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ORIGINAL ARTICLE

Expression of a novel endothelial marker, C-type lectin 14A, in epithelial ovarian cancer and its prognostic significance

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Abstract
Objective The purpose of this study was to evaluate microvessel density (MVD) as assessed by C-type lectin 14A (CLEC14A), which is a new marker for endothelial cells, and compare its expression to CD31 and CD105 in epithelial ovarian cancer (EOC).
Methods MVD was evaluated in tumors ($n = 50$) from patients with EOC who underwent primary surgery and in patients with EOC who received preoperative chemotherapy ($n = 49$) using immunohistochemistry with antibodies to CLEC14A, CD31 and CD105. The median duration of follow-up was 24.5 months (range 1−101 months). The effect of prognostic factors on event-free survival (EFS) and overall survival (OS) was assessed using the Cox regression model.
Results The amount of residual disease was found to be an independent prognostic factor in multivariate analysis with respect to EFS ($P = 0.009$) and OS ($P < 0.001$). The mean MVD of CLEC14A (MVD = 6), in tumors from patients who underwent primary surgery, was significantly lower than that of CD31 (MVD = 25, $P < 0.0001$) and CD105 (MVD = 11, $P = 0.018$). However, there was no significant correlation between MVD as detected by these markers and clinical outcome. There was no expression of CLEC14A in tumors from patients who received preoperative chemotherapy and the MVD of CD31 and CD105 was significantly reduced ($P = 0.001$ and 0.006, respectively) in this set of patients.
Conclusion This study demonstrates MVD as detected by CLEC14A in EOC. Treatment with chemotherapy reduces tumor blood vessels significantly. We suggest that CLEC14A may be a more specific endothelial marker to assess tumor angiogenesis.

Keywords C-type lectin 14A (CLEC14A) · Endothelial cells · Microvessel density · Epithelial ovarian cancer

Introduction

Angiogenesis is required for the growth and maintenance of tumors. The concept of anti-angiogenic therapy was initially demonstrated by Dr. Judah Folkman [1]. Tumor blood vessels are characterized by their structural and functional abnormalities in contrast to their normal counterpart. The endothelial cells in normal blood vessels are homogeneous, whereas tumor blood vessels are leaky, tortuous, and heterogeneous with irregular branching. To date, no marker that is strictly specific for tumor blood vessels has been found. Intense research is underway to identify more specific tumor endothelial markers which can be used to develop vascular targeting agents. However, characterization of many potential and viable tumor endothelial markers constitutes a major area of research [2]. Furthermore, the origin of blood vessels within a tumor can also be by multiple mechanisms [3].

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Tumor vascularization in solid tumors can be assessed by evaluating microvessel density (MVD) [4]. The commonly used antibodies to evaluate MVD are against antigens, CD31, CD34, CD105, and von Willebrand factor [5]. Although there are many reports evaluating MVD in different tumor types, their correlation with clinical outcome is still controversial. It has recently been reported by a systematic review and meta-analysis that high MVD, as assessed by CD34, correlated with poorer overall survival (OS) and progression-free survival (PFS) in patients with epithelial ovarian cancer (EOC). However, in this study, there was no significant correlation between MVD as detected by antibodies against CD31 or CD105 and clinical outcome in patients with EOC, although they were evaluated by different research groups [6]. Therefore, an endothelial marker which specifically detects tumor blood vessels remains an area of further research. In particular, in EOC the correlation between MVD and outcome remains to be proven conclusively.

Recently, a member of the C-type lectin family, CLEC14A, was reported to be a novel specific marker expressed in the blood vessels of tumors. The authors identified this protein through bioinformatics and data mining [7]. In this report, real-time polymerase chain reaction (PCR) analysis showed that CLEC14A was expressed in endothelial cells [human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells] but absent in other primary isolates. On immunostaining of the tumor and adjacent normal tissue, CLEC14A was found to be expressed in the vessels in the tumors while there was no expression in the adjacent normal tissue. Furthermore, immunohistochemical analysis of tissue microarrays demonstrated that CLEC14A was overexpressed in the blood vessels of different solid tumors such as breast, prostate, kidney and thyroid while there was no expression of this protein in the respective normal tissue [7]. Other reports have demonstrated a major role for this protein in angiogenesis [8–12]. It is a single-pass transmembrane protein and is a member of the C-type lectin family. The chromosomal location of this gene is 14q21.1. The CLEC14A protein has 490 amino acids of which the first 22 amino acids (1–22) code for a signal peptide. The extracellular portion involves a C-type lectin and epidermal growth factor-like domain [7]. The C-type lectin domain (CTLD) of CLEC14A has been shown to be the major player in controlling cell migration and filopodia formation [8]. The si-RNA-mediated knockdown of CLEC14A resulted in reduced migration of endothelial cells and defective formation of tubular structures on matrigel [9]. Recombinant human antibodies targeting the CTLDs of human and mouse CLEC14A specifically blocked migration and tube formation of endothelial cells [10]. It has been demonstrated that CLEC14A is a component of the extracellular matrix (ECM) in HUVECs and binds to MMRN2, FN1 and LAMA4, which regulate angiogenesis. The authors have also reported that CLEC14A is strongly expressed in blood vessels in tumors compared to their normal counterpart [11]. It was also demonstrated by in vivo studies that CLEC14A and MMRN2 are essential for endothelial cell morphogenesis. These molecules are deregulated during the progression of the tumor [12].

CD31 (also known as platelet endothelial cell adhesion molecule-1) is a commonly used blood vessel marker in immunohistochemistry (IHC) [13]. The antibody recognizing CD31 has been found to detect the antigen in capillaries, veins and arteries of normal tissue [14]. Endoglin (CD105), a potential blood vessel-specific marker, is a homodimeric transmembrane glycoprotein with a molecular weight of 180 kDa [15]. It is a receptor for transforming growth factor-β1 (TGF-β1) and TGF-β3, and other growth factors such as activin A, and bone morphogenic protein-7. The chromosomal location of endoglin is 9q34 in humans. The expression of CD105 protein is mainly found in endothelial cells of proliferating tissue or tumors. Endoglin is up-regulated in endothelium within brain, breast, prostate, gastric and cervical cancer [16]. CD105 knockout mice exhibited defects in the development of normal blood vessels and died of defective vascular development during the early embryonic stage [17]. It is now apparent that CD105 is the responsible gene and is mutated in hereditary hemorrhagic telangiectasia type 1, which is an autosomal-dominant inherited disease characterized by arteriovenous malformations and bleeding [16]. A functional antibody against CD105 (clone SN6h) known as TRC105 has been evaluated in phase 1 trials [18–20]. Mab E9, another monoclonal antibody to CD105, has been used for ex vivo immunoscintigraphy in resected renal tumors from patients with renal cell carcinoma. The technetium ($^{99}Tc^{m}$)-labelled CD105 antibody was specifically localized in endothelial cells [21]. The expression of CD105 on the proliferating endothelial cells of the tumor vasculature implies that CD105 may be a good target for imaging and therapy.

The expression profile of CD31, CD105 and CLEC14A in normal tissue is described in Table 1. The data for CD105 and CLEC14A have been summarized from The Human Protein Atlas (http://www.proteinatlas.org/). In malignant tissues, three of the antigens are expressed primarily by endothelial cells. None of these three antigens are expressed in lymphatic vessels. However, CD105 has been reported to be expressed in malignant cells when examined by a polyclonal antibody from Sigma [22]. Hence, our objective was to study the expression of CLEC14A for evaluation of MVD in ovarian tumors and its correlation with clinical outcome. The MVD as detected by the expression of CLEC14A was also compared with that of the conventionally used endothelial markers, CD31 and CD105.

Table 1 Expression profile of CD31, CD105 and CLEC14A in normal tissue

Tissue	CD31[a]	CD105[b]	CLEC14A[b]
Cerebral cortex	Capillaries, large vessels	Endothelial cells	Endothelial cells
Hippocampus	NA	Negative	Neuronal cells
Spleen	Sinus, red pulp capillaries, arteria centralis,trabecular veins and arteries	Negative	Negative
Liver	Sinusoidal endothelium, central and portal veins, portal arteries	Negative	Negative
Stomach	Endothelial cells	Glandular cells	Glandular cells
Colon	Endothelial cells	Glandular cells, endothelial cells	Glandular cells, endothelial cells
Lung	Alveolar septal capillaries, venules, veins and arteries	Negative	Macrophages
Uterus	Endothelial cells	Cells in endometrial stroma	Glandular cells
Cervix	Endothelial cells	Negative	Glandular cells
Ovary	Small vessels and capillaries, large arteries and veins	Follicles cells, ovarian stroma cells	Negative
Placenta	Endothelium of blood vessels	Trophoblastic and decidual cells	Negative
Testis	Capillaries, venules, large veins and arteries	Cells in seminiferous ducts, leydig cells	Cells in seminiferous ducts, leydig cells
Kidney	Glomerular and interstitial capillaries	Cells in glomeruli, tubules	
	Interlobular vessels, large veins and arteries	Cells in glomeruli	Cells in glomeruli, tubules
Skin	Vessels in papillary dermis, vessels in deep dermis	Melanocytes	Langerhans
Thyroid	Capillaries, large veins and arteries	Glandular cells	Negative
Parathyroid	NA	Glandular cells	Negative
Adrenal gland	Capillaries, post capillary venules, larger veins and arteries	Glandular cells	Negative
Pancreas	Capillaries, venules, large veins and arteries	Negative	Glandular cells
Lymph node	Sinuses, other capillaries and veins, trabecular and hilar veins, arteries	Negative	Negative
Thymus	Medullar and cortical capillaries, HEV, tissue veins and arteries	NA	NA
Tonsil	HEV, other capillaries and veins, larger arteries and veins	Negative	Negative
Breast	Endothelial cells	Glandular cells, endothelial and myoepithelial cells	Negative

NA data not available, *HEV* high endothelial venules

[a] Data summarized from [14]

[b] Data have been summarized from the Human Protein Atlas (http://www.proteinatlas.org/)

Materials and methods

Patient data

Clinical data for this study were collected from patients diagnosed with EOC between 2004 and 2007 who underwent surgery and treatment at the Cancer Institute, Amrita Institute of Medical Sciences, Cochin, India. The study included patients with known follow-up data and biopsies with sufficient tissue blocks available for IHC. The patients were staged according to the International Federation of Gynecology and Obstetrics classification (FIGO). Overall, 109 patients who presented during this period were included in the study. Only 99 patients had representative tumor paraffin blocks. Biopsies from patients ($n = 50$) who had epithelial ovarian tumors of any stage (confirmed by histology) and who had undergone primary surgery were retrieved for analysis. Subsequently, the patients were treated with carboplatin and paclitaxel. Paraffin blocks from 49 additional patients with ovarian cancer who

received neoadjuvant chemotherapy (carboplatin ± Taxol) followed by interval surgery were also chosen for the study. All patients subsequently received platinum therapy based on tumor stage. None of our patients received bevacizumab. The median duration of follow-up was 24.5 months (range 1−101 months). The study was conducted after obtaining approval from the Institutional Ethics Committee. All slides were reviewed for confirmation of pathology. The grading of tumors was performed according to the WHO 2003 classification as follows—Grade 1 = well-differentiated tumors and Grade 3 = poorly differentiated tumors. Grade 2 = moderate and intermediate between Grades 1 and 3. The tumors which upon review showed pseudomyxoma histology were not included in the statistical analysis of prognostic factors.

IHC and antibodies

The sections were deparaffinized with three changes of xylene and rehydrated in a descending isopropanol series. The CD31 antigen was retrieved by heat-induced epitope retrieval (HIER) at 95 °C in a microwave oven with citrate buffer (pH 6) for 10 min. No antigen retrieval was performed in the case of CD105. For CLEC14A, the tissues were subjected to HIER at 95 °C in a water bath for 10 min, followed by cooling the tissue slides to room temperature. The primary antibodies used were mouse anti-human monoclonal antibody, CD105 (Dako, Clone SN6 h) at a dilution of 1:5, and mouse anti-human monoclonal antibody, CD31 (pre-diluted, Biogenex, clone JC70). The tissues were incubated with these antibodies for 90 min at room temperature followed by washing with phosphate-buffered saline (PBS) and further incubated with anti-mouse secondary antibody (Biogenex), conjugated with horseradish peroxidase, for 1 h at room temperature. After washing with PBS, the tissues were incubated with diaminobenzidine for 15–30 min. For CLEC14A, the primary antibody was sheep polyclonal (dilution 1:40, R&D Systems) and incubation was overnight at 4 °C. Further staining was performed according to the manufacturer's instructions for the IHC Detection Kit (R&D Systems). The nuclei were stained with hematoxylin. The slides were dehydrated with xylene and mounted with a mixture of distyrene, a plasticizer, and xylene.

Quantitative analysis of blood vessel density

The slides were scanned at low magnification and the areas with the highest MVD were identified as 'hot spots'. The microvessels were counted at 200× magnification by the Chalkley method [4]. Five hot spots were identified for each tissue section and the number of blood vessels was counted. The MVD was calculated for CLEC14A, CD31and CD105 by taking the mean count from five hot spots.

Statistics

OS and event-free survival (EFS) were the outcome measures. An event was defined as local or distant recurrence, doubling of CA125 on more than two occasions, at least one week apart. OS was defined as the period from diagnosis until the time of death from any cause. EFS was defined as the period from diagnosis until the occurrence of an event as defined above.

The Cox proportional hazards model was used to evaluate the prognostic factors and to assess any impact on EFS and OS [23]. Univariate and multivariate analyses were performed to evaluate these factors. The survival curve was also plotted using the Kaplan and Meier method [24]. All statistical analyses were performed using SPSS version 11. A P value <0.05 was considered to be significant throughout the analysis.

Results

Demographic data of patients

One hundred and nine consecutive patients diagnosed with EOC between 2004 and 2007 were analyzed. Clinicopathological data are presented for 109 patients with a median age of 54 years (Table 2). The majority of patients had serous (92/109) carcinoma and had stage III disease (101/109). Of 109 patients, 53 had undergone primary surgery and 56 had received neoadjuvant chemotherapy followed by interval debulking based on a discussion at a multidisciplinary tumor board.

MVD of CLEC14A, CD105 and CD31 in EOC

Staining with the three antibodies, CD31, CD105 and CLEC14A, was observed only in the blood vessels. In tissue sections from the normal ovary, CLEC14A stained fewer vessels compared to CD31 and CD105 (Fig. 1). In ovarian tumors, the number of blood vessels identified by the expression of CLEC14A was lower than that of the other two markers. CD31 stained more vessels than CD105 and CLEC14A, indicating that CD105 and CLEC14A are probably staining tumor endothelial cells (Fig. 2). Evaluation of CLEC14A, CD31 and CD105 staining was performed on both sets of patients, i.e., those (n = 50) who had undergone primary surgery and those (n = 49) who had received preoperative chemotherapy. In the patients who had undergone primary surgery, the mean MVD for CLEC14A was 6

Table 2 Clinicopathological characteristics of patients

Characteristic	All patients
Total no.	109
Age	19–82
Median age (years)	54
FIGO stage	
Stage I/II	2
Stage III	99
Stage IV	8
Histological type	
Serous	92
Mucinous	6
Endometroid	2
Clear cell	3
Pseudomyxoma	3
Others	3
Grade	
Grade I	9
Grade II	56
Grade III	44
Treatment	
Primary surgery	53
Neoadjuvant chemotherapy	56
Cytoreduction	
Optimal	77
Suboptimal	32
Chemotherapy received	
Platinum-based chemotherapy	105
No chemotherapy	4

(range 0−to 34) which was lower than that for CD31 (MVD 25, range 0–66.3) and CD105 (MVD 11, range 0–67.3). In this instance, the mean MVD values alone were used as the cut-off for statistical analysis. We chose the mean MVD as the cut-off score, i.e., 6 for CLEC14A, 25 for CD31 and 11 for CD105. Tumours whose MVD was less or more than 6 (CLEC14A), 25 (CD31) or 11 (CD105) were categorized as low or high respectively. The mean MVD of CLEC14A was significantly lower than that of CD31 ($P < 0.0001$) and CD105 ($P = 0.018$). MVD was also determined in tumors obtained at interval debulking ($n = 49$). We evaluated the expression of all three endothelial markers in these tumors. The number of stained vessels for CD31 (0–15) and CD105 (0–14) was low in this set of patients ($n = 49$), probably due to tumor regression subsequent to chemotherapy. The mean MVD as assessed by CD31 and CD105 was significantly reduced (2 for CD31, $P = 0.001$ and 1.7 for CD105, $P = 0.006$) in this set of patients. The expression of CLEC14A was completely absent in the tumors after chemotherapy ($n = 10$).

Association between clinicopathologic factors and expression of endothelial markers and clinical outcome

Univariate and multivariate analyses were performed to evaluate the prognostic factors in 106 patients. Of 109 patients, three with pseudomyxoma histology were not included in the statistical analysis. In univariate analysis (Table S1), grade ($P = 0.03$ and $P = 0.009$, respectively) and residual disease ($P = 0.001$ and $P < 0.001$, respectively) were significantly associated with a shorter EFS and OS. The histology or the serum CA125 levels did not show any impact on EFS or OS. Since the majority of patients had advanced stage disease (104/106), statistical analysis based on stage as a variable was not performed. In multivariate analysis (Table S2), only optimal debulking correlated with OS ($P < 0.001$) and EFS ($P = 0.009$).

Correlation between expression of endothelial markers and survival in patients who underwent primary surgery

IHC was performed on tissues from 50 patients who underwent primary surgery. The effect of MVD on the EFS and OS was evaluated by Kaplan–Meier survival curves. The EFS of patients with tumors with low and high MVD for CLEC14A was not statistically significant ($P = 0.526$). In addition, MVD as detected by CLEC14A, did not correlate with OS in this set of patients ($P = 0.959$) (Fig. 3a). The MVD for CD105 ($P = 0.717$ and 0.424, respectively) and CD31 ($P = 0.698$ and $P = 0.776$, respectively) also did not correlate significantly with both EFS and OS (Fig. 3b, c). The prognostic significance of CLEC14A, CD31 and CD105 was evaluated by univariate and multivariate analyses according to the Cox regression model. In univariate analysis (Table 3), residual disease and grade were significant prognostic factors ($P = 0.01$ and $P = 0.04$, respectively). Therefore, we chose two models, each with residual disease and grade as a factor to evaluate the prognostic impact on the outcome by multivariate analysis (Table 4). When the residual disease was taken into account with the MVD of CLEC14A, CD31 and CD105 in a Cox proportional hazards model, the amount of residual disease was the strongest independent prognostic factor for OS ($P = 0.01$) and EFS ($P = 0.01$). Similarly, when tumor grade was considered along with the MVD of CLEC14A, CD31 and CD105, the tumor grade was the only factor which was significantly correlated with EFS ($P = 0.04$) and OS ($P = 0.02$).

Correlation between expression of endothelial markers and survival in patients who underwent preoperative chemotherapy

In univariate analysis (Table S3), residual disease was a significant factor when correlated with EFS and OS

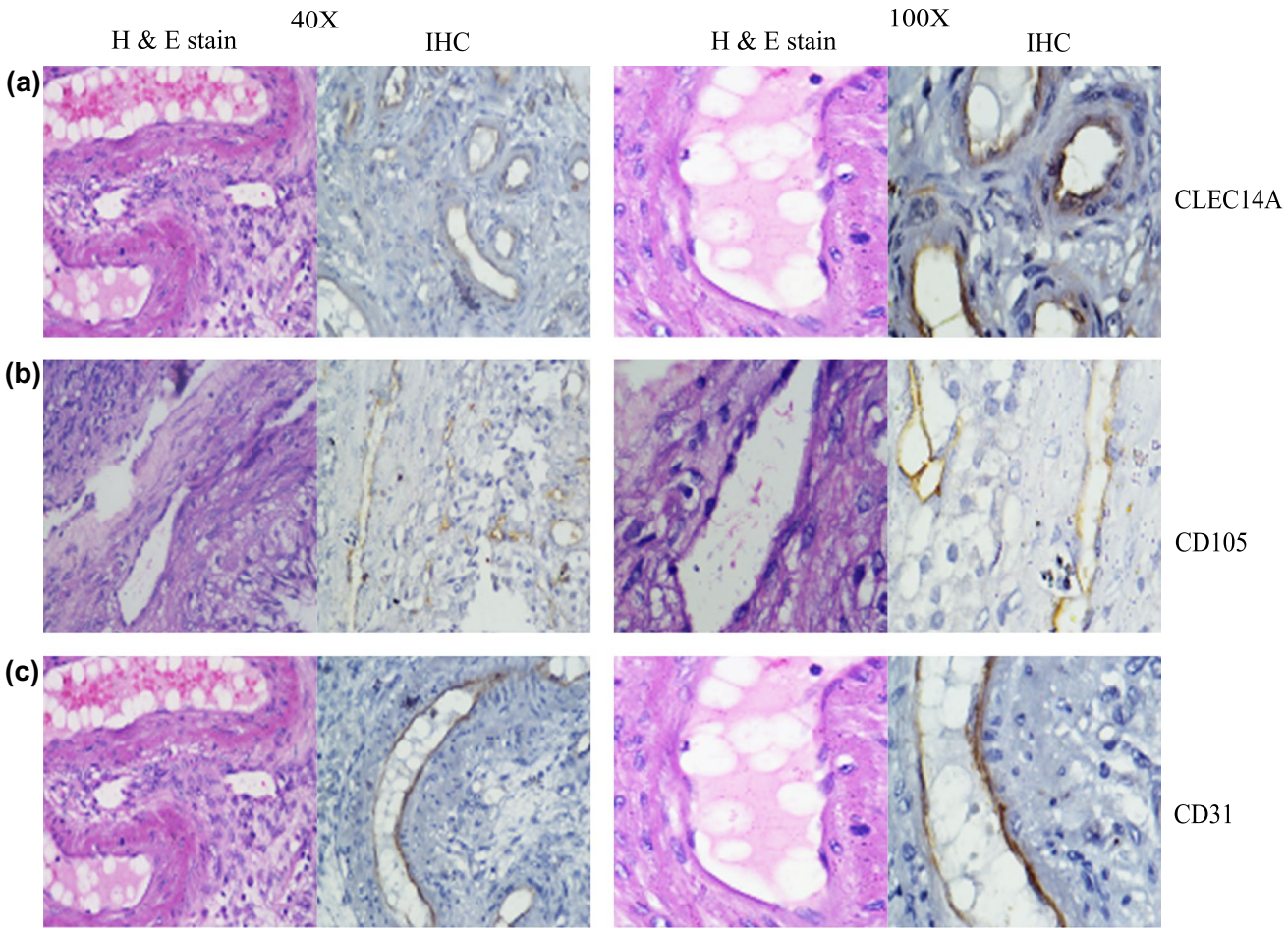

Fig. 1 Immunostaining of tissue from normal ovary with antibodies. Representative images showing the expression of **a** CLEC14A, **b** CD105 and **c** CD31 in normal ovary. CD31 was expressed in more blood vessels compared to CD105 and CLEC14A

($P = 0.023$ and $P = 0.036$, respectively). In multivariate analysis (Table S4), residual disease was entered into a model and showed a significant association with EFS ($P = 0.019$) and OS ($P = 0.027$) in this model.

Discussion

The recent identification of endothelial markers that detect tumor blood vessels has helped us to understand tumor angiogenesis. We investigated MVD in EOC in order to evaluate tumor angiogenesis. This is the first report evaluating the MVD of CLEC14A in a solid tumor. Moreover, this is the first study of MVD as measured by CLEC14A, CD105 and CD31 in patients with ovarian cancer from India.

This study found that on univariate analysis, both EFS and OS decreased significantly in patients with higher grade tumors and in those with residual disease after surgery. MVD was not a significant factor in both sets of patients. A previous study in ovarian cancer ($n = 58$) showed that cytoreduction ($P = 0.02$) and MVD determined by CD105 ($P = 0.04$) were significant when correlated with survival [25]. Our study varied from this report in several respects. First, we included patients who had undergone primary surgery as well as those who had received preoperative chemotherapy. Second, we used three specific endothelial markers to evaluate MVD. We used a monoclonal antibody for CD105 (clone SN6 h, Dako) for immunohistochemical staining. In the above-mentioned report, the authors used CD31 (Clone JC/70A; Neomarker, Fremont, CA, USA) and CD105 (clone 105 CO2; Neomarker).

A recent study by the Gynecological Oncology Group ($n = 50$) found that high MVD assessed using CD105 appeared to be an independent prognostic factor for poor PFS ($P = 0.02$) in women with advanced EOC [26]. MVD as determined by the expression of CD105 has been found to be a prognostic factor in other cancers such as colon [27], prostate [28], upper urinary tract [29] and breast [30, 31]. However, a previous study by our group ($n = 100$) found

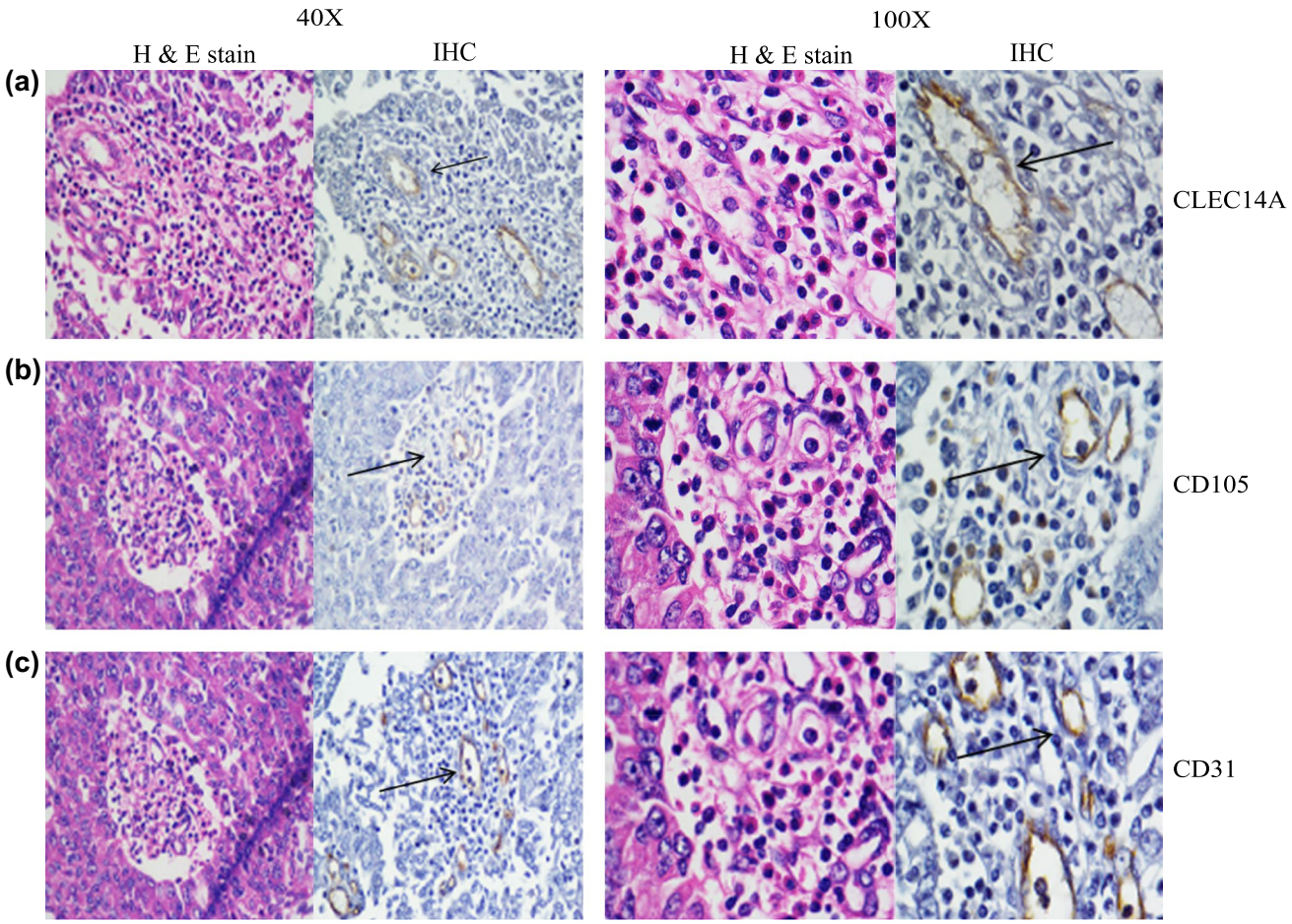

Fig. 2 Immunostaining of tissue from epithelial ovarian cancer with antibodies. Representative images showing the expression of **a** CLEC14A **b** CD105 and **c** CD31 in the primary ovarian tumor at 40× and 100× magnification. CD31 was expressed in the majority of blood vessels compared to CD105 and CLEC14A. The *arrows* show the positively stained blood vessel by each marker. *H&E stain* hematoxylin and eosin stain, *IHC* immunohistochemical staining with each marker

that the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) and thymidine phosphorylase and MVD (determined by CD31) did not correlate with survival [32]. Recently, it has been reported that the MVD of CD34 and CD105 did not help in estimating survival or the chance of recurrence in patients with ovarian cancer [33]. It was demonstrated in another study evaluating the expression of endoglin and its clinical relevance in effusions, primary tumors, and solid metastatic lesions in ovarian serous carcinoma, that the expression of endoglin did not correlate with survival [34]. Similarly, the correlation between MVD and poor prognosis was contradicted by another study which showed that the level and control of angiogenesis may differ among ovarian tumor types [35]. A recent meta-analysis of 22 studies showed that the prognostic significance of MVD is variable across different reports. MVD, as detected by CD34 antibody, showed a significant impact on survival, whereas MVD, detected by other antibodies such as CD31, CD105 and Factor VIII did not correlate with survival. It was also demonstrated that there was no significant correlation between MVD and survival after initial chemotherapy [6]. However, microarray data from The Cancer Genome Atlas on 557 samples showed a significant correlation between expression of CLEC14A and disease-free survival [36, 37]; however, this correlation is with expression of mRNA rather than MVD.

This is the first study in ovarian cancer comparing the expression of CLEC14A, CD31 and CD105 in patients who had undergone primary surgery with those who had received neo-adjuvant chemotherapy. In our study, 49 tumors from patients who had undergone neo-adjuvant chemotherapy were assessed for MVD for CD31 and CD105. The expression of both CD31 and CD105 had reduced considerably in these tumors probably due to the reduction of the viable tumor after chemotherapy. Another report on the study of the relationship between endoglin and response to neoadjuvant chemotherapy in breast cancer compared the expression of CD105 on pretreatment

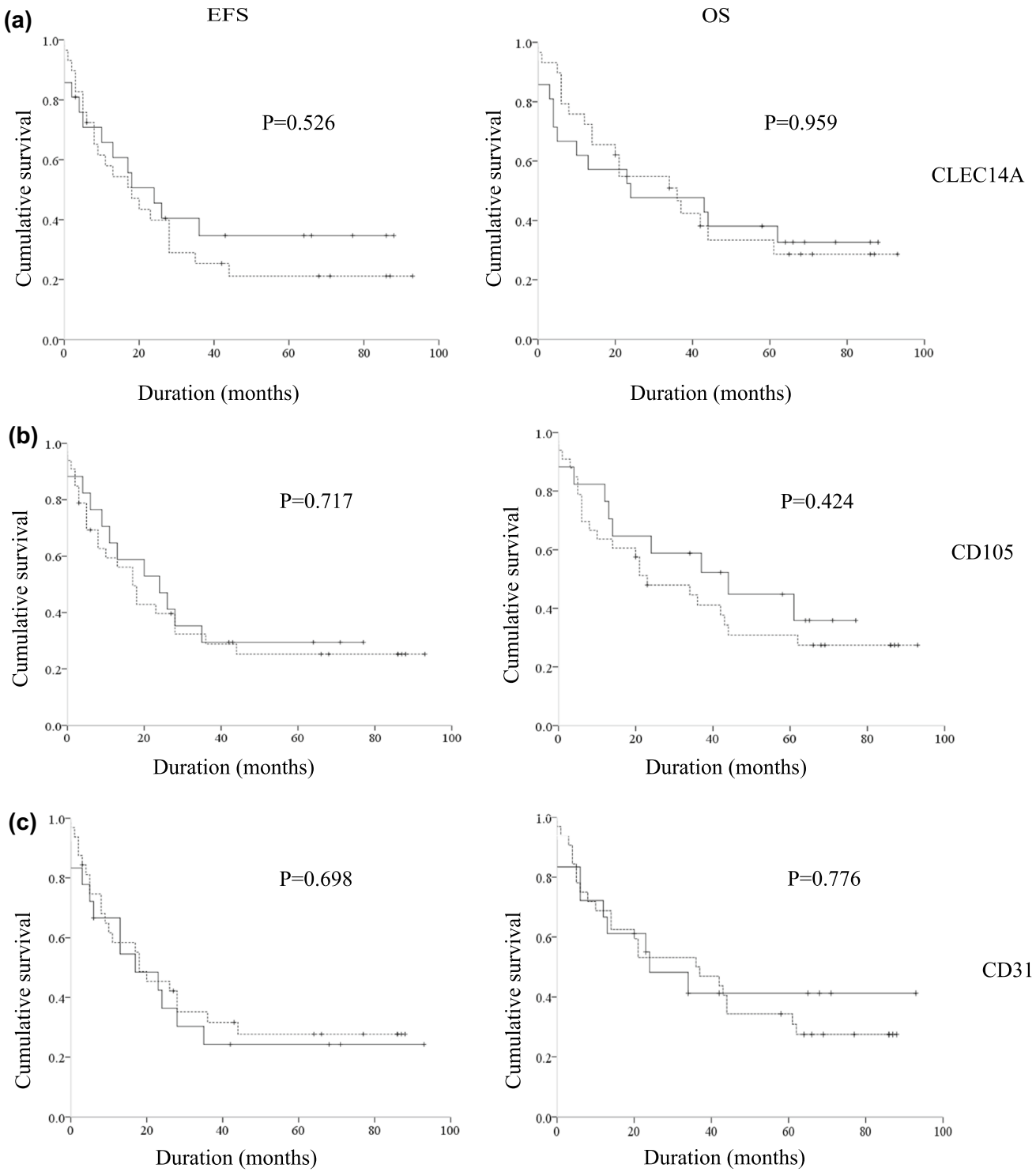

Fig. 3 Correlation between microvascular density (MVD) and event-free survival (EFS) and overall survival (OS) in patients who had undergone primary surgery. Kaplan–Meier survival plots of MVD for **a** CLEC14A, **b** CD105 and **c** CD31 in ovarian cancer as an outcome measure for EFS and OS. The *dotted line* represents patients with low MVD and the *solid line* represents patients with high MVD for the respective markers. The difference in EFS and OS between patients with low and high MVD was not statistically significant

Table 3 Univariate analysis of vascular density and clinical parameters of patients who had undergone primary surgery

Variable	Number	Event-free survival			Overall survival		
		HR	95 % CI	*P* value	HR	95 % CI	*P* value
Age							
≤50	16	Baseline			Baseline		
>50	34	1.219	0.572–2.597	0.609	1.915	0.628–5.842	0.254
Stage	NA						
Histology							
Serous	35	Baseline			Baseline		
Mucinous	7	2.254	0.948–5.36	0.066	1.42	0.461–4.38	0.54
Others	8	0.934	0.277–3.148	0.912	0.868	0.195–3.86	0.85
Grade							
Low/intermediate	27	Baseline			Baseline		
High	23	1.872	0.945–3.710	0.072	2.107	1.033–4.296	0.04*
Residual disease							
Optimal	24	Baseline			Baseline		
Suboptimal	26	2.459	1.229–4.919	0.011*	2.564	1.254–5.243	0.01*
CLEC14A ≤6	29	Baseline			Baseline		
CLEC14A >6	21	0.804	0.405–1.598	0.534	1.018	0.508–2.041	0.959
CD31 ≤25	32	Baseline			Baseline		
CD31 >25	18	1.143	0.575–2.27	0.703	1.113	0.528–2.347	0.779
CD105 ≤10	33	Baseline			Baseline		
CD105 >10	17	0.881	0.438–1.772	0.722	0.741	0.352–1.560	0.431

HR hazard ratio, *CI* confidence interval, *NA* not applicable

* Statistically significant ($P < 0.05$)

biopsies and post-treated surgical specimens and found that the lower the microvessel count for CD105 before treatment, the better the clinical response to chemotherapy [38].

Although there are no specific endothelial markers that detect new blood vessels in a tumor, one can speculate that selective expression of a marker prior to treatment that disappears completely after treatment, may be significant. The ability to detect only tumor blood vessels as opposed to normal blood vessels by any surface marker has proven difficult to establish with certainty. CLEC14A, as opposed to CD31 or CD105, identifies fewer blood vessels in tumors, and this expression is the first to disappear after chemotherapy. The above data along with that published previously suggest that CLEC14A may be more specifically identifying blood vessels in a tumor [7]. The initial report, which identified CLEC14A as an angiogenic molecule demonstrated that this protein is expressed in blood vessels of tumor tissue such as breast, liver, prostate, kidney and thyroid. However, this marker was not expressed in the blood vessels of respective normal tissue [7]. It has also been shown by another group that human antibodies specific to CTLDs blocked endothelial cell migration and tube formation. In addition to this, antibodies specific to CTLDs cross-link with CLEC14A and significantly downregulated the expression of CLEC14A on the surface of endothelial cells, which implies that CLEC14A−CTLD may be a key domain in angiogenesis [10].

It is important to understand if the same blood vessels expressed CD31, CD105 or CLEC14A. In order to study this, we attempted to evaluate the co-expression of CD31 and CD105 on vessels by double IHC. However, we were not able to show the simultaneous expression of both these antigens on blood vessels, although they were expressed individually by IHC in the same experiment (unpublished data). The inability to show simultaneous expression of all markers in blood vessels may be due to their expression in the same subcellular location in endothelial cells, namely the plasma membrane.

The relationship between the MVD of tumors as determined by CD31 and survival has not been consistent based on previous reports [35, 39–41]. Similarly, MVD, as determined by CD105, has also not been consistent as a prognostic factor in this study and previous reports [6]. However, CD105 is a good target for imaging vessels in vitro. Furthermore, an antibody against CD105 has undergone clinical trials (phase 1) [18]. VEGF is present abundantly in patients with malignant ascites due to ovarian cancer,

Table 4 Multivariate analysis of vascular density and clinical parameters of patients who had undergone primary surgery

Variable	Number	Event-free survival			Overall survival		
		HR	95 % CI	*P* value	HR	95 % CI	*P* value
Model I grade							
Low/intermediate	27	Baseline			Baseline		
High	23	2.035	1.007–4.113	0.048*	2.240	1.087–4.619	0.029*
CLEC14A ≤6	29	Baseline			Baseline		
CLEC14A >6	21	0.788	0.382–1.627	0.520	0.937	0.450–1.951	0.863
CD31 ≤25	32	Baseline			Baseline		
CD31 >25	18	1.151	0.547–2.422	0.710	0.947	0.43–2.082	0.893
CD105 ≤10	33	Baseline			Baseline		
CD105 >10	17	0.761	0.36–1.609	0.475	0.671	0.312–1.444	0.307
Model II residual disease							
Optimal	24	Baseline			Baseline		
Suboptimal	26	2.534	1.244–5.162	0.010*	2.514	1.222–5.174	0.012*
CLEC14A ≤6	29	Baseline			Baseline		
CLEC14A >6	21	0.775	0.375–1.605	0.493	0.955	0.458–1.990	0.901
CD31 ≤25	32	Baseline			Baseline		
CD31 >25	18	1.092	0.520–2.293	0.817	0.982	0.444–2.169	0.964
CD105 ≤10	33	Baseline			Baseline		
CD105 >10	17	1.042	0.496–2.187	0.913	0.834	0.391–1.777	0.638

HR hazard ratio, *CI* confidence interval

* Statistically significant ($P < 0.05$)

and bevacizumab, which is a VEGF-specific monoclonal antibody, has been shown to inhibit ascites and tumorigenesis in xenograft models of ovarian cancer [42]. MVD in ovarian tumors may not be relevant as the pattern of spread is possibly transcoelomic. Evaluation of lymphatic vascular density was also not significant [32]. Despite the debatable relationship between MVD and prognosis in ovarian cancer, recent clinical trials have shown that administration of bevacizumab (antibody against VEGF) in conjunction with chemotherapy as maintenance improved survival significantly [43, 44]. This lends hope that anti-vascular therapy may still find a role in the management of ovarian cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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