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Quantification of angiogenesis by the Chalkley method and its prognostic significance in epithelial ovarian cancer ☆

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ABSTRACT

Aim: The aim of the present study was to clarify prognostic role of angiogenesis in epithelial ovarian cancer.
Methods: Quantification of angiogenesis was performed by the Chalkley method after immunostaining of 175 epithelial ovarian cancer specimens with an antibody against CD34.
Results: The Chalkley count was categorised into two groups according to the median value: low <8 or high ⩾8. The low Chalkley count correlated significantly with serous and clear cell histological subtype of the tumour ($p < 0.0005$), whereas there existed no association with FIGO (International Federation of Gynecology and Obstetrics) stage, histological grade, presence of primary residual tumour, age at diagnosis, or chemotherapy response. In univariate analysis, the high Chalkley count predicted poor overall survival in the subgroup of patients with FIGO stages III–IV tumours ($p = 0.007$) but not in the entire study cohort. However, in multivariate analysis, the Chalkley count was found to be an independent predictor of death from ovarian cancer in the entire study cohort ($p = 0.044$, RR = 1.50, 95% CI 1.01–2.21) as well as in the subgroup of FIGO stages III–IV tumours ($p = 0.046$, RR = 1.58, 95% CI 1.01–2.46) together with the presence of primary residual tumour ($p < 0.0005$, RR = 5.10, 95% CI 3.02–8.62, and $p = 0.002$, RR = 4.28, 95% CI 1.34–13.73, respectively).
Conclusions: The Chalkley count seems to be suitable for evaluation of angiogenesis and to have prognostic significance in ovarian cancer.

1. Introduction

Angiogenesis means the formation of new capillaries from the existing vascular network, and is essential for tumour growth and metastatic capacity. Neovascularisation of a tumour is required to provide essential nutrients beyond the limit of simple diffusion, and allow for tumour growth beyond 2 mm^3,[1] although tumours may be able to grow also without

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neovascularisation if they find a suitable preexisting vascular bed.[2] The regulation of angiogenesis is complex, and controlled by the balance between inhibitors and stimulators of angiogenesis.[3]

Association of angiogenesis with patient outcome has been studied widely in different carcinomas, including ovarian cancer, but mainly with relatively small materials, which have left the prognostic significance of tumour angiogenesis still controversial in epithelial ovarian cancer also in studies including more than 90 patients.[4–7] The discrepancies in results may originate at least partly from differences in the study materials. In addition, a lack of standardised immunohistochemical techniques hampers the comparison of studies. For example, antibodies to CD34,[4–6,8–10] CD31,[7,11,12] von Willebrand factor (Factor VIII)[13,14] and Ulex[15] have been used to detect vascular structures in prognostic ovarian carcinoma studies. CD34 is a cell surface protein that is selectively expressed by human hematopoietic progenitor cells and vascular endothelial cells,[16,17] and it has been shown to mark tumour vessels also in the solid cancers of ovary.[18] Since Weidner and colleagues estimated the microvessel density in the most vascularised area ("hot-spot") in their pioneering work in 1991,[19] the same technique with slight modifications has been used widely to assess the prognostic value of angiogenesis in various types of carcinomas. Afterwards, the Chalkley method based on Chalkley eyepiece graticule was introduced to provide a quicker and more objective procedure for measuring tumour vascularity.[20] Currently, the Chalkley assay with CD34 immunostaining has been suggested as a standard method for angiogenesis quantification in solid tumour sections in an international consensus report,[21] although the basis for the consensus has been questioned by others.[22] No previous studies on angiogenesis in ovarian cancer were found in the literature, analysed with the original Chalkley method. Therefore, we decided to study the expression of CD34 and its prognostic significance using the Chalkley method in epithelial ovarian cancer.

2. Materials and methods

2.1. Patients

We analysed 175 specimens from patients treated and diagnosed for epithelial ovarian malignancy at Kuopio University Hospital and Jyväskylä Central Hospital, Finland, between 1976 and 1992, with a follow-up until January 2004. Patients who were given any treatment before operation were excluded, as were also patients who were not operated on. Patients who died because of any post-operative complications (deaths during one month after operation) were not included in survival analyses. In addition, 32 metastatic samples were available for CD34 analysis. The research was approved by the research ethical committee of Kuopio University and Kuopio University Hospital.

Staging of the tumours was based on standards of the International Federation of Gynecology and Obstetrics (FIGO). In addition to operative therapy, 152 (87%) patients received postoperative chemotherapy, 3 (2%) patients received postoperative radiotherapy, 17 (9%) patients received both of these adjuvant therapies and 3 (2%) patients had no other treatment. In January 2004, 38 (22%) patients were still alive, and from these patients disease recurrence was observed in 6 patients (16%), no recurrence in 31 patients (82%), and data on recurrence were missing from one patient (2%). The median follow-up time for all patients (n = 175) was 23 months (range, 1–327 months), and for patients still alive (n = 38) 122 months (range, 71–327 months). The clinicopathological characteristics of the patients are summarised in Table 1.

2.2. Histology

Histological type and grade were reevaluated according to the WHO (World Health Organization) classification, as described previously.[23]

Table 1 – Clinicopathological characteristics of the patients (N = 175)

Variable	N	%
Age, years		
Median	61	
Range	21–83	
FIGO stage		
I	31	18
II	27	15
III	95	54
IV	22	13
Histological type		
Serous	71	40
Mucinous	17	10
Endometrioid	46	26
Clear cell	17	10
Miscellaneous[a]	24	14
Histological grade		
1	23	13
2	63	36
3	89	51
Primary residual tumour		
None	53	30
⩽2 cm	36	21
>2 cm	70	40
No data	16	9
Adjuvant chemotherapy		
Platinum-containing therapy	155	89
Non-platinum therapy	14	8
None	6	3
Chemotherapy response		
PR	28	16
CR	86	49
SD	14	8
PD	38	22
No chemotherapy	6	3
No data	3	2
End state		
Dead	137	78
Alive	38	22

Abbreviations: PR, partial response; CR, complete response; SD, stable disease; PD, progressing disease.
a Includes 9 mixed epithelial and 15 unclassified epithelial.

2.3. Immunohistochemistry

Five-μm-thick paraffin-embedded tissue sections of all tumours were stained immunohistochemically. After deparaffinisation and rehydration, the sections were heated in a microwave oven for 3 × 5 min in a citrate buffer (pH 6.0), then incubated in the citrate buffer for 18 min and washed twice for 5 min with phosphate buffered saline (PBS). 5% hydrogen peroxide was used for 5 min to block endogenous peroxidase activity, followed by washings with water for 2 × 5 min and with PBS for 2 × 5 min. Non-specific binding was blocked with 1.5% normal horse serum in PBS for 25 min at room temperature. The samples were incubated overnight at 4 °C with the primary antibody for CD34 (mouse monoclonal anti-human CD34, Anti-HPCA-1, clone My10, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA, 1:200 dilution). Instead of the primary antibody, the negative control was incubated with 1% BSA in PBS. Next, the slides were washed with PBS for 2 × 5 min and incubated with the biotinylated secondary antibody (anti-mouse IgG; ABC Vectastain Elite kit, Vector Laboratories, Burlingame, CA, USA) for 35 min at room temperature. After this, slides were washed with PBS for 2 × 5 min, incubated for 45 min in preformed avidin-biotinylated peroxidase complex (ABC Vectastain Elite kit, Vector Laboratories, Burlingame, CA, USA) and washed twice for 5 min with PBS. The colour was developed with diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO, USA). The slides were counterstained with Mayer's haematoxylin, washed, dehydrated, cleared and mounted with DePex (BDH, Poole, UK). Hemangioma was used as an external positive control showing strong staining. The negative control sample (primary antibody omitted) did not show any signal.

2.4. Evaluation of angiogenesis by the Chalkley counting

Angiogenesis in ovarian cancer samples was evaluated by two observers (KAS and MAA) without knowledge of the clinical outcome. We evaluated tumour vascularisation using the Chalkley method, which has been described in detail previously.[20] Briefly, the CD34-stained sections were scanned at low magnification (12.5× ocular, 4× and 10× objective) for the most vascular areas within the tumour section, and three areas of the highest vascularity (hot spots) were chosen subjectively. A 25-point Chalkley eyepiece graticule (Graticules, Pyser-SGI Limited, United Kingdom) was applied to each hot spot at higher magnification (12.5× ocular and 20× objective, corresponding to an area of 0.322 mm^2), and oriented to permit the maximum number of points to hit on or within immunohistochemically stained microvessels. The Chalkley count was expressed as the mean value of the three counts for each tumour and further divided into two groups according to the median value of Chalkley count: low <8 or high ⩾8. Reproducibility of the method was investigated by reanalysing 50 randomly chosen tumour samples. The assessment of each tumour took approximately 10 min.

2.5. Statistical analysis

Statistical analyses were carried out using the SPSS 11.5 for Windows program package (SPSS Inc., Chicago, IL, USA). The associations of the Chalkley count with other clinicopathological parameters were tested by the Spearman correlation test and Wilcoxon test for continuous variables, and frequency tables were analysed using a χ^2 test. We used the coefficient of variation (CV) for analysing the reproducibility data from the Chalkley method. CVs for intra- and interobserver differences were calculated. Univariate survival analyses were performed with the Kaplan–Meier method, and the differences between survival curves were compared by the log-rank test. Multivariate survival analysis was evaluated by means of Cox's proportional hazards model in a forward stepwise manner with the log-likelihood ratio significance test. Overall survival (OS) was defined as the time interval between the date of surgery and the date of death due to ovarian cancer. Recurrence-free survival (RFS) was defined by the time interval between the date of surgery and the date of recurrence. Probability values less than 0.05 were regarded as significant.

3. Results

3.1. CD34 expression

A total of 175 primary ovarian cancers and their 32 metastases were analysed for CD34 expression by the Chalkley method. The median Chalkley count was 7.67 for primary tumours

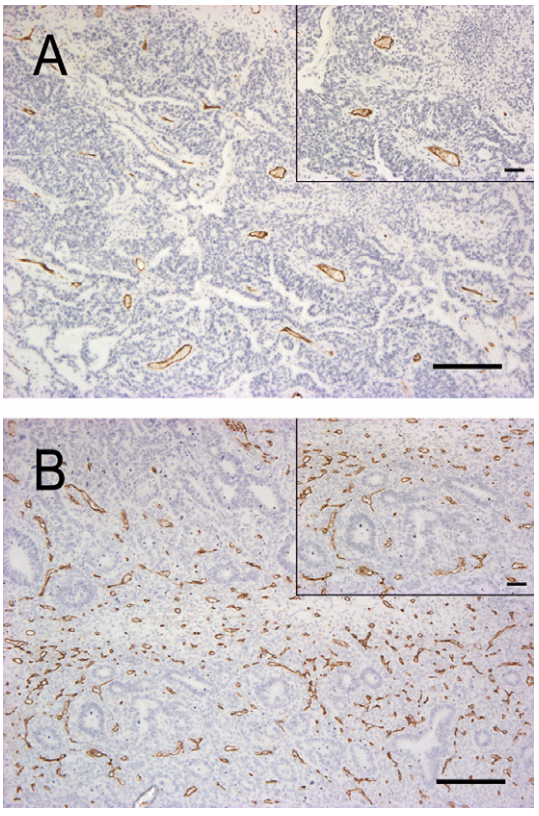

Fig. 1 – CD34 expression in epithelial ovarian cancer. (A) A serous ovarian cancer with low CD34 expression in terms of the Chalkley count. (B) An endometrioid ovarian cancer showing high expression of CD34. Scale bars = 200 μm; insets 50 μm.

and 8.17 for metastases (range 4.00–19.00 and 6.00–15.00, respectively). Ninety one of 175 (52%) primary samples were categorised into low (Fig. 1A), and 84 samples (48%) into high (Fig. 1B) expression group. There was no significant difference in the Chalkley count between primary tumours and metastases ($z = -1.9$; $p = 0.057$). Coefficient of variation for intraobserver variability was 12% and for interobserver variability 22%.

3.2. *Relation of CD34 expression to clinicopathological factors*

A low Chalkley count was associated with serous and clear cell histology ($\chi^2 = 25.3$, $p < 0.0005$). No association was found between CD34 expression and FIGO stage of the tumour, histological grade of the tumour, presence of primary residual tumour, age at diagnosis, chemotherapy response, cancer recurrence or death of the patients (Table 2).

Table 2 – Distribution of clinicopathological variables within CD34 expression categories (crosstabulation)

Variable	CD34 expression (Chalkley count)				
	Low (<8)		High (⩾8)		*P*
	N	%	N	%	
FIGO stage					
I	14	45	17	55	0.98
II	14	52	13	48	
III	56	59	39	41	
IV	7	32	15	68	
Histological grade					
1	12	52	11	48	0.78
2	34	54	29	46	
3	45	51	44	49	
Histological type					
Serous	51	72	20	28	<0.0005
Mucinous	4	24	13	76	
Endometrioid	17	37	29	63	
Clear cell	11	65	6	35	
Miscellaneous	8	33	16	67	
Primary residual tumour					
None	26	49	27	51	0.57
⩽2 cm	19	53	17	47	
>2 cm	38	54	32	46	
Chemotherapy response					
PR	12	43	16	57	0.66
CR	47	55	39	45	
SD	8	57	6	43	
PD	18	47	20	53	
Age at diagnosis					
<50	14	47	16	53	0.11
50–65	39	47	44	53	
>65	38	61	24	39	
Recurrence					
Yes	26	59	18	41	0.60
No	23	54	20	46	
End state					
Dead	68	50	69	50	0.23
Alive	23	61	15	39	

Abbreviations: PR, partial response; CR, complete response; SD, stable disease; PD, progressing disease.

3.3. *Survival*

Significant predictors of overall (OS) and recurrence-free survival (RFS) in univariate analysis of the entire cohort are presented in Table 3. The Chalkley count was not significant in predicting OS or RFS in the entire study cohort (Fig. 2A). Instead, the high Chalkley count was found to predict poor overall survival in FIGO stages III–IV tumours ($p = 0.007$) (Fig. 2B).

CD34 expression as the Chalkley count as well as histological type and grade, FIGO stage, primary residual tumour, age at diagnosis, and adjuvant chemotherapy were entered into the Cox's multivariate analysis. The Chalkley count reached statistical significance in predicting OS in the entire study group ($N = 156$; $p = 0.044$, RR = 1.50, 95% CI 1.01–2.21) but was not a significant predictor of RFS. The results of Cox's multivariate analysis in the entire study group are presented in Table 4. In addition, the Chalkley count independently predicted OS in the subgroup analysis of FIGO stages III–IV tumours ($N = 99$; $p = 0.046$, RR 1.58, 95% CI 1.01–2.46) together with the presence of primary residual tumour ($p = 0.002$, RR = 4.28, 95% CI 1.34–13.73).

4. Discussion

While the Chalkley method has been suggested as a preferred method in estimating the prognostic significance of angiogenesis in breast cancer,[24] and recommended for angiogenesis quantification in solid tumour sections in an international consensus report,[21] its applicability in ovarian cancer has not been studied. In the present pilot study, the method turned out to be reliable, and indicated that the high Chalkley count is an independent prognostic marker for poor survival in epithelial ovarian cancer.

Quantitating angiogenesis by the Chalkley method represents a relative area estimate of the vessels rather than a true vessel count. This has been thought to be an advantage by improving the objectivity of evaluation because the method abolishes one of the highly observer-dependent steps in microvessel density measuring: the decision whether two immunostained and adjacent structures were the reflection of one single or two separate blood vessels.[21] Supporting this assumption, the Chalkley method has been shown to have less observer variation than estimation of microvessel density in breast cancer.[24] An important observer-dependent step still remains in the selection of the densely vascularised areas, "vascular hot-spots" for microvessel quantitation. However, the reproducibility is not necessarily optimised by choosing the same hot-spot area.[24] In our study we found moderate intraobserver reproducibility with coefficient of variation of 12%, which is slightly lower than the 18% observed in breast cancer.[24] Interobservationally, coefficient of variation in the present study was 22%, whereas in breast cancer it was reported to be 20% with isolated contribution of only 8–9% from observers to the variance.[24] The Chalkley method has been considered also rapid in contrast to time-consuming nature

Table 3 – Univariate overall and recurrence-free survival analyses of the patients

Variable	Surviving at 10 years			Recurrence-free at 10 years		
	N	%	P[a]	N	%	P[a]
Age, years						
	174		0.31	87		0.36
<50	30	43		17	59	
50–65	82	29		42	40	
>65	62	29		28	57	
Histological grade						
	174		0.013	87		0.29
1	23	48		13	62	
2	62	40		34	59	
3	89	21		40	38	
Histological type						
	174		0.41	87		0.027
Serous	71	24		30	30	
Mucinous	17	47		12	75	
Endometrioid	46	37		23	61	
Clear cell	16	44		10	60	
Miscellaneous	24	25		12	42	
FIGO stage						
	174		<0.00005	87		0.0059
I	31	74		26	77	
II	27	41		20	50	
III	94	20		37	32	
IV	22	9		4	25	
Primary residual tumour						
	158		<0.00005	82		0.0001
None	53	66		44	70	
⩽2 cm	36	22		22	27	
>2 cm	69	16		16	31	
Adjuvant chemotherapy						
	174		0.88	87		0.87
Platin-based	155	30		77	49	
Non-platin	14	50		7	57	
None	5	20		3	33	
CD34 expression (Chalkley count)						
	174		0.15	87		0.58
<8	90	34		49	47	
⩾8	84	29		38	53	

a Log-rank analysis.

of counting microvessel density, but according to our experience it takes approximately 10 min per section as presented also for microvessel counting,[24,25] differing from 2 to 5 min reported earlier for the Chalkley counting procedure.[24,25]

In our study material, angiogenesis as evaluated with the Chalkley method did not associate with FIGO stage or histological grade, a finding in accordance with many previous ovarian cancer studies with different evaluation methods.[5,7,9,13–15] However, angiogenesis has been shown to relate to histological grade[12] and tumour stage[8,12] in some studies, whereas in the study of advanced ovarian cancer by Hollingsworth and colleagues[8] the association with tumour stage was not present when studied with the modified Chalkley method instead of the vessel counting method. In the present study angiogenesis was associated only with histological type, as angiogenesis was significantly higher in mucinous and endometrioid tumours compared to serous and clear cell histological types. Despite many reports of missing association between angiogenesis and histological type,[5,8,9,12] our finding is corroborated with earlier observations by others,[7] and may reflect the distinct molecular genetic alterations underlying the pathogenesis of different histological subtypes of ovarian cancer.[26]

Prognostic significance of angiogenesis quantified by the Chalkley method has been studied previously in other carcinomas. Especially in breast cancer the association of poor outcome of patients with increasing angiogenesis has been shown,[22,25,27,28] although missing associations have also been reported.[29,30] In other tumours, the method has been shown to have prognostic significance or lack the significance[22] probably at least partly because of methodological problems. Indeed, the lack of standardised techniques, emerging as differences in antibodies, modifications of the Chalkley evaluation method, and cut-offs, makes comparisons between

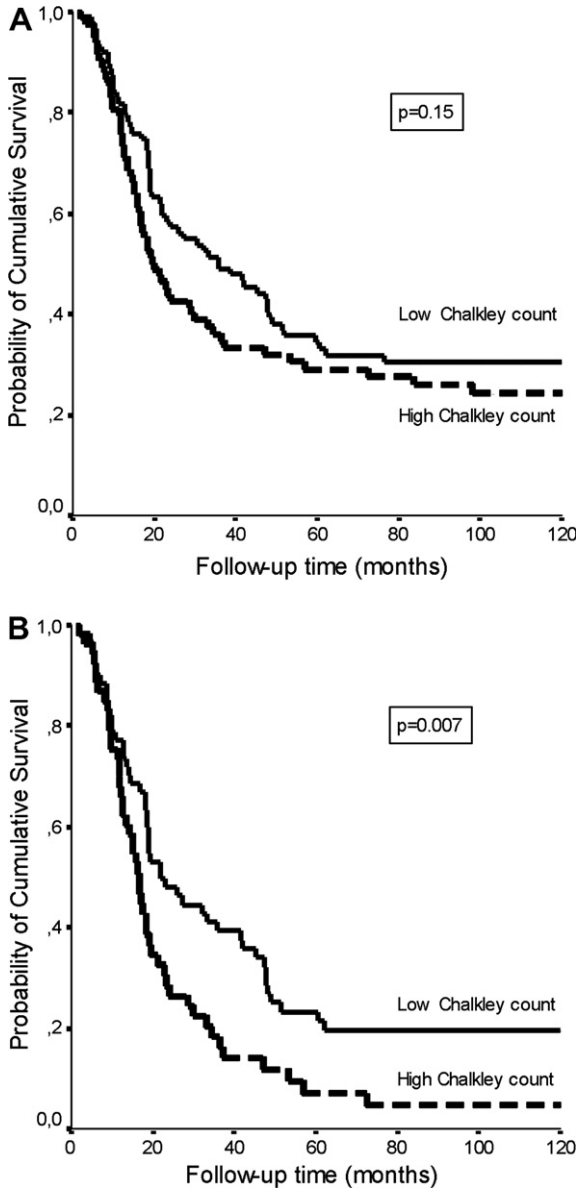

Fig. 2 – (A) Overall survival of the patients in the entire study group according to low (N = 90) and high (N = 84) CD34 expression. (B) Overall survival of the patients with FIGO stages III–IV tumours according to low (N = 62) and high (N = 54) CD34 expression as evaluated by the Chalkley counting procedure (log-rank analysis).

the studies difficult. In addition, the degree of angiogenesis seems to vary in different carcinomas. Nakayama and colleagues studied different features of angiogenesis between ovarian and breast carcinoma, and found that angiogenesis in ovarian carcinoma was lower than in breast carcinoma when evaluated by intratumoural microvessel density.[31] In the current study, the median Chalkley count (7.67) was higher than that in breast cancer studies,[22,25,27–30] but considering the wider graticule area used our study with the Chalkley method supports the findings by Nakayama and colleagues.[31]

Multivariate analysis indicated that the high Chalkley count was an independent predictor of poor overall survival in the entire study group, as well as in the subgroup with advanced stage tumours. In line with the present findings, Hollingsworth and colleagues showed with a different evaluation method that increased angiogenesis independently predicted poor disease-free survival in advanced ovarian cancer.[8] However, with their considerably smaller study group, no association between angiogenesis and patients' outcome by the modified Chalkley method was found.[8] In addition to that, prognostic significance of angiogenesis analysed by different methodology has been studied in numerous small ovarian cancer materials with contradictory results. Studies including more than 90 ovarian cancer patients have found that increased angiogenesis predicts either poor[4] or improved survival,[5] or have shown no association between angiogenesis and survival in ovarian carcinoma.[6,7] The differences in the methods used in the previous studies on ovarian cancer angiogenesis make it difficult to compare the results with those of the present study.

Despite advances achieved in ovarian cancer treatment, chemotherapy resistance still hampers the effective treatment of ovarian cancer patients.[32] Increased angiogenesis has been speculated to improve delivery of chemotherapeutic agents, and reported to associate with improved response to chemotherapy in advanced stage ovarian carcinoma patients.[9] On the other hand, also inverse association between vascularity and response to platinum-based chemotherapy has been suggested.[7] In our study, statistically significant association between chemotherapy response and the Chalkley count was not found, including patients with advanced-stage tumours and those who had received platinum-based chemotherapy (data not shown). This may be explained by a lack of functional lymphatic system in tumours as well as structural and functional abnormalities of tumour blood vessels, contributing to chaotic blood flow, and making certain

Table 4 – The independent prognostic factors in Cox's multivariate analysis for overall survival (OS; N = 156) and recurrence-free survival (RFS; N = 82)

Factor	Category	RR	95% CI	P[a]
OS				
Primary residual tumour	Positive vs. negative	5.10	3.02–8.62	<0.0005
CD34 expression (Chalkley count)	High vs. low	1.50	1.01–2.21	0.044
RFS				
Primary residual tumour	Positive vs. negative	3.32	1.66–6.62	<0.0005
Histological type	Others vs. serous	0.47	0.25–0.89	0.022

a Log-likelihood ratio significance test.

regions of the tumour inaccessible to drugs.[33] Malignant cells, including those from ovarian cancer, may also participate in vascular channel formation independent of endothelial cells.[34] These kinds of structures may not stain or may stain discontinuously with endothelial cell markers,[35] which may underestimate the real capacity for chemotherapeutic agents' delivery. Our findings suggest that determination of angiogenesis may not help to find the patients who benefit from anticancer drug therapies. However, even if there exists vasculogenic mimicry in ovarian cancer, it does not annul the fact that also quantification of angiogenesis based on CD34 seems to be significant in predicting the patients' outcome.

In conclusion, we show for the first time that high angiogenesis measured by the Chalkley method predicts poor overall survival in the whole study group and in the advanced-stage ovarian cancers. To define the clinical significance of this finding in more detail, further studies on other patient materials and perhaps comparison with other evaluation methods of angiogenesis in ovarian cancer are suggested.

Conflict of interest statement

The authors have no conflicts of interests with relation to the study methodology, results, or other sections of the study.

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