TUMOR/NORMAL COUNTERPART MICROVESSEL DENSITY RATIO HAS A BETTER CORRELATION WITH CLINICOPATHOLOGIC PARAMETERS IN ENDOMETRIAL CARCINOMA THAN TUMOR MICROVESSEL DENSITY ALONE

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Introduction

Endometrial cancer is the most common gynecologic malignancy in the Western world, and the second most common in Taiwan. The estimated incidence increases in more industrialized countries, so endometrial cancer will play an increasingly important role in gynecologic malignancies in the next decades. The clinical course and prognosis of endometrial cancer are mainly associated with tumor stage, histologic subtype and grade, age, and hormone receptor status [1]. Clinical and pathologic evidence for endometrial carcinoma have led to the development of two classifications: type I (estrogen-related) and type II (non-estrogen-related) [2]. Type I is found in relatively young, perimenopausal women with an estrogen-related risk history and a history of endometrial hyperplasia [2]. In contrast, type II is seen in older, postmenopausal women with no estrogen-related risk history and is not preceded or accompanied by hyperplasia [2,3]. Angiogenesis status and/or angiogenic regulators related to the different pathogeneses of endometrial carcinomas, especially in the highly variable expression of microvessel density (MVD), are not known.

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is a physiologic process in adult cyclic endometrial changes [4], which are highly controlled processes mediated by angiogenic and anti-angiogenic factors, under the direct or indirect influence of ovarian hormones [5]. Angiogenesis is also important for disease processes including dysfunctional uterine bleeding, response to exogenous hormonal treatment, bleeding associated with intrauterine contraceptive devices, uterine leiomyomata, endometriosis, complex endometrial hyperplasia and endometrial carcinoma [6]. Tumor angiogenesis is essential for tumor growth and metastasis [7]. In order to grow beyond minimal size and to metastasize, tumors need to induce the growth of new blood vessels. Intratumoral angiogenesis status (measured by MVD) has been proposed as a prognostic factor in endometrial carcinoma [7–10], as have angiogenic factors (e.g. vascular endothelial growth factor [VEGF]) and anti-angiogenic factors (e.g. thrombospondin-1 [TSP-1]) [10,11]. Down-regulation of angiogenesis inhibitors plays a critical role in the angiogenic switch in several tumor types, including bladder cancer, breast cancer, glioblastoma and fibrosarcoma [12–14]. We found that MVD in normal cervical epithelia and low-grade squamous intraepithelial lesions (LSIL) were significantly lower than those in high-grade squamous intraepithelial lesions (HSIL) and squamous cell carcinoma [15]. MVD is negatively associated with the expression of the angiogenesis inhibitor TSP-1. Down-regulation of an angiogenesis inhibitor and the switch to the angiogenic phenotype occurs during the transition from LSIL to HSIL [15].

The aim of this study was to correlate angiogenesis status, as measured using the MVD of the tumor alone (MVD-T) or the tumor/normal counterpart MVD ratio (MVD T/N ratio), with the clinicopathologic parameters of endometrial carcinoma with innate highly variable expression of MVD. We also tried to understand the relationship between estrogen receptor (ER) and progesterone receptor (PR) status and angiogenesis, because angiogenesis regulators are controlled by progesterone and estrogen [5].

Materials and Methods

From July 2000 to June 2002, patients in the Department of Obstetrics and Gynecology of Chi Mei Foundation Hospital with endometrial carcinoma were recruited into this study. All patients underwent total abdominal hysterectomy, bilateral salpingo-oophorectomy and pelvic and/or para-aortic lymph node dissection or sampling. Patients with lymph node metastasis or deep myometrial invasion (more than one-half depth) also received postoperative adjuvant irradiation. Stage was determined using the International Federation of Gynecology and Obstetrics classification [16]. Patients with tumors of histologic types other than adenocarcinoma and adenoacanthoma were excluded. Histologic grade used a three-level system: grade 1 (> 95% glandular formation), grade 2 (5–50% solid growth pattern) and grade 3 (> 50% solid pattern). Other clinicopathologic parameters including myometrial invasion, cervical involvement, lymph node metastasis, menopausal status, histologic type, and ER and PR status were recorded.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded tissue blocks were cut into serial sections of 4 μm thickness. One section from each sample was stained with hematoxylin and eosin to confirm the histologic diagnosis, and adjacent sections were stained with anti-34 antibody (1:50; Serotec, Raleigh, NC, USA), ER and PR antibodies (1:30; Novocastra, Newcastle-upon-Tyne, UK) using standard immunoperoxidase methods. Slides were incubated with the BioGenex supersensitive immunodetection system (San Ramon, CA, USA). Diaminobenzidin tetrahydrochloride was used as the chromogen. Human placenta, which expresses CD-34, was used as the positive control. A negative control, in which the primary antibody was substituted with the same concentration of the appropriate immunoglobulin G (IgG), was included in each staining run. All slides were reviewed by a pathologist (CCT) and associates, who were blinded to the patients' status.

Microvessel density

Microscopic sections were scanned at low magnification (× 40) to identify areas with a high density of capillaries. Using anti-34 antibody immunostaining to highlight endothelial cells, MVD was determined by counting the number of vessels in a × 100 field (× 10 objective lens and × 10 ocular lens, approximately 0.29 mm^2/field). The number of vessels per field was calculated by averaging the total number obtained from six fields in two different blocks for each tumor. Vessels were defined as any positively stained single cell or cluster of cells. Both tumor and normal tissue were counted for comparison. The MVD T/N ratio was defined as the ratio of MVD of tumor lesions and normal counterparts obtained in two high-power-fields from the tumor lesion site from the same patient. The relationships among MVD-T, MVD T/N ratio and clinicopathologic parameters were analyzed.

Statistical analysis

All values are reported as mean ± standard deviation (SD). Due to the small sample size and ordinal property of some parameters, the MVD and MVD T/N ratio were subjected to non-parametric data analysis. The Wilcoxon rank sum test was used to evaluate the significance between different subgroups. A *p* value of less than 0.05 was considered statistically significant.

Results

The mean age of the 20 women was 54.5 years with a SD of 10.8 years. The tumor MVD was 46.8, the MVD of the normal counterpart was 39.9, giving an MVD T/N ratio of 1.17 (Table 1). Most cases (60%) were histologic grade 1, most (70%) had myometrium invasion of less than one-half, and most (95%) had no cervical involvement (Table 2). Lymph node metastasis was absent in nine cases (45%) and present in four (20%); no data were available for seven cases (35%). Most patients (80%) did not have lymphovascular space involvement. Seven patients (35%) were premenopausal and 13 (65%) were postmenopausal. Most patients (90%) had endometrioid tumor and most (65%) had surgicopathologic stage Ib disease. The distributions of ER and PR status were similar (Table 2).

Table 1. Microvessel densities (MVDs) of the tumor (MVD-T) and the ratio of the tumor and normal counterpart MVD ratio (MVD T/N ratio)

Variable	Mean ± SD
Age	54.5 ± 10.8
MVD-T	46.8 ± 18.7
MVD-N	39.9 ± 14.3
MVD T/N ratio	1.17 ± 0.21

SD = standard deviation; N = normal counterpart.

Table 2. Clinicopathologic parameters among the 20 patients

	n (%)
Histologic grade	
1	12 (60)
2	3 (15)
3	5 (25)
Myometrial invasion	
–	1 (5)
< 1/2	14 (70)
≥ 1/2	5 (25)
Cervical involvement	
–	19 (95)
+	1 (5)
Lymph node involvement	
–	9 (45)
+	4 (20)
N/A	7 (35)
Lymphovascular space involvement	
–	16 (80)
+	4 (20)
Menopausal status	
Pre	7 (35)
Post	13 (65)
Type	
Endometrioid	18 (90)
Serous	2 (10)
Stage	
Ia	1 (5)
Ib	13 (65)
Ic	1 (5)
II	3 (15)
III	2 (10)
ER	
+	9 (45)
–	11 (55)
PR	
+	9 (45)
–	11 (55)
ER, PR	
+, +	7 (35)
–, –	9 (45)
(+, –) or (–, +)	4 (20)

– = negative; + = positive; N/A = not available; ER = estrogen receptor; PR = progesterone receptor.

MVD and clinicopathologic parameters

The relationship between MVD and clinicopathologic parameters are shown in Table 3. There was no significant relationship between MVD-T (× 200 field) and any clinicopathologic parameter. However, the MVD T/N ratio was significantly higher in tumors of higher histologic grade (grade 2 and 3; $p = 0.010$), with lymphovascular space involvement ($p = 0.029$) and of serous type ($p = 0.021$), compared with tumors of histologic grade 1, without lymphovascular space involvement and of endometrioid type. The MVD T/N ratio was higher in ER-negative ($p = 0.002$), PR-negative ($p = 0.016$) and both receptor-negative tumors ($p = 0.002$) compared with ER-positive, PR-positive and both receptor-positive tumors.

Characteristic pictures are shown in the Figure. Microvessels were much less common in endometrioid tumors (Figure 1A) than in serous tumors (Figure 1B). Most endometrioid tumors were PR-positive (Figure 1C) while both serous tumors were PR-negative (Figure 1D).

Discussion

MVD counting techniques have been widely used to assess the vasculature in tumors. MVD counts assess the presence of blood vessels but do not give an indication of the degree of angiogenesis and the functional status of the tumor neovasculature [17]. MVD has been reported to be associated with unfavorable histopathologic features, or as a predictor of tumor progression or metastasis [18,19]. However, we did not see any dif-

Table 3. Correlation of clinicopathologic parameters and microvessel density (MVD)

	N	MVD-T		T/N ratio	
		Mean ± SD	*p*	Mean ± SD	*p*
Histologic grade					
1	12	40.3 ± 15.9	0.057	1.07 ± 0.19	0.010
2/3	8	56.6 ± 19.3		1.31 ± 0.18	
Myometrial invasion					
< 1/2	15	45.5 ± 20.1	0.349	1.16 ± 0.18	0.933
≥ 1/2	5	50.8 ± 14.9		1.18 ± 0.32	
Lymph node involvement					
–	9	49.3 ± 25.3	0.710	1.13 ± 0.20	0.604
+	4	52.3 ± 16.8		1.23 ± 0.35	
Lymphovascular space involvement					
–	16	42.7 ± 15.6	0.099	1.13 ± 0.22	0.029
+	4	63.5 ± 23.1		1.32 ± 0.06	
Menopausal status					
Pre	7	38.2 ± 7.79	0.157	1.09 ± 0.18	0.183
Post	13	51.5 ± 21.4		1.21 ± 0.22	
Type					
Endometrioid	18	45.4 ± 19.2	0.211	1.13 ± 0.19	0.021
Serous	2	59.6 ± 4.95		1.48 ± 0.15	
Stage					
I	15	46.0 ± 20.0	0.612	1.14 ± 0.18	0.349
> I	5	49.4 ± 16.0		1.24 ± 0.31	
ER					
–	11	50.8 ± 18.7	0.370	1.30 ± 0.13	0.002
+	9	42.0 ± 18.5		1.01 ± 0.18	
PR					
–	11	49.3 ± 20.1	0.710	1.26 ± 0.21	0.016
+	9	43.9 ± 17.6		1.05 ± 0.17	
ER, PR					
+, +	7	45.4 ± 19.9	0.606	1.02 ± 0.17	0.002
–, –	9	53.5 ± 19.7		1.33 ± 0.13	

T = tumor; T/N ratio = tumor/normal counterpart ratio; SD = standard deviation; – = negative; + = positive; ER = estrogen receptor; PR = progesterone receptor. Wilcoxon rank sum test was used to evaluate the correlation of clinicopathologic findings and MVD, or MVD T/N ratio.

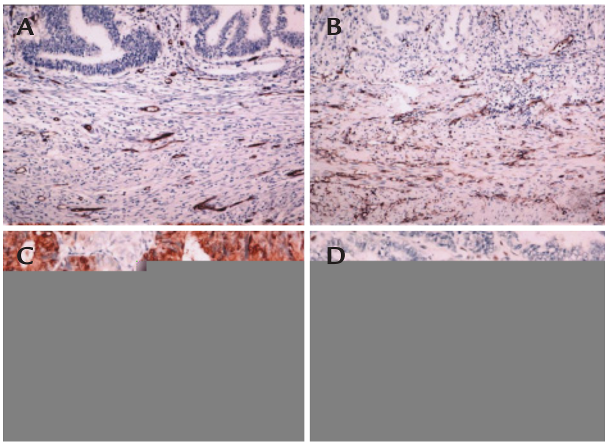

Figure. Representative immunohistochemistry staining showing microvessel density (MVD) and progesterone receptor (PR) status of endometrioid (A, C) and serous endometrial cancer (B, D). Note the low tumor/normal counterpart MVD ratio (MVD T/N ratio) in endometrioid (A) and the high MVD T/N ratio in serous endometrial cancer (B). Endometrioid endometrial cancer is positive for PR (C) while serous endometrial cancer is negative for PR (D). (A, B × 100; C, D × 200.)

ference among our patients. This may be because of the inborn heterogeneity in endometrial cancer and the limited case numbers. Highly variable expression of MVD in different surgical specimens is not uncommon [20]. In this situation, some novel techniques and methods can be used to adjust for highly variable angiogenesis. Recently, color Doppler ultrasound has been used as an *in vivo* model to correlate histologic and biologic findings with angiogenesis status [21]. In the MVD T/N ratio measurement system, the normal counterpart can be used as an internal control to adjust for inborn variation. We found that MVD T/N ratios were significantly related to clinicopathologic parameters including histologic grade, lymphovascular space involvement, histologic type and ER and PR status, and were better predictors of clinicopathologic parameters in endometrial cancer than MVDs of tumor lesions only.

The timing of the angiogenic switch during endometrial carcinogenesis remains unknown. In this study, MVD T/N ratios were significantly higher in tumors of high histologic grades (grade 2 and 3) than of low grade (grade 1). Thus, we postulate that the angiogenic switch happens during the transition from grade 1 to higher grades. Angiogenesis is a critical factor in the progression of many solid tumors. In many cancer models, the angiogenic switch occurs some time during tumorigenesis [22]. When enough tumor cells become angiogenic, the tumor can expand progressively and shed metastatic cells. This angiogenic switch has recently been quantified for human breast cancer, as well as for prostate cancer [23]. In cervical multi-step carcinogenesis, MVD is significantly higher in more advanced lesions (CIN III) than low-grade lesions (condyloma and CIN I) [24]. This means that the angiogenic switch in cervical carcinogenesis occurs during the transition from LSIL to HSIL [15]. The angiogenic switch during endometrial carcinogenesis may manifest in a similar pattern.

Lymphovascular space involvement was significantly associated with MVD, which is compatible with the phenomenon that the degradation of extracellular matrix is a crucial step in angiogenesis [25]. Iurlaro et al evaluated MVD and matrix metalloproteinase-2 (MMP-2, gelatinase A) and MMP-9 (gelatinase B) mRNA using immunohistochemistry and *in situ* hybridization. Angiogenesis and extracellular matrix degradation occurred simultaneously with endometrial upgrading and advancing depth of invasion [26]. The changes in angiogenesis and expression of extracellular matrix-degrading enzymes were substantiated during tumor changeover and progression.

Endometrioid and serous cancers have different underlying pathogenesis, usually either estrogen-related (endometrioid) or non-estrogen-related (serous) [2]. Estrogen dependency in uterine endometrial cancers involves complicated tumor biology [27]. Our MVD T/N ratio results may explain, at least in part, the different pathogenesis.

Angiogenesis is regulated by angiogenesis activators such as VEGF and angiogenesis inhibitors such as thrombospondins, which are under the control of the ovarian hormones estrogen and progesterone [5,28]. Both angiogenic and anti-angiogenic factors are responsible for angiogenic balance [29]. Angiogenesis regulators (e.g. TSP-1) may affect the behavior of endometrial cancer, e.g. cervical and lymphovascular space involvement, and are associated with an angiogenic phenotype and a prognostic marker in endometrial cancer [30]. The expression of angiogenesis regulators is controlled by progesterone [5]. The production of TSP-1 at later stages of the endometrial cycle is linked to the inhibition of vessel formation and is progesterone-dependent in this tissue [5]. VEGF can be up-regulated by estrogen and down-regulated by progesterone [28]. The expression of VEGF is involved in the promotion of angiogenesis in endometrial cancer. In addition, VEGF contributes to the aggressive potential of high-grade tumors or certain histologic subtypes with unfavorable prognosis through the induction of angiogenesis [31]. A high level of ER-α is believed to be favorable in the prognosis and treatment of certain female cancers [32]. Unlike estradiol, high levels of ER-α significantly inhibit the growth of tumors xenografted from endometrial cancer cells, because of its inhibitory effect on angiogenic pathways [32].

In conclusion, to know specific angiogenic patterns of various disease processes might improve anti-angiogenic medications in therapies for benign and neoplastic diseases of the endometrium [6].

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