as serous carcinoma, are found in 3-10% of cases. Endometrial clear cell carcinoma accounts for <5% of diagnoses and both latter types belong to the group of non-endometrioid endometrial cancers (NEECs) (2). In recent years, this simplistic pathological classification of EC types has been challenged by high-quality molecular data available from large genome databases. For example, four clinically distinct EC types are defined by The Cancer Genome Atlas database. These types have been selected on the basis of their overall gene mutational status, which includes specific p53, polymerase ε (a DNA polymerase involved in DNA replication and repair) and phosphatase and tensin homolog protein mutations, along with microsatellite instability and histology assessments (3). Additionally, novel histologic categories of endometrial hyperplasia (EH) types, with clear prognostic implications as possible EC precursors, have been proposed (4). Furthermore, it is becoming evident from an epidemiologic point of view that metabolic factors may serve an important role in EC (5). Although consensus in various aspects of EC diagnosis and treatment has been recently reached by the European Society for Medical Oncology, the European Society of Gynaecological Oncology and the European Society of Radiation Oncology (6), there is a need for novel, robust, prognostic and predictive biomarkers of EC and EH that could be used in the near future to improve therapeutic decisions.

One notable histological parameter is tumor budding (TB), and its prognostic significance was first described by Hase *et al* (7) in 1993 in colorectal cancer. TB is defined as single cells or clusters of up to four cells at the margin of the tumor front (7). This specific phenomenon has been observed in various types of cancer in which the invasive parts of the tumors send finger-like projections called 'buds' into adjacent tissues (8). During local cancer growth, some of these cell clusters detach from the main tumor body and invade the neighboring stroma. This phenomenon is regarded as a histological basis of metastasis formation and further tumor invasion. In colorectal cancer, TB has been demonstrated to be a novel prognostic factor that may be used to better define the risk of adverse outcomes (8). Additionally, Yamaguchi *et al* (9) have revealed that TB is a distinct morphological feature that has biologic and prognostic significance in adenocarcinoma of the lung. Gujam *et al* (10) have found that, in patients with invasive ductal breast cancer, TB is a significant predictor of survival. Furthermore, it is independent of adverse pathological characteristics and components of the tumor microenvironment (10). Lugli *et al* (11) have proposed a three-tier system that should be used along with budding count in order to facilitate risk stratification in patients with colorectal cancer. Since TB and tumor grade are not the same and TB is now a well-described and standardized prognostic factor, these authors concluded that TB should be included in guidelines and protocols for colorectal cancer reporting (11). Interestingly, this histopathological feature can be identified by usual routine pathological examination in different types of cancer (12).

TB may be further divided into peritumoral budding (PTB), where tumor buds are counted at the tumor front, and intratumoral budding (ITB), where clustered cancer cells representing tumor buds are observed and counted in the tumor center (13). PTB can only be assessed in endoscopic or surgical resection specimens, whereas ITB can be assessed in both colorectal cancer biopsies and resection specimens. Both ITB and PTB have been considered to be morphological markers of epithelial-mesenchymal transition (EMT) (14). EMT can be found in physiological and pathological conditions, and it has been defined as the transformation of an epithelial cell into a spindle cell (14). Using immunohistochemistry (IHC), the loss of membrane E-cadherin expression and the appearance of mesenchymal cell markers can be demonstrated (15). Notably, an association between loss of E-cadherin and TB has been identified in EC (16). TB can be studied with the use of IHC and specific markers, such as E-cadherin or laminin. The latter is the main active element of various basal membranes, including the perivascular basal lamina (17). Laminin promotes attachment, spreading, scattering and migration of non-tumorigenic epithelial cells. Previous studies have revealed that the expression levels of laminin subchain, namely laminin-5γ2-chain (L5γ2), could be a specific marker for invasive tumors because it is frequently expressed as a monomer in several types of cancer cells in association with a lack of simultaneous expression of other laminin chains, such as L5-α3 and L5-β3 (18,19). Furthermore, immunohistochemical experiments have revealed that laminin 5-γ2 is expressed at the invasive front of TB cells (20).

Most solid tumors that grow beyond 2-3 mm in size require angiogenesis (21). Normal endometrium secretes angiogenic factors, including mainly vascular endothelial growth factor, during the menstrual cycle and in early pregnancy (22). Elevated concentrations of proangiogenic factors secreted by malignant tumors, such as EC, along with activation of tissue matrix metalloproteinases induce the formation of the microvascular network (23). This increased vascularity is frequently observed in aggressive EC and could be used as a specific target in anticancer therapy (24). Blood vessels in cancer exhibit various structural and functional abnormalities, including unusual leakiness which enables the dissemination of tumor cells into the bloodstream. Furthermore, malignant tumor microvessel density (MVD) is heterogeneous; the highest values are found in the invading tumor edge, where the density may be 4-10 times greater than inside the tumor. Additionally, the arrangement of vessels in the center of a tumor is much more chaotic than at its edges (25). Both MVD and TB can be examined by light microscopy and histopathological examination with or without IHC. These methods are much cheaper to perform than molecular analyses and are readily available in most pathology units. Based on a sufficient number of standardized cases, they may offer novel indications for a more accurate classification of the removed endometrial tumors.

To the best of our knowledge, no previous studies have attempted to associate tumor angiogenesis with TB and with various clinical and pathological parameters in patients with EC. Therefore, the present pilot study aimed to analyze the association between TB and selected clinicopathological features in female patients with EC.

Materials and methods

Patients and tumor samples. The present study included 137 female patients, among them 117 had EEC and 3 had NEEC.

Additionally, the present study included 8 cases of simple EH and 9 cases of atypical endometrial hyperplasia (AEH). The patients received surgery at the Ist Department of Gynecological Oncology and Gynecology of the Medical University of Lublin (Lublin, Poland) between January 2011 and January 2014.

Data collected included patient age at diagnosis, tumor stage and histological grade. Postmenopausal status was considered as when a woman had no periods for 12 consecutive months prior to surgery. The histological type and grade of the tumors was classified according to the criteria of the World Health Organization (4). Malignant tumor staging was established according to the International Federation of Gynecology and Obstetrics (FIGO 2009) criteria (26). In some, but not all, patients, typical coexisting diseases, such as obesity, diabetes and/or hypertension, were noted. According to previously published data (5,6,24,25,27), the present study did not regard these diseases as potentially confounding variables of both tumor angiogenesis and/or TB. All patients included in the present study were treated with a total abdominal or laparoscopic hysterectomy with adnexectomy with or without pelvic lymph node resection according to the FIGO guidelines. All analyzed samples were obtained by excision. None of the patients in the study groups were treated with cytostatics, since the material was always collected after surgery but before any chemotherapy was initiated.

The median age of the patients was 63±9.7 years (range, 40-83 years) at the time of diagnosis, which was representative of the general population with EC. The Medical University of Lublin Ethics Committee approved the study protocol. All participants were informed of the nature of the study and provided informed oral consent.

IHC. Immunohistochemical analysis was performed using paraffin wax-embedded representative tumor tissue sections fixed in 10% neutral buffered formalin. Sections (4-μm thick) of the formalin-fixed, paraffin-embedded tissues were mounted on silanized slides (Dako; Agilent Technologies, Inc.). The slides were then air-dried, and the tissues were deparaffinized and rehydrated. The following primary antibodies were used: CD34 (mouse, monoclonal IgG Class II, Clone QBEnd 10, DAKO Cytomation; catalog number M7165; dilution 1:50); laminin-5, gamma-2 chain Clone 4G1 (catalog number:M7262; non-conjugated mouse monoclonal antibody DAKO Cytomation dilution1:50) for tumor budding index (TBI) calculation. Following deparaffinization, rehydration and antigen retrieval with the Target Retrieval solution at pH=9.0 (Dako; Agilent Technologies, Inc.), three cycles of heating in a microwave oven (5 min each; 750 W) were performed. Tissue sections were incubated with the primary antibody for 1 h at room temperature. After washing in Wash Buffer (Dako; Agilent Technologies, Inc.), the slides were incubated with the secondary antibody conjugated to streptavidin-biotin-peroxidase complex (Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse - PL Code: K5007. and a color reaction was developed using 3'-3-diaminobenzidine tetrahydrochloride (Dako; Agilent Technologies, Inc.) according to the manufacturer's protocol. The sections were counterstained with Mayer's hematoxylin. For each case, the negative control was applied by replacing the antibody with PBS or nonimmune serum.

Variables

MVD. The average number of microvessels within selected tumor areas was determined according to the Weidner method (28) as previously described (27). Briefly, tumor sections were first examined at low magnifications (x40 and x100) to identify the most vascular areas of the invasive front 'hotspots', i.e., the area(s) with most intense CD34 staining and the highest apparent density of microvessels. Subsequently, as a rule, 10 fields were examined, except in a few cases where less tumor tissue was available. The counts were expressed as the average of all fields examined at high magnification [x200; high power field (HPF)]. MVD was presented as the mean number of vessels per one HPF.

TB and TBI. TB was defined as dissociated single cancer cells or clusters of up to four cancer cells with cytoplasmic L5γ2 immunostaining, ahead of the invasive tumor front. First, the area was scanned at low power (x100) to identify the region displaying maximal budding. Subsequently, tumor buds were counted in high-power fields (x400; 0.49 mm^2) in the area at the invasive front. The present study compared two widespread methods of quantification of tumor buds. First, as proposed by Ueno *et al* (8), the assessment was performed by counting buds in a region of interest spanning one microscope high-power field (1-HPF) and displaying maximal budding. Classification was dichotomic and scored as 'negative' (<5 buds) or 'positive' (≥5 buds). The '10-HPF' method proposed by Karamitopoulou *et al* (29) was the second method of counting tumor buds used in the present study. According to this method, the average number of the 10 counts was taken as the final TBI.

Statistical analysis. Statistical analysis was performed using Statistica software v.10.0 (StatSoft, Inc.). The association between categorical variables was examined by Pearson's χ^2 test or Fisher's exact test, as deemed appropriate. The Shapiro-Wilk test was used to assess the normality of data distribution. Mann-Whitney nonparametric tests were used to compare categorical with continuous tumor variables when there were two categories, whereas the Kruskal-Wallis nonparametric test was used when there were more than two categories. P<0.05 was considered to indicate a statistically significant difference.

Results

Most of the examined patients were postmenopausal (126/137; 92%) and the mean body mass index (BMI) in the study group was 31.7±6.1 (range, 21.8-44.6). Table I presents selected clinical and pathological features of the studied population. There were statistically significant differences in age between the group of patients with EC and the group of patients with benign endometrial lesions (H, 9.1; P=0.01 as per Kruskal-Wallis test by ranks). The differences in BMI values between EC/NEEC and EH/AEH groups were not statistically significant (P=0.3). Histological analysis of the removed specimens revealed 25% cases of low grade (G1) EC; most of the remaining malignancies were grade 2 and only 12% of ECs were undifferentiated high grade (G3) or undifferentiated. The majority of the endometrial cancers were classified as clinical FIGO stage I (73 cases; 61%), and 30 cases (25%) were FIGO stage II. The morphologic

Table I. Selected clinical and pathological features of the studied population.

Feature	N (%)
Type of endometrial pathology	
EC	117 (85.4)
NEEC	3 (2.2)
EH	8 (5.8)
AEH	9 (6.6)
Histopathological grading	
G1	35 (29)
G2	74 (61)
G3	11 (10)
Clinical staging (FIGO)	
I	70 (58)
II	33 (27)
III	16 (13)
IV	1 (1)

EC, endometrial endometrioid cancer; NEEC, non-endometrial endometrioid cancer; EH, endometrial hyperplasia; AEH, atypical endometrial hyperplasia.

features that best distinguished endometrial hyperplasia or EC from normal endometrium included glandular crowding that was well distinguished from normal glands and abnormal architecture of the glands, with their long axes pointing in different directions or being parallel to the endometrial surface. Other histological features used for the discrimination between malignant and benign lesions included irregularly-shaped glands that were dilated, densely packed non-secretory glands, including budding or branching glands and nuclear atypia in cases of atypical hyperplasia, and EC with cribriform or confluent glands in cases of carcinoma. Selected clinical and pathological features of the study population are presented in Table I.

MVD-CD34 assessment. Table II shows median values of MVD-CD34 according to clinical and histological features in endometrial lesions. In the patients included in the present study, the median value (MD) of MVD-CD34 was 19/HPF (range, 13-29). Statistically significant differences were identified between patients with malignant lesions and patients with nonmalignant endometrial lesions (P=0.01). The present study revealed that the median MVD-CD34 in patients with EEC was higher than that in patients with EH/AEH and NEEC. MVD was associated with histological grade and well-differentiated tumors in which MVD was significantly lower than in undifferentiated tumors (MD, 14 vs. 24.9 in G1 and G3, respectively; P=0.001). Age, menopausal status and BMI were not associated with tumor MVD. MVD, as assessed using the anti-CD34 antibody, did not differ between the groups with low and high clinical FIGO stage of EC (MD, 18.4 in the low-stage group; and MD, 17.6 in the high-stage group; P=0.2).

TB assessment using laminin L5γ2 expression. Laminin L5γ2 expression in tumor buds was identified in 120 (84%) patients with endometrial lesions, and 89 of them were classified as TB-positive. Table III shows the results of TB quantification in relation to clinical and histopathological features in endometrial lesions assessed using the '1-HPF' scoring method. Examples of laminin 5γ2-chain expression in tumor buds in EC are shown in Figs. 1 and 2. More TB-positive lesions were observed in patients with EC compared with in patients with non-malignant hyperplasia (P=0.003). Table IV presents median values of TBI according to clinical and histopathological features in endometrial lesions assessed using the '10-HPF' scoring method. The median value of TBI was 9.2 (range, 1.2-16.8) and it was significantly associated with malignant endometrial lesions (P=0.002). Benign endometrial lesions had a TBI ranging among 0.4 for EH, 1.1 for AEH and 14.1 for NEEC. Figs. 3 and 4 present typical patterns of TB in the invasive front of EC. The median TBI was 10.7 in women with EC. Most of the high grade endometrial tumors (G3) were positive for the TB phenomenon (13/15; P=0.006). Additionally, high TBI values were more often observed in high-grade tumors than in low grade malignant tumors (P=0.01). Fig. 5 presents median values of TBI according to

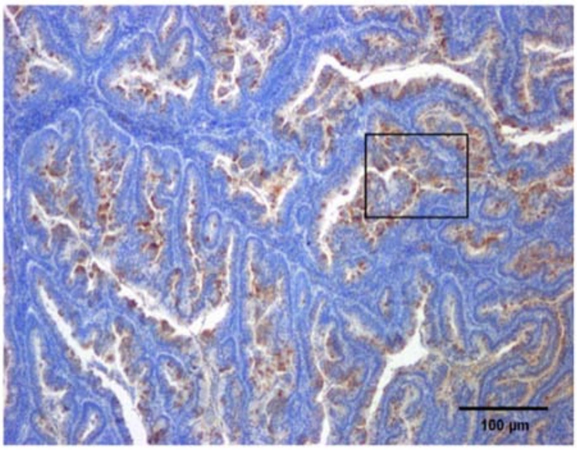

Figure 1. Example of laminin 5γ2-chain expression (brown color) in tumor buds in endometrial carcinoma (magnification, x40). Scale bar, 100 µm. The black rectangle indicates area magnified and presented on Fig. 2.

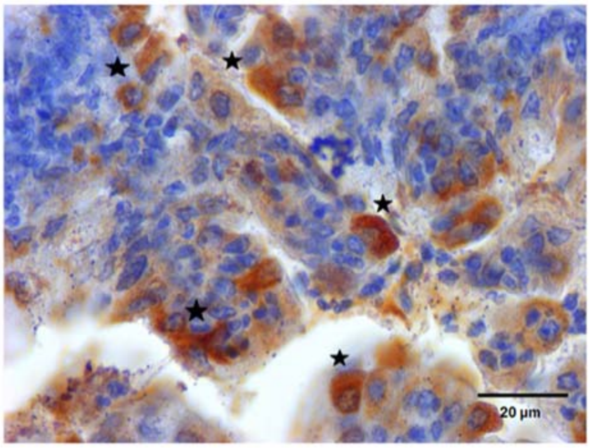

Figure 2. Example of laminin 5γ2 expression in selected region of EC sample (presented on Fig. 1 in square; magnification x400). Scale bar, 20 µm. Black stars indicate single and clusters of tumor budding.

Table II. Median values of MVD-CD34 according to clinical and histological features in endometrial lesions.

Feature	Median value (range)	P-value
All patients	19 (13-29); min-max 2-49	
Type of endometrial lesion		H=11.2; P=0.01
EC (n=117; 85.4%)	19 (13.8-28.8)	
NEEC (n=3; 2.2%)	10 (2.1-14.8)	
EH (n=8; 5.8%)	16 (8.4-30)	
AEH (n=9; 6.6%)	9 (7-18.9)	
Menopausal status		Z=0.6; P=0.6
Before menopause (n=11; 8%)	20.7 (10.8-27.8)	
After menopause (n=126; 92%)	18.8 (12.7-28.9)	
Histopathological grading		H=13.4; P=0.001
G1 (n=31; 25%)	14 (11.8-20.8)	
G2 (n=77; 63%)	18.6 (14.7-30.9)	
G3 (n=15; 12%)	24.9 (19.1-33.7)	
Clinical stage of the disease (FIGO staging)		H=4.2; P=0.2
I (n=70; 60%)	18.4 (14.3-27.8)	
II (n=30; 25%)	23.6 (15.3-33.7)	
III+IV (n=17; 13%)	17.6 (14.6-23.7)	

EC, endometrial endometrioid cancer; NEEC, non-endometrial endometrioid cancer; EH, endometrial hyperplasia; AEH, atypical endometrial hyperplasia; MVD-CD34, ,icrovessel denstity assessed with CD-34 antibody; HPF, high power field.

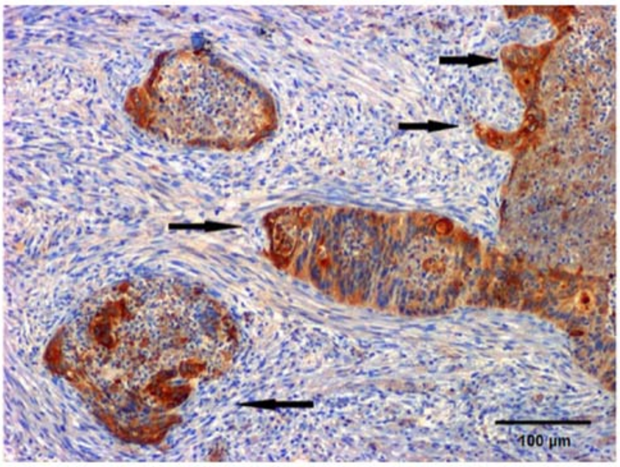

Figure 3. Example of endometrial cancer tumor buds where the primary tumor sends numerous fingerlike projections (black arrows) towards the neighboring stroma, (magnification, x100). Scale bar, 100 μm.

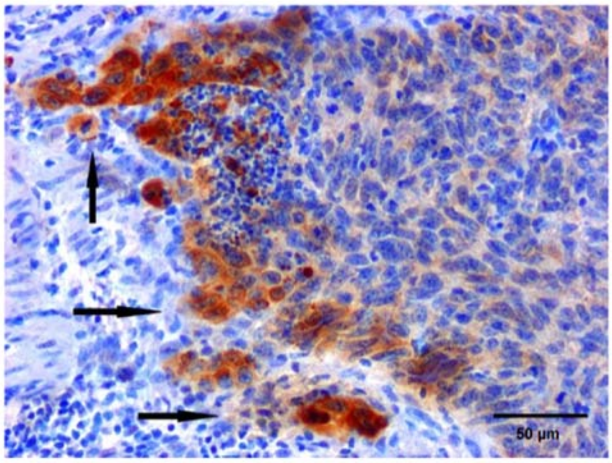

Figure 4. Examples of tumor buds (black arrows) some of which eventually detach from the main tumor mass as small cell clusters (magnification, x200). Scale bar, 50 μm.

histological grade of EC. The median values of TBI in patients with G2 and G3 EC were 12.1 and 16.2, respectively. These indices were markedly higher than TBI values in low grade tumors which had a median TBI of 4.5. No significant associations between TB and the clinical stage of EC were found. However, advanced malignant endometrial tumors (FIGO stage III and IV) tended to be TB-positive more often (13/17). The median values of TBI did not differ between low clinical FIGO stage (I and II) and high clinical stage (III and IV) tumors. The TBI values were 8.8 in low FIGO stage EC and 10.3 in high-stage EC (P=0.2). TBI was markedly associated with MVD (P=0.0002) and TB-positive tumors had a markedly higher MVD than TB-negative EC. Furthermore, menopausal status was associated with TB, and TB-positive tumors were found more frequently in postmenopausal patients (P=0.03). In the group of premenopausal patients, the TBI was significantly higher compared with that in postmenopausal women (TBI, 0.3 vs. 11.1, respectively; P<0.005). No association was identified between TBI and patient age (P=0.1) or BMI (P=0.12). Fig. 6 presents the median values of MVD in TB positive and negative ECs. The association between MVD and TBI in EC is presented in Fig. 7.

Table III. The results of tumor budding quantification in relation to clinical and histopathological features in endometrial lesions (1-HPF scoring method).

Feature	Negative (≤5 budds\HPF) N=47 (34.6%)	Positive (<5 budds\HPF) N=89 (65.4%)	P-values (χ^2 or Z)
Type of endometrial pathology			18.8; P=0.0003
EC (n=117; 85.4%)	33 (28%)	83 (72%)	
NEEC (n=3; 2.2%)	1 (33%)	2 (67%)	
EH (n=8; 5.8%)	8 (100%)	0 (0%)	
AEH (n=9; 6.6%)	5 (56%)	4 (44%)	
Menopausal status			4.4; P=0.03
Before menopause (n=11; 8%)	7 (15%)	4 (5%)	
After menopause (n=126; 92%)	40 (85%)	85 (95%)	
Histopathological grade of tumor (grading)			10.2; P=0.006
G1 (n=31; 25%)	15 (44%)	16 (18%)	
G2 (n=77; 63%)	18 (53%)	59 (67%)	
G3 (n=15; 12%)	1 (3%)	13 (15%)	
Clinical stage of the desease (FIGO staging)			2.4; P=0.4
I (n=73; 61%)	24 (70%)	48 (57%)	
II (n=30; 25%)	6 (18%)	24 (28%)	
III+IV (n=17; 14%)	4 (12%)	13 (15%)	
Microvessel density (MVD-CD34)			
Median (range)	14.4 (9.9-22.7)	19.1 (14.8-30.8)	-3.01; P=0.002
BMI			
Median (range)	31.6 (26.5-35.6)	31 (26.9-34.8)	0.17; P=0.8

EC, endometrial endometrioid cancer; NEEC, non-endometrial endometrioid cancer; EH-endometrial hyperplasia; AEH, atypical endometrial hyperplasia; MVD-CD34, microvessel denstity assessed with CD-34 antibody.

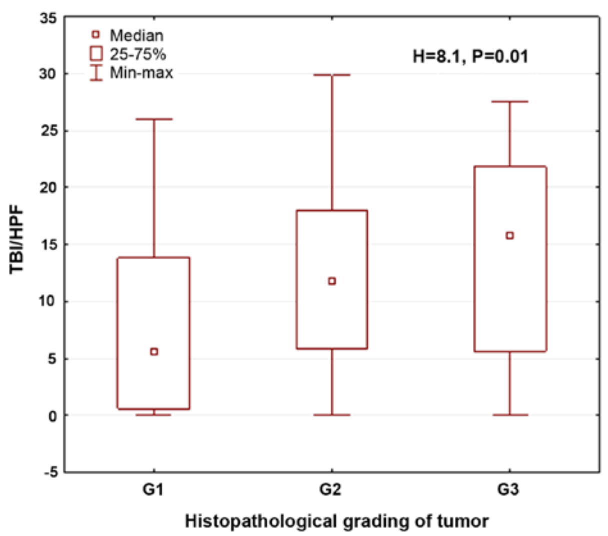

Figure 5. Median values of TBI according to histopathological grade of endometrial cancer. TBI, tumor budding index. HPF, high power field (microscopic).

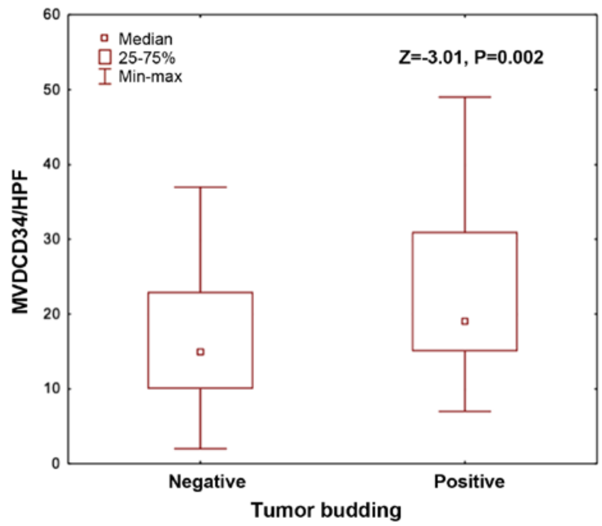

Figure 6. Median values of MVD in negative and positive for tumor budding endometrial cancers. TBI, tumor budding index; MVD-CD34, microvessel density measured with antibody to CD34; HPF, high power field.

Discussion

Most EC cases are diagnosed in early stages, but 15-20% of women with aggressive cancer types have an increased risk of occult malignancy dissemination and tumor recurrence despite chemo- and radiotherapy (30). Currently, tumor staging according to the FIGO criteria remains the basic method used to stratify women with EC into prognostic

Table IV. Median values of Tumor Budding Index (TBI) acording to clinical and histopathological features in endometrial neoplasia (10-HPF scoring method).

Feature	Median (range)	P-value
All groups	9.2 (1.2-16.8); min-max: 0-29.8	
Histological type of endometrial lesion		H=15.2; P=0.002
EC (n=117; 85.4%)	10.7 (3.2-17.7)	
NEEC (n=3; 2.2%)	14.1 (0-28)	
EH (n=8; 5.8%)	0.4 (0-1.7)	
AEH (n=9; 6.6%)	1.1 (0.5-13.1)	
Menopausal status		Z=3.3; P=0.0009
Before menopause n=11 (8%)	0.3 (0-6.1)	
After menopause n=126 (92%)	11.1 (2.3-17.7)	
Histological grade of tumor (grading)		H=8.1; P=0.01
G1 (n=32; 23%)	4.5 (0.6-13.5)	
G2 (n=77; 56%)	12.1 (5.8-17.9)	
G3 (n=15; 11%)	16.2 (5.8-21.6)	
Clinical stage of the desease (FIGO staging)		H=5.1; P=0.2
I (n=82; 60%)	8.8 (2.5-15.7)	
II (n=27; 20%)	14.5 (5.5-18.8)	
III+IV (n=13; 9%)	10.3 (2.6-18.7	

EC, endometrial endometrioid cancer; NEEC, non-endometrial endometrioid cancer; EH, endometrial hyperplasia, AEH, atypical endometrial hyperplasia; MVD-CD34, microvessel denstity assessed with CD-34 antibody; HPF, high power field; BMI, Body Mass Index.

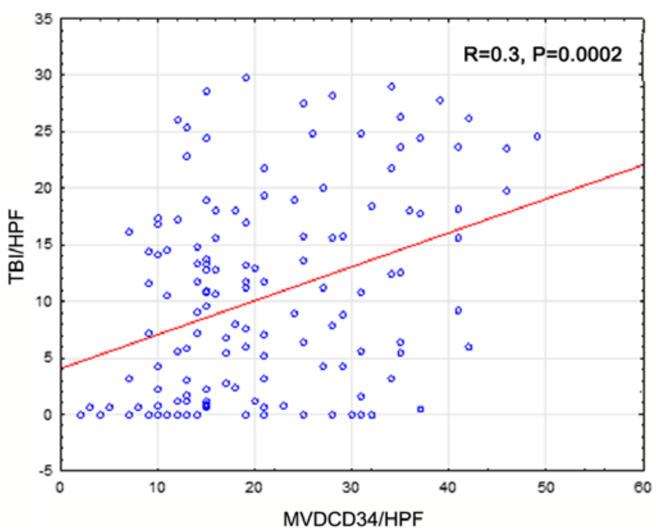

Figure 7. Correlation between microvessel density and tumor budding index in endometrial cancers. TBI, tumor budding index; MVD-CD34, microvessel density measured with antibody to CD34; HPF, high power field.

groups that could benefit from different types of surgery and chemo- or radiotherapy. Factors controlling growth of EC and its interactions with the surrounding uterine stromal microenvironment have recently gained increasing attention. Little is known about the regulation of TB and MVD in EC. Furthermore, in most of the studied cancer types such as lung, breast, colorectal and endometrial endometrioid cancers, the presence of tumor budding phenomenon was associated with lower survival rates (8-11).

A putative connection between TB and neoangiogenesis at the invasive tumor front has not been investigated yet. The present study revealed that TB, in terms of L5γ2 expression, increased gradually in endometrial lesions as they progressed from benign endometrial hyperplasia to AEH and finally to EC (EEC and NEEC). When examining tumor sections stained for L5γ2, clusters of undifferentiated malignant cells were observed in the tumor stroma, and these were located mainly in close proximity and ahead of the invasive front of the tumor. It was speculated that L5γ2 expression in tumor buds at the invasive front of endometrial neoplasia may be associated with the process of tumor differentiation. The present results indicated that there was a link between intratumoral MVD and endometrial tumor cell proliferation or TB. This was expected, since an adequate blood vascular system is required for effective tumor cell growth. Furthermore, when activated, endothelial cells can release various paracrine growth factors important for cancer cells, such as collagenases, urokinases and plasminogen activators (25). These factors enable tumors to spread into adjacent tissues and lymphovascular spaces.

Tumor buds could be regarded as a more invasive subpopulation of cells disseminated from the mass of the tumor. Therefore, they may have acquired the ability to invade the lymphatic system and metastasize to distant nodes. This hypothesis is in line with the results of a previous study by Koyuncuoglu *et al* (16) which reported a high prognostic value of TB in both endometrioid and non-endometrioid EC. In this study, cytokeratin C11 was used for improved visualization of numerous buds fused with stromal fibroblasts, yielding three- to four-fold higher tumor bud calculations compared with those using only histological hematoxylin and eosin (H&E)

staining. TB was detected by both H&E and cytokeratin C11 staining methods in 95 patients with primary EC. The authors demonstrated that a high TB count was strongly associated with undifferentiated tumors, advanced stage and decreased postoperative survival. Park *et al* (12) recently demonstrated that TB is associated with depth of invasion and higher FIGO grade in patients with EC, suggesting reduced histologic differentiation, lymphovascular invasion and lymph node involvement. The presence of TB is an independent parameter for the prediction of lymphovascular invasion in multivariate analysis and a significant factor for the prediction of lymph node metastasis in univariate analysis (12). Another similar study by Huang *et al* (31) demonstrated the prognostic significance of TB in early-stage cervical cancer (ESCC). They revealed that TB is an independent and unfavorable prognostic factor for patients with ESCC. The authors have suggested that following radical surgery, TB assessment could be promising for improved recurrence risk stratification. Marangon *et al* (20) evaluated L5γ2 expression in 57 patients with oral squamous cell carcinoma (OSCC) and its association with the intensity of TB and the density of stromal myofibroblasts. In their study, higher laminin-5 γ2 expression was associated with high-intensity TB and with a higher density of stromal myofibroblasts, suggesting that TB is associated with the establishment of an invasive phenotype of neoplastic cells and a permissive environment for tumor invasion in OSCC. Several lines of evidence suggest that the presence of TB may indicate the process of EMT, which is commonly associated with increased expression of molecules related to tumor invasion, such as matrix metalloproteinases, presence of L5γ2 in tumor cells and activation of the Wnt signaling pathway (32,33).

Despite numerous apparent molecular differences between EC types, histological criteria with or without selected IHC markers are still widely used for the initial diagnosis of a tumor type. However, since this cancer is a heterogeneous disease, there is a need to effectively stratify patients according to further treatment. A comprehensive characterization of the endometrial malignant tumors and their microenvironment is required for improved prediction of the effects of current treatment methods. However, the main obstacle is the absence of a consensus methodology, which is why it remains problematic to evaluate the real prognostic significance of TB in these malignant tumors. The most frequently used staining technique is the H&E method which may have some limitations and cannot without difficulty discriminate between real buds and other structures, where tissue disintegration gives the false impression of budding and these fragments should be excluded from counting (13,19).

Another important issue is associated with the counting method that should be applied. The present results indicated that the observed significant associations between clinical and histological variables were comparable for both counting methods. Nevertheless, it seems that quantification of 10 HPF should be used for optimal viewing of surgical samples, while the more restrictive '1 HPF' method may be reserved for small samples, such as endometrial biopsies (29).

To the best of our knowledge, the present study is one of the few that have reported the possible significance of TB estimation in patients with EC. The present results indicated for the first time that L5γ2 expression in tumor buds could be useful for the microscopic assessment of EC cell invasion. Previously, it has been demonstrated that the invasive front of the lesions exhibits a striking disorganization at the tumor architecture which may have been related to EMT and stem cell activation (32-34). In particular, the changes included loss of glandular features for differentiated cancer and loss of the trabecular characteristic for undifferentiated carcinoma (9). Most likely, this phenomenon helps to mobilize the EC cells from the main tumor mass, which is followed by invasion of host tissues through movement and their angiogenic activity (35). Our previous study revealed that the invasive potential of EC is related to the angiogenic phenotype of tumor tissues, and the ability of a tumor to develop its own microvascular network (27). In the current study, it was observed that, in EC, MVD increased with an increase in the number of TB structures. An implication of these observations is the possibility that at least in some endometrial malignant tumors, neoangiogenesis as assessed by MVD and TB are associated, and could be used as potential prognostic factors.

In patients with malignant endometrial lesions, a possibility of biopsy material examination is usually obtained prior to the decision on the type of surgery being made. It is not yet known if samples from the uterine cavity could be sufficient for both histology and TB assessment. Recently, Almangush *et al* (36) have reviewed all published reports on TB in diagnostic biopsies and matching cancer surgical specimens. They found that not only did all these studies show that evaluation of TB is easily applicable, but also that there is a significant association between the expression of TB in both surgical specimens and their corresponding biopsies specimens. Furthermore, the assessment of the TB phenomenon in diagnostic biopsies enabled a more accurate prognosis of lymphatic spread beyond the uterus and decreased survival of patients with EC. Unfortunately, to the best of our knowledge, there has been no study that compared TB in endometrial biopsies and material obtained after hysterectomy.

The strengths of the present study include the relatively large group of patients who received surgery for EC at one institution. We also are aware that the group with endometrial hyperplasias was much smaller and that it's usually much better to have 60-65 participants in both conditions rather than 17 in one condition and 120 in the other - even though the total number of participants is much greater in the second set-up. However, simple randomization can cause serious imbalances and in fact, theoretically, it's possible to end up with no participants in one of the groups. Prior to the present study, it was difficult to make predictions about if and how EMT and TB could influence neoangiogenesis in malignant endometrial tumors. This was the first study to report an association between the phenomenon of TB in EC and MVD assessment. The presented results could be important in furthering our understanding of the role of malignant EC cell interactions in the uterine stroma. Furthermore, since EMT inhibitors are already available, future studies should address the question if TB measurements could serve as potential markers for targeted anticancer therapy in patients with EC.

There were potential sources of bias in the present study, and the findings of the present study were subject to at least three limitations. First, the intensity of TB was arbitrarily categorized into low and high intensity. TB counts are also prone to subjective and interobserver variability. However, in the present study, only one experienced researcher (SC) was responsible for IHC microscopic preparations, detection, counting and reporting of the relevant data. Therefore, interobserver variability was not a possible confounding factor. Second, the present study used only a small number of different types of benign endometrial lesions. It was attempted to show the results in a relatively large group of EC cases, but it was also considered interesting to make comparisons with several cases of endometrial hyperplasia, both simple and atypical. It was considered unnecessary to discard data in order to perfectly balance the datasets, although the simple randomization used in the present study had less power, i.e. a lower chance to find systematic differences between the studied conditions. Third, despite investigating 120 EC cases, the present analysis remained hampered by a lack of survival analysis due to the relatively small number of non-endometroid EC cases. An explanation for omitting this parameter is that the survival data have already been published in two other studies (12,16), and that the survival analysis in cases of EC must take into consideration >10 years since the collection of data. All the aforementioned limitations mean that the findings of the present study need to be interpreted cautiously.

Despite the relatively limited sample size, the present study also offers valuable insights into EC microangiogenesis and its possible association with the TB phenomenon. As has been suggested in colorectal cancer, TB may be applied in the future as an additional quantitative prognostic factor to facilitate the management of patients with EC in three possible clinical scenarios. First, if TB and/or increased MVD are identified in uterine endometrial samples prior to surgery, the patients could benefit from pelvic lymph node dissection. Second, the presence of intensive high-grade TB may be considered as an additional indication that neoadjuvant chemotherapy could increase the chances of survival of a patient. Third, the results of preoperative endometrial biopsy and the finding of intensive TB could be used to recommend neoadjuvant chemotherapy for patients and maybe, if validated, could predict the regression of these malignant tumors (37,38). TB grade could potentially help discriminate patients into groups with worse or better prognosis, even in cases of advanced-stage EC. Further studies are required to examine the molecular factors and mechanisms of TB and its possible association with microangiogenesis at the invasive front of EC.

In summary, it was concluded that TB assessment using laminin expression combined with MVD measurements using a CD34 antibody provided novel insights into whether these markers could be novel and valuable indicators of tumor aggressiveness in patients with EC. Additionally, it was hypothesized that the present results highlight the potential usefulness of the TB phenomenon and appeared to identify the behavior of aggressive EC. An implication of this is the possibility that both markers combined could be further applied in patients with endometrial malignant tumors to facilitate improved and personalized treatment planning.

Acknowledgements

The authors would like to thank Dr Jadwiga Sierocińska-Sawa, Head of the Department of Pathology of the Clinical Hospital No. 1 in Lublin (Lublin, Poland), for her excellent expert assistance with the tumor immunopathological studies and Professor Agata Smolen, Head of the Department of Biostatistics of the Medical University of Lublin for her assistance with the statistical analysis. This abstract was presented at the 2016 International Gynecological Cancer Society Meeting in Lisbon, Portugal (October 29-31, 2016), and was published at 16th Biennial Meeting of the International Gynecologic Cancer Society, International Journal of Gynecologic Cancer 2016; 26:1-1193.

Funding

The current study was supported in part by a grant (grant no. DS119) from the Medical University of Lublin.

Availability of data and materials

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

Authors' contributions

TK was involved in project development, data collection, data analysis, manuscript preparation and editing. TL participated in project development, data collection, data analysis and manuscript preparation. NS was involved project development, data analysis and manuscript editing. GG developed the project and analyzed the data. SC performed data collection, immunohistochemical studies and data analysis. MC was involved in project development, data collection and manuscript editing. AC participated in project development, data analysis, manuscript editing and supervision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Bioethical Committee of the Medical University of Lublin. Oral patient consent was obtained for participation.

Patient consent for publication

Oral patient consent was obtained for publication.

Competing interests

The authors declare that they have no competing interests.

References

1. Torre LA, Islami F, Siegel RL, Ward EM and Jemal A: Global cancer in women: Burden and trends. Cancer Epidemiol Biomarkers Prev 26: 444-457, 2017.
2. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2018. CA Cancer J Clin 68: 7-30, 2018.
3. Suarez AA, Felix AS and Cohn DE: Bokhman redux: Endometrial cancer 'types' in the 21st century. Gynecol Oncol 144: 243-249, 2017.
4. Emons G, Beckmann MW, Schmidt D and Mallmann P; Uterus commission of the Gynecological Oncology Working Group (AGO): New WHO Classification of Endometrial Hyperplasias. Geburtshilfe Frauenheilkd 75: 135-136, 2015.

5. Felix AS, Yang HP, Bell DW and Sherman ME: Epidemiology of endometrial carcinoma: Etiologic importance of hormonal and metabolic influences. Adv Exp Med Biol 943: 3-46, 2017.
6. Colombo N, Creutzberg C, Amant F, Bosse T, González-Martín A, Ledermann J, Marth C, Nout R, Querleu D, Mirza MR, *et al*; ESMO-ESGO-ESTRO Endometrial Consensus Conference Working Group: ESMO-ESGO-ESTRO consensus conference on endometrial cancer: Diagnosis, treatment and follow-up. Int J Gynecol Cancer 26: 2-30, 2016.
7. Hase K, Shatney C, Johnson D, Trollope M and Vierra M: Prognostic value of tumor 'budding' in patients with colorectal cancer. Dis Colon Rectum 36: 627-635, 1993.
8. Ueno H, Murphy J, Jass JR, Mochizuki H and Talbot IC: Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. Histopathology 40: 127-132, 2002.
9. Yamaguchi Y, Ishii G, Kojima M, Yoh K, Otsuka H, Otaki Y, Aokage K, Yanagi S, Nagai K, Nishiwaki Y, *et al*: Histopathologic features of the tumor budding in adenocarcinoma of the lung: Tumor budding as an index to predict the potential aggressiveness. J Thorac Oncol 5: 1361-1368, 2010.
10. Gujam FJ, McMillan DC, Mohammed ZM, Edwards J and Going JJ: The relationship between tumour budding, the tumour microenvironment and survival in patients with invasive ductal breast cancer. Br J Cancer 113: 1066-1074, 2015.
11. Lugli A, Kirsch R, Ajioka Y, Bosman F, Cathomas G, Dawson H, El Zimaity H, Fléjou JF, Hansen TP, Hartmann A, *et al*: Recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) 2016. Mod Pathol 30: 1299-1311, 2017.
12. Park JY, Hong DG, Chong GO and Park JY: Tumor budding is a valuable diagnostic parameter in prediction of disease progression of endometrial endometrioid carcinoma. Pathol Oncol Res 25: 723-730, 2019.
13. Lugli A, Vlajnic T, Giger O, Karamitopoulou E, Patsouris ES, Peros G, Terracciano LM and Zlobec I: Intratumoral budding as a potential parameter of tumor progression in mismatch repair-proficient and mismatch repair-deficient colorectal cancer patients. Hum Pathol 42: 1833-1840, 2011.
14. Grigore AD, Jolly MK, Jia D, Farach-Carson MC and Levine H: Tumor budding: The name is EMT. Partial EMT. J Clin Med 5: E51, 2016.
15. Gurzu S, Turdean S, Kovecsi A, Contac AO and Jung I: Epithelial-mesenchymal, mesenchymal-epithelial, and endothelial-mesenchymal transitions in malignant tumors: An update. World J Clin Cases 3: 393-404, 2015.
16. Koyuncuoglu M, Okyay E, Saatli B, Olgan S, Akin M and Saygili U: Tumor budding and E-Cadherin expression in endometrial carcinoma: Are they prognostic factors in endometrial cancer? Gynecol Oncol 125: 208-213, 2012.
17. Hallmann R, Horn N, Selg M, Wendler O, Pausch F and Sorokin LM: Expression and function of laminins in the embryonic and mature vasculature. Physiol Rev 85: 979-1000, 2005.
18. Miyazaki K: Laminin-5 (laminin-332): Unique biological activity and role in tumor growth and invasion. Cancer Sci 97: 91-98, 2006.
19. Masuda R, Kijima H, Imamura N, Aruga N, Nakazato K, Oiwa K, Nakano T, Watanabe H, Ikoma Y, Tanaka M, *et al*: Laminin-5γ2 chain expression is associated with tumor cell invasiveness and prognosis of lung squamous cell carcinoma. Biomed Res 33: 309-317, 2012.
20. Marangon Junior H, Rocha VN, Leite CF, de Aguiar MC, Souza PE and Horta MC: Laminin-5 gamma 2 chain expression is associated with intensity of tumor budding and density of stromal myofibroblasts in oral squamous cell carcinoma. J Oral Pathol Med 43: 199-204, 2014.
21. Folkman J: Role of angiogenesis in tumor growth and metastasis. Semin Oncol 29 (Suppl 16): 15-18, 2002.
22. Demir R, Yaba A and Huppertz B: Vasculogenesis and angiogenesis in the endometrium during menstrual cycle and implantation. Acta Histochem 112: 203-214, 2010.
23. Mahecha AM and Wang H: The influence of vascular endothelial growth factor-A and matrix metalloproteinase-2 and -9 in angiogenesis, metastasis, and prognosis of endometrial cancer. OncoTargets Ther 10: 4617-4624, 2017.
24. Viallard C and Larrivée B: Tumor angiogenesis and vascular normalization: Alternative therapeutic targets. Angiogenesis 20: 409-426, 2017.
25. Nagy JA and Dvorak HF: Heterogeneity of the tumor vasculature: The need for new tumor blood vessel type-specific targets. Clin Exp Metastasis 29: 657-662, 2012.
26. Zalewski K, Doniec J, Baranowski W and Bidziński M: The revised FIGO staging system for uterine malignancies. Ginekol Pol 8: 778-782, 2010.
27. Czekierdowski A, Czekierdowska S, Czuba B, Cnota W, Sodowski K, Kotarski J and Zwirska-Korczala K: Microvessel density assessment in benign and malignant endometrial changes. J Physiol Pharmacol 59 (Suppl 4): 45-51, 2008.
28. Weidner N: Tumour vascularity and proliferation: Clear evidence of a close relationship. J Pathol 189: 297-299, 1999.
29. Karamitopoulou E, Zlobec I, Kölzer V, Kondi-Pafiti A, Patsouris ES, Gennatas K and Lugli A: Proposal for a 10-high-power-fields scoring method for the assessment of tumor budding in colorectal cancer. Mod Pathol 26: 295-301, 2013.
30. de Boer SM, Powell ME, Mileshkin L, Katsaros D, Bessette P, Haie-Meder C, Ottevanger PB, Ledermann JA, Khaw P, Colombo A, *et al*; PORTEC study group: Toxicity and quality of life after adjuvant chemoradiotherapy versus radiotherapy alone for women with high-risk endometrial cancer (PORTEC-3): An open-label, multicentre, randomised, phase 3 trial. Lancet Oncol 17: 1114-1126, 2016.
31. Huang B, Cai J, Xu X, Guo S and Wang Z: High-grade tumor budding stratifies early-stage cervical cancer with recurrence risk. PLoS One 11: e0166311, 2016.
32. Zlobec I and Lugli A: Epithelial mesenchymal transition and tumor budding in aggressive colorectal cancer: Tumor budding as oncotarget. Oncotarget 1: 651-661, 2010.
33. Mirantes C, Espinosa I, Ferrer I, Dolcet X, Prat J and Matias-Guiu X: Epithelial-to-mesenchymal transition and stem cells in endometrial cancer. Hum Pathol 44: 1973-1981, 2013.
34. Koelzer VH, Zlobec I, Berger MD, Cathomas G, Dawson H, Dirschmid K, Hädrich M, Inderbitzin D, Offner F, Puppa G, *et al*: Tumor budding in colorectal cancer revisited: Results of a multicenter interobserver study. Virchows Arch 466: 485-493, 2015.
35. Mazurek A and Kuć P: Angiogenesis-prognostic factor in patients with endometrial cancer. Ginekol Pol 76: 838-845, 2005 (In Polish).
36. Almangush A and Youssef O, M. Pirinen M, Sundström J, Leivo I and Mäkitie AA: Does evaluation of tumour budding in diagnostic biopsies have a clinical relevance? A systematic review. Histopathology 74: 536-544, 2019.
37. Papa A, Zaccarelli E, Caruso D, Vici P, Benedetti Panici P and Tomao F: Targeting angiogenesis in endometrial cancer - new agents for tailored treatments. Expert Opin Investig Drugs 25: 31-49, 2016.
38. Lheureux S and Oza AM: Endometrial cancer-targeted therapies myth or reality? Review of current targeted treatments. Eur J Cancer 59: 99-108, 2016.