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ORIGINAL ARTICLE

CD105 is a more appropriate marker for evaluating angiogenesis in urothelial cancer of the upper urinary tract than CD31 or CD34

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Abstract Angiogenesis plays an important role in cancer progression in many types of cancer. Evaluation of angiogenesis is often performed, but the optimal methodology for human cancer has not been agreed upon. As adequate evaluation of angiogenesis in cancer tissues might be important for prediction of prognosis and treatment decisions, we evaluated angiogenesis semiquantitatively by assessing microvessel density (MVD) in urothelial cancer of the upper urinary tract (UC-UUT). We compared the performance of three endothelial cell markers (CD31, CD34, and CD105) on formalin-fixed tissues from 122 patients diagnosed with UC-UUT without metastasis. Vascular endothelial growth factor (VEGF)-A expression was also evaluated immunohistochemically. Correlations between MVD with each marker and pT stage, grade, survival, and VEGF-A expression were investigated. Mean (standard deviation) MVD as estimated by immunohistochemical staining with anti-CD31, anti-CD34, and anti-CD105 were 47.1 (17.9)/high-power field (HPF), 70.9 (19.5)/HPF, and 31.2 (16.7)/HPF, respectively. Although all MVDs were significantly associated with pT stage and grade, CD105-MVD showed the strongest association. Similarly, CD105-MVD showed the strongest correlation with VEGF-A expression ($r=0.530$, $p<0.001$). Although all MVDs were associated with metastasis-free survival and cause-specific survival on univariate analysis, only CD105-MVD was retained as an independent predictor in multivariate analysis including pT stage and grade. CD105-MVD may be the preferred marker for semiquantitative assessment of angiogenesis in patients with UC-UUT.

Keywords Angiogenesis · CD105 · CD31 · CD34 · Urothelial cancer of the upper urinary tract

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Introduction

Angiogenesis is an important step for tumor growth and progression in almost all malignancies [1]. Numerous investigations have focused on the pathological role, clinical significance, and predictive value of angiogenesis in patients with cancer. In human cancer tissues, the most representative method for (semi-)quantification of angiogenesis is the measurement of microvessel density (MVD) using endothelium-specific markers. This approach is highly suitable for histodiagnostic applications because it can be performed on formalin-fixed and paraffin-embedded specimens. There is a general agreement that MVD offers an important and useful prognostic marker of tumor progression and survival in various types of cancer.

Urothelial cancer (UC) frequently recurs and metastasizes despite adequately performed surgery [2]. Many investigators have investigated the pathological significance and prognostic role of angiogenesis in patients with UC of the urinary bladder and upper urinary tract (UUT), but conflicting results have been published regarding the relationship between MVD and malignant potential, clinicopathological features, and prognosis. Although some reports have described positive correlations between MVD and malignant potential cancer cell

progression and poor survival [3–5], others have shown no significant relationship between MVD and tumor progression, and others even considered MVD as a favorable predictor for survival [6, 7]. Such contradictory results are most likely attributable to the use of different markers for detecting endothelial cells in different studies.

To detect neovascular microvessels, various markers have been used for immunohistochemical staining of endothelial cells. CD31, CD34, and CD105 have often been used on human cancer tissues, including UC [8–14]. The biology of these markers differs, and no single endothelial marker might be perfect under all conditions. Studies comparing these markers have been performed on different cancer types in recent years [8–12]. MVD assessment using CD105 as marker (CD105-MVD) has been proposed as a better predictor of progression and prognosis than that using CD31 or CD34 in a variety of cancers [8, 9, 12–14]. Several studies have investigated the clinical significance and pathological role of CD105-MVD in bladder cancer patients [15, 16]. However, reports on the relationship between CD105-MVD and clinicopathological features, tumor progression, and survival in patients with UC-UUT have not been published as yet.

The main aim of this study was to elucidate the role in pathological assessment and clinical significance of CD105-MVD in UC-UUT. We therefore evaluated CD105-MVD in UC-UUT patients without metastasis and compared the results with those for CD31-MVD and CD34-MVD. Furthermore, we analyzed the correlations with expression of vascular endothelial growth factor (VEGF)-A for CD31-MVD and CD34-MVD.

Materials and methods

Patients and tissue samples

Specimens from 122 consecutive patients who had been diagnosed with non-metastatic UC-UUT were examined. Patients who had received any preoperative therapy were excluded. Specimens with carcinoma in situ were also excluded because MVD was difficult to evaluate in some specimens. All histological characteristics, including tumor grade and pT stage, were determined using formalin-fixed paraffin-embedded specimens from the resection specimen, and both staging and grading were assessed using the 2002 tumor–node metastasis classification. Cancer grade was divided into three grades (i.e., G1, G2, and G3), according to the World Health Organization classifications. A single pathologist performed all pathological examinations. Median duration of follow-up was 50 months (range, 2–250 months). The study protocol was approved by the human ethics review committee of Nagasaki University Hospital.

Immunohistochemistry and evaluation

Five-micrometer thick sections were deparaffinized stepwise in xylene and rehydrated in graded solutions of ethanol. Antigen retrieval was performed at 95 °C for 40 min in 0.01 M sodium citrate buffer (pH 6.0). All sections were then immersed in 3 % hydrogen peroxide for 30 min to block endogenous peroxidase activity. Primary antibodies were obtained from Novocastra (New Castle, UK; anti-human CD31 antibody), DakoCytomation (Glostrup, Denmark; anti-human CD34 antibody), Vector Laboratories (Burlingame, CA; anti-human CD105 antibody), and Santa Cruz Biotechnology (Santa Cruz, CA; anti-VEGF-A antibody). Sections were incubated overnight with primary antibodies at 4 °C. After incubation with primary antibody, sections were washed extensively and treated with peroxidase using the labeled polymer method with Dako EnVision™ peroxidase (Dako, Carpinteria, CA) for 60 min. The peroxidase reaction was visualized using a liquid DAB substrate kit (Zymed Laboratories, San Francisco, CA). Sections were counterstained with hematoxylin, dehydrated stepwise through a graded alcohol series, and cleared in xylene before mounting. Consecutive sections from each sample processed without the primary antibody were used as negative control. Positive controls for all antibodies comprised of kidney tissues including renal cell carcinoma. Methods of immunohistochemical staining for all antibodies except CD105 have been described previously [4, 17, 18].

All analyses of immunohistochemically stained sections were performed using light microscopy within the tumor area. Expression levels of VEGF family members were assessed semiquantitatively from the percentage of VEGF-expressing carcinoma cells (from ≥500 carcinoma cells) using a method similar to that described previously in detail [4, 17, 18]. Two investigators (S.W. and Y.M.) who were blinded to clinical features and survival data independently performed semiquantitative analyses and immunostaining interpretations. The disagreement rate for analyses between these two investigators was less than 10 %, and results from both investigators were averaged for statistical analyses.

To determine the MVD, tumor sections stained with each antibody were examined under a Nikon E-400 bright-field microscope (Nikon, Tokyo, Japan), and images were captured using a digital camera (DU100; Nikon) at ×200 objective lens magnification. For each tumor section, 3–5 fields with the greatest density of positively stained vessels (hot spots) were evaluated, irrespective of the tumor region. MVD was defined as the number of positively stained vessels per high-power field (HPF) estimated using computer-aided image analysis (WinROOF version 6.4; Mitani, Fukui, Japan). In our system, the field area of 1 HPF corresponded to 0.392 mm^2.

Statistical analyses

Normality was evaluated by normal distribution and histograms for each variable, and results are expressed as mean ± standard deviation (SD) unless otherwise stated. Student's *t* test was performed for continuous variables. The Scheffé's test was used for multiple comparisons of data. Pearson's correlation was used to evaluate relationships between continuous variables and the correlation coefficient (r). Corresponding p values are shown. Spearman's rank correlation coefficient was calculated to confirm Pearson's correlation. In survival analyses, variables that achieved statistical significance in univariate analyses were subsequently entered into multivariate analysis using Cox proportional hazards analysis. In this study, each MVD was measured as a continuous variable. We therefore performed survival analyses using two models as follows: MVD as a continuous variable (model A), and MVD values less than or equal to the median versus MVD values above the median (model B). All statistical tests were two-sided, and significance was defined as $p < 0.050$. All statistical analyses were performed on a personal computer using the StatView for Windows statistical package (version 5.0; Abacus Concepts, CA).

Results

The study population comprised of 92 men and 30 women, with a median age at the time of surgery of 68 years (range, 39–87 years). The pathological features are shown in Table 1. The most frequent pT stage was pT1 in 42 patients (34.7 %), followed by pT3 in 34 patients (27.3 %). In our study population, the number of patients with muscle invasion (68, 55.7 %) was higher than that without muscle invasion (54, 44.3 %). The most frequent grade in our study population was high grade (G3) in 47 patients (38.5 %).

Representative examples of CD31-, CD34-, and CD105-stained vessels in non-muscle-invasive cancer tissues are shown in Fig. 1a–c, respectively. Representative examples of CD31-, CD34-, and CD105-stained vessels in invasive tumor tissues are shown in Fig. 1d–f. Mean n ± SD (median) CD31-, CD34-, and CD105-MVDs were 47.1±17.9 (58.4)/HPF, 70.9±19.5 (69.0)/HPF, and 31.2±16.7 (22.3)/HPF, respectively. CD34-MVD was significantly higher than the other values ($p < 0.001$). On the other hand, CD105-MVD was the lowest of the three.

Significant differences were apparent between MVDs in low pT stage (non-muscle-invasive, pTa +1) and high (muscle-invasive, pT 2–4) ($p < 0.001$, Table 1). Similar results were also found in the relationship between MVDs in low-grade (G1 + 2) and high-grade (G3) tumors (Table 1). Detailed analysis of MVD (Table 1) showed for CD31-MVD a significant difference between pTa and pT1 and between pT3 and pT4. For CD34-MVD, a significant difference was only found between pT2 and pT3. For CD105-MVD, a significant difference was found between pTa and pT1, between pT1 and pT2, and between pT3 and pT4. In addition, CD105-MVD was markedly lower in pTa than in pT1, despite both representing non-muscle-invasive stages. Similar analyses for grade (Table 1) showed that CD31-MVD and CD34-MVD were significantly higher in G3 tumors than in G2 tumors. However, no such significant difference was detected between G1 and G2 tumors. In contrast, CD105-MVD was closely associated with grade. With none of the markers, striking differences in the location of "hot spots" for MVD, as for example between the invasive front and tumor center, were apparent.

Correlations between each MVD and VEGF-A expression are shown in Fig. 2. In the whole cohort, all MVDs correlated significantly with VEGF-A expression (Fig. 2a–c), but this was strongest for CD105-MVD. In muscle-invasive disease, similar results were found for all MVDs (Fig. 2c–e). However, in non-muscle-invasive disease, CD105-MVD correlated with VEGF-A expression (Fig. 2h), whereas no such difference was found for CD31- or CD34-MVD (Fig. 2f, g).

We analyzed the prognostic value of each MVD for metastasis after primary treatment (Table 2). Univariate analysis showed that all MVDs significantly associated with metastasis. However, CD31-MVD and CD34-MVD were not identified as independent significant predictors in a multivariate analysis model including pT stage, grade, and adjuvant therapy. For cause-specific survival, similar results were shown for both uni- and multivariate analyses (Table 2). Models A and B showed similar results in all survival analyses.

Discussion

In this study, we compared MVDs determined by immunohistochemical staining of vessels using three antibodies in tissues from non-metastatic UC-UUT: anti-CD31, anti-CD34, and anti-CD105. Our results show that staining with anti-CD105 more accurately reflects pathological features than that with the other antibodies and represents the most useful predictor of outcome.

CD105, endoglin, is a cell membrane glycoprotein that modulates angiogenesis by regulating cellular proliferation, differentiation, and migration [19]. Several investigators have reported preferential expression of CD105 in activated endothelial cells participating in neoangiogenesis particularly in cancer, with no or only weak expression in blood vessels of most normal tissues [13, 14]. Several reports have positively associated CD105-MVD with cancer cell invasion and metastasis in malignancies including cervical and head and neck cancers [20, 21]. In addition, increased CD105-MVD has been associated with worse survival in several types of cancer, including breast and head and neck cancer [13, 21]. Our findings are in line with these reports. On the other hand,

Table 1 Relationship between each microvessel density (MVD) and pathological features

	N	CD31-MVD		CD34-MVD		CD105-MVD	
		Mean (SD)	*p* value	Mean (SD)	*p* value	Mean (SD)	*p* value
pT stage							
Low	54	36.5 (8.6)	<0.001	60.6 (13.7)	<0.001	18.5 (7.7)	<0.001
High	68	55.3 (19.0)		78.6 (19.6)		41.0 (14.6)	
Ta	12	35.0 (9.0)	0.996	58.7 (12.8)	0.994	9.3 (3.8)	0.028
T1	42	37.0 (8.6)	0.093	61.2 (14.3)	0.860	20.5 (5.3)	<0.001
T2	23	47.5 (16.6)	0.338	65.9 (14.0)	0.002	34.0 (12.7)	0.119
T3	34	55.8 (17.0)	0.042	84.0 (20.9)	0.793	42.0 (11.8)	0.010
T4	11	71.6 (20.4)		91.2 (8.9)		54.3 (16.1)	
Grade							
Low	75	41.9 (15.2)	<0.001	65.7 (17.9)	<0.001	24.4 (12.6)	<0.001
High	47	55.5 (18.8)		79.3 (19.3)		41.2 (17.2)	
G1	23	37.5 (9.4)	0.326	62.3 (12.3)	0.581	17.4 (7.6)	0.012
G2	52	43.8 (16.9)	0.003	67.2 (19.8)	0.006	28.0 (13.3)	<0.001
G3	47	55.5 (18.8)		79.3 (19.3)		41.2. (17.2)	

MVD microvessel density, *P p* value, *G* grade

earlier reports regarding CD105-MVD and pT stage in bladder cancer patients with non-muscle-invasive disease showed no difference in CD105-MVD between pTa and pT1 tumors [15]. We have no explanation for these discrepancies, but the choice of the antibody, method of unmasking, tissue characteristics, and microvessel definition might be involved.

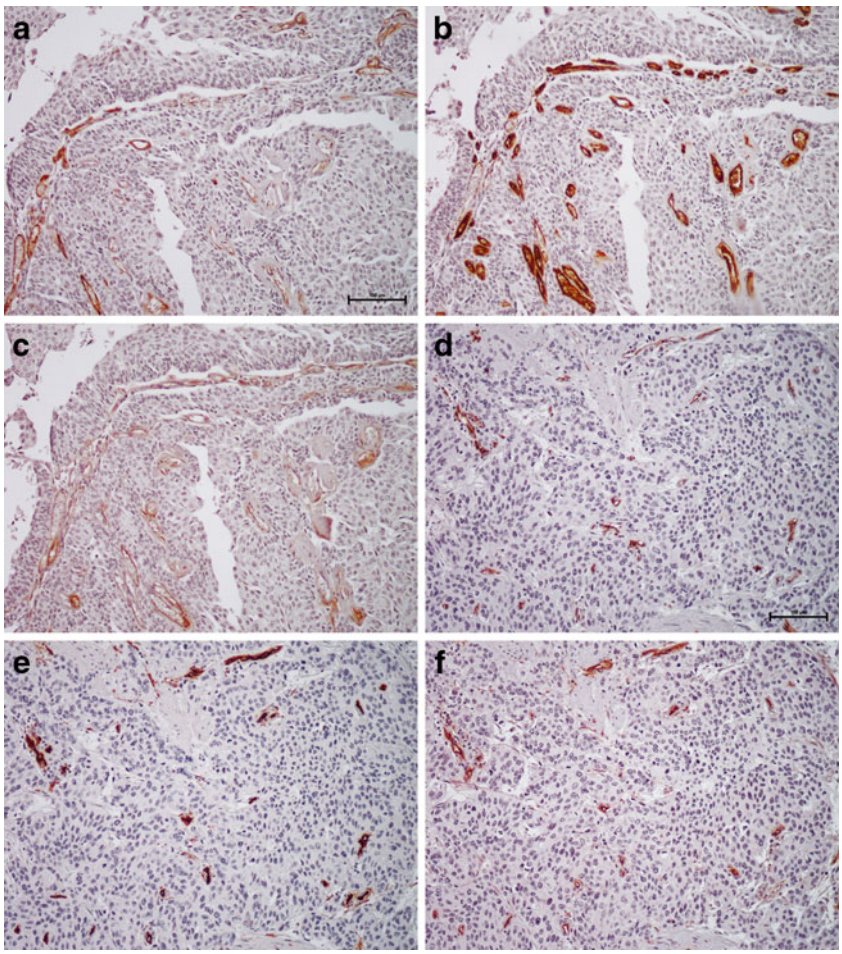

Fig. 1 **a**–**c** Representative tissues from non-muscle-invasive urothelial cancer of the upper urinary tract with staining for CD31 (**a**), CD34 (**b**), and CD105 (**c**). Some vessels stained strongly for CD31 and CD34. However, such strongly stained vessels were relatively lower in CD105 compared to CD31 and CD34. **d**–**f** Representative tissues from muscle-invasive cancer with staining for CD31 (**d**), CD34 (**e**), and CD105 (**f**). With regard to staining intensity, CD34 was clearly stained in both non-muscle-invasive and muscle-invasive disease. On the other hand, CD105 clearly showed a wider difference between non-muscle-invasive and muscle-invasive disease. (×200 magnification; bar=100 μm)

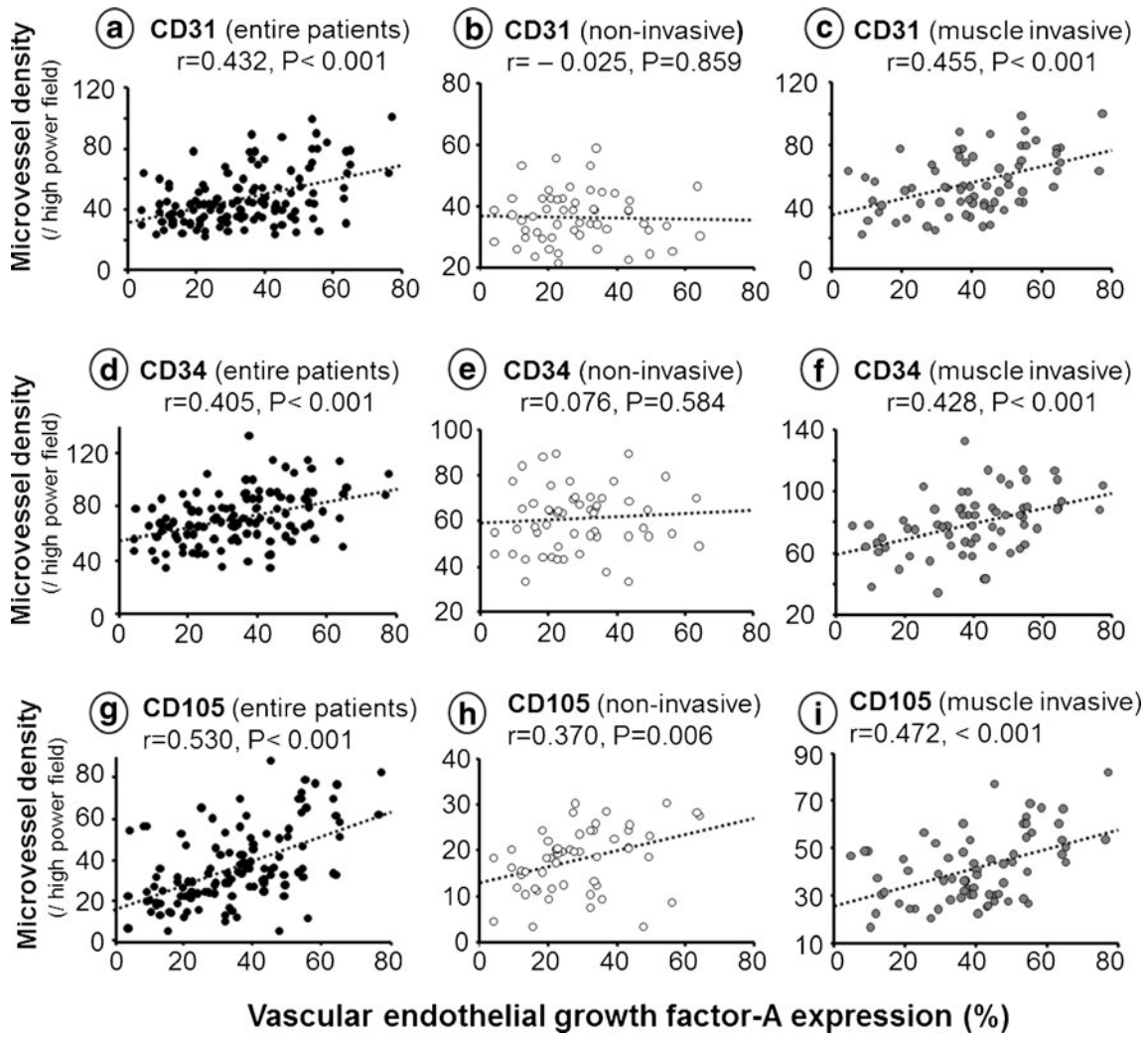

Fig. 2 Correlations between VEGF expression and each type of microvessel density (*MVD*) are shown. CD31-MVD (**a**–**c**) correlated significantly with VEGF expression in the overall patient population (**a**) and in patients with muscle-invasive disease (**c**), but not in patients with non-muscle-invasive disease (**b**). Similar findings were found for CD34-MVD (**d**–**f**). CD105-MVD correlated with all conditions, including overall population (**g**), non-muscle-invasive disease (**h**), and muscle-invasive disease (**i**)

Our results also show that CD31-MVD and CD34-MVD are significantly higher in muscle-invasive than in non-muscle-invasive disease and are associated with progression and survival in patients with UC-UUT. Similar findings have been described in previous reports [3, 4]. We consider these MVDs to still be useful for evaluating angiogenic status. MVD as measured using these antigens has been evaluated in several previous reports on human UC specimens, providing useful information in terms of clinical significance of angiogenesis [4, 5, 15, 22]. However, our multivariate analyses show that CD31-MVD and CD34-MVD are not independent significant predictors of outcome. We propose that CD31-MVD and CD34-MVD do not adequately reflect malignant potential and should be considered insufficient for predicting prognosis in patients with non-metastatic UC-UUT.

CD31, a platelet endothelial cell adhesion molecule 1, is an endothelial cell antigen. Anti-CD31 antibody stains macrophages and plasma cells in paraffin-embedded specimens [23], but this staining can be reduced by microwave antigen retrieval [24]. CD34, a transmembrane glycoprotein, is a hematopoietic progenitor cell antigen. Anti-CD34 antibodies stain endothelial cells in neoplasia more strongly than normal endothelium, and constitute a very sensitive marker of endothelial differentiation [25]. However, anti-CD34 antibodies also stain fibroblasts, adipocytes, and lymphatic endothelial cells [26], which might interfere with an MVD count, notably as regards lymphatic vessels. As a consequence, in all earlier reports, MVD-CD34 was higher compared to MVD-CD31 and/or MVD-CD105 [10, 12, 15]. Using anti-CD31 and anti-CD105, staining intensity seems to be higher in muscle-invasive than in non-muscle-invasive cancer but not with anti-CD34, which supports the notion that anti-CD34 detects various normal as well as cancer-related endothelial cells.

Various findings, thus, support the hypothesis that CD105-MVD might reflect pathological and prognostic significance

Table 2 Each microvessel density (MVD) and outcome after primary treatment

	Univariate analysis			Multivariate analysis		
	HR	95 % CI	*p* value	HR	95 % CI	*p* value
For metastasis						
(Model A)						
CD31-MVD	1.044	1.024–1.064	<.0001	1.022	0.998–1.047	0.070
CD34-MVD	1.031	1.013–1.048	0.001	1.014	0.994–1.035	0.212
CD105-MVD	1.053	1.038–1.069	<0.001	1.031	1.002–1.061	0.038
(Model B)						
CD31-MVD	3.71	1.65–8.36	0.016	1.47	0.59–3.69	0.409
CD34-MVD	2.59	1.21–5.54	0.015	1.06	0.43–2.57	0.911
CD105-MVD	10.56	3.67–30.41	<0.001	5.40	1.34–21.8	0.018
For CSS						
(Model A)						
CD31-MVD	1.042	1.020–1.063	<0.001	1.015	0.406–2.192	0.254
CD34-MVD	1.056	1.033–1.079	<0.001	1.016	0.993–1.040	0.167
CD105-MVD	1.034	1.015–1.055	0.001	1.032	1.001–1.064	0.041
(Model B)						
CD31-MVD	2.39	1.04–5.12	0.040	0.94	0.41–2.19	0.828
CD34-MVD	2.54	1.12–5.73	0.025	0.93	0.36–2.38	0.875
CD105-MVD	12.89	3.84–43.27	<0.001	4.30	1.07–17.25	0.040

Model A continuous variables of MVD were used for analyses

Model B over median levels was defined as risk

HR hazard ratio, *CI* confidential interval, *P p* value, *MVD* microvessel density, *CSS* cause-specific survival

of angiogenesis more accurately than CD31-MVD or CD34-MVD. In line with our findings on non-metastatic UC-UUT, in breast, lung, and ovarian cancer, CD105-MVD associated with worse prognosis [12–14], but CD34-MVD did not. This is contested by other reports, e.g., in cervical cancer, with high CD31-MVD predicting progression-free survival and overall survival, whereas high CD105-MVD only showed a trend for worse survival [11]. It is suggested that CD31-positive endothelial cells are in more stable blood vessels, while CD105-positive endothelial cells reflect newly formed leaky vessels, less efficient in delivery of oxygen and nutrients, but providing a more efficient route of metastatic spread. Another report showed that in multivariate analyses, CD34-MVD correlated with survival for lung cancer, whereas CD105-MVD did not [27]. Further detailed investigations are needed to establish optimal conditions to evaluate MVD in cancer, including marker and antibody choice, definition of microvessels and blood vessels, and microscopy conditions.

Of note, all of our MVDs showed a positive correlation with VEGF-A expression in the whole cohort (Fig. 2). Interestingly, when analyzed separately in non-muscle-invasive and muscle-invasive tumors, CD31- and CD34-MVD correlated significantly with VEGF-A expression in muscle-invasive but not in non-muscle-invasive disease, whereas for CD105-MVD, this difference was not found. We ruled out a pT stage bias as explanation. VEGF-A expression has been higher in high-stage UC than in low-stage UC [22, 28]. There is a general agreement that VEGF-A expression is closely associated with tumor-associated angiogenesis. We speculate that CD31-MVD and CD34-MVD reflects angiogenic status only in muscle-invasive disease when VEGF-A is highly expressed. CD105-MVD might be more closely associated with the proangiogenic functions of VEGF-A in human UC-UUT tissues, as has been suggested for other cancer types. For example, CD105-MVD closely associated with VEGF-A expression in gastric cancer [29] and hepatocellular carcinoma [9]. Furthermore, VEGF-A expression associated with CD105-MVD, but with neither CD31-MVD in anaplastic astrocytoma nor CD34-MVD in endometrial cancer [8, 30].

In conclusion, our results show that CD105-MVD is more closely associated with malignant potential and tumor growth than CD31-MVD or CD34-MVD. Only CD105-MVD was an independent predictor for tumor progression and survival on multivariate analysis. Therefore, CD105 is the pan-endothelial marker of choice for evaluating cancer-related angiogenesis in UC-UUT.

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