RESEARCH ARTICLE

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Endocan-microvascular Density in Primary Ovarian Carcinoma

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Abstract

Background: Epithelial ovarian cancer is among the leading causes of death in women and is driven by angiogenesis. Microvascular density (MVD) can be used to evaluate angiogenesis in carcinomas and thus it can be used as a potential biomarker for ovarian cancer. This study is aimed to establish the association between endocan-MVD with clinicopathological factors in primary epithelial ovarian cancer. **Methods:** The clinicopathological characteristics were acquired from the medical records filed between January 2008 and December 2018 of 89 epithelial ovarian cancer cases in Hospital Universiti Sains Malaysia, Kelantan, Malaysia. Sectioned samples were analyzed for endocan through immunohistochemistry followed by the quantification of MVD. The association between clinicopathological characteristics and endocan-MVD was analyzed using the Pearson chi-square test and Fischer's exact test. **Results:** All cases of epithelial ovarian carcinomas were positive for endocan. The mean ± standard deviation value of endocan-MVD level was 21.6±14.60 microvessels per 200x field. A total of 53 (59.6%) cases had low and 36 (40.04%) had high endocan-MVD values. High endocan-MVD level had a significant association with the older age group (p-value = 0.009), smaller tumor size (p-value<0.001), type II tumor (p-value<0.001), high-grade tumor (p-value<0.001), advanced FIGO stage (p-value=0.002), and presence of tumor recurrence (p-value=0.017). No significant association was found between endocan-MVD and the other clinicopathological characteristics such as race, pre-operative serum CA-125 level, presence of diabetes mellitus, endometriosis, lymph node involvement, distant metastasis, and family history of malignancy. **Conclusion:** Endocan-MVD showed a significant association with age, tumor size, tumor type, tumor grade, FIGO stage, and recurrence in primary epithelial ovarian cancer. Thus, endocan-MVD could be implemented as a reliable marker to predict prognosis in epithelial ovarian cancer in the future.

Keywords: Endocan-microvascular density- epithelial ovarian cancer- angiogenesis

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Introduction

Ovarian cancer is the eighth most common cancer and the ninth leading cause of cancer-related mortality in women worldwide. The Global Cancer Statistic 2018 reported that ovarian cancer contributed 3.4% of new cancer cases (295,414 cases) and 4.4% of cancer-related mortality in women (184,799 deaths) (Bray et al., 2018). According to the latest Malaysian National Cancer Registry Report, ovarian cancer is the fourth most common cancer in women in Malaysia and ranked second in female genital tract cancer with a total of 3,575 new cases reported between 2012 and 2016 as compared to 3,472 cases reported between 2007 and 2011 (Azizah et al., 2019).

The majority of ovarian cancer cases (56.3%) in Malaysia are diagnosed at an advanced stage (stage III or stage IV), with a little prospect of treatment, primarily due to late presentation (Azizah et al., 2019). This may be attributable to the symptomless nature of the early stage of ovarian cancer. Therefore, it remains undiagnosed until it reaches a significant severity.

Endocan is an endothelium-derived soluble dermatan sulfate (DS) proteoglycan secreted by tip cell, a specialized subset of endothelial cells known to mediate vessel growth during the angiogenesis process (Marion-Audibert et al., 2003). Endocan is a gene product located in the proximal region of the long arm of chromosome 5 (5q11.2). Inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and pro-angiogenic growth factors such as VEGF and FGF-2 strongly increase the expression, synthesis, or the secretion of endocan by human endothelial cells (Chang et al., 2016). Endocan promotes tumorigenesis via the hepatocyte growth factor / scatter factor (HGF/SF) pathway (Imao et al., 2004; Chang et al., 2016; Priya et al., 2017). The DS chain of endocan binds and activates HGF in vitro (Chang et al., 2016). The HGF is a multifunctional growth factor that can bind to both heparan sulfate (HS) and dermatan sulfate

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glycosaminoglycans (DS GAGs). It subsequently activates the Met receptor, which plays significant roles in many pathophysiological processes, including development, wound healing, and tumor progression (Chang et al., 2016). Endocan also regulates cancer cell survival by suppressing the cancer cell apoptosis via the NF-κB signaling pathway (Chang et al., 2016; Priya et al., 2017).

Endocan detection is the most appropriate tool to discriminate between the 'good' (the resting vessel in normal tissue), the 'bad' (the emerging vessel dedicated tumor angiogenesis), and the 'ugly' (the proliferating multi-layered capillary as seen in a highly aggressive tumor of the brain) (Chang et al., 2016). The immunodetection of endocan in tissues may reveal aggressive behaviour or recurrence depending on the type of tumor. For instance, endocan expression by endothelial cells in hepatocellular carcinoma is associated with unfavorable prognostic features (He et al., 2015). Similarly, a multivariate analysis showed that endocan-MVD was an independent prognostic marker for the overall survival of epithelial ovarian cancer (Priya et al., 2017).

Despite the interest, to the best of our knowledge, there are limited studies on endocan-MVD in epithelial ovarian cancer (Lal et al., 2017; Ziol et al., 2013; Priya et al., 2017). This is the first clinical study to explore the expression of endocan in epithelial ovarian cancer in Malaysia. Thus, this study contributes to knowledge on endocan-MVD and its association with clinicopathological parameters in primary epithelial ovarian cancer patients.

Materials and Methods

Case and sample selection

Through a cross-sectional study conducted in Hospital Universiti Sains Malaysia (USM), Kelantan, Malaysia, a total of 89 cases of primary epithelial ovarian cancer diagnosed between 1st January 2008 and 31st December 2018 were enrolled. The retrospective cases were retrieved from the computerized registry data (LIS and PATHORS) of the Department of Pathology, Hospital USM. The search for cases was done through PATHORS and LIS using keywords '*ovarian carcinoma*,' '*ovarian adenocarcinoma*,' '*ovarian cancer*,' '*cystadenocarcinoma*,' and '*ovary*'.

All primary epithelial ovarian cancer cases with at least one representative paraffin-embedded tissue block available was included in the study. Patients with secondary epithelial ovarian cancer cases, referral cases to Hospital USM, inadequate clinical history and insufficient tissues on two serial sections were excluded. The sample size was calculated based on single proportion formula with precision of 5% and power of study of 80%. The estimated sample size was 91 cases, after including an anticipated dropout rate of 10%.

The patient's clinicopathological data were obtained by reviewing the medical report from the medical record unit, Hospital USM. All samples were retrieved from the archived formalin-fixed paraffin-embedded (FFPE) tissue blocks diagnosed by independent pathologists in the Department of Pathology, Hospital USM. The FFPE block with the most viable and a good percentage of tumour tissue examined under the haematoxylin and eosin (H&E) stain was chosen for the immunohistochemistry study. Only one representative tissue block was selected from each case. The information was entered in the data collection form.

Immunohistochemistry procedures

The selected FFPE tissue blocks containing primary epithelial ovarian cancer were sectioned into 3 µm thickness slices and mounted on poly-L-lysine pre-coated adhesive glass slides. The slides were baked on the hot plate for one hour at 60°C to dry the water. Following that, the slides were incubated inside the Dako PT Link instrument (Agilent, USA) to perform an automated water bath antigen retrieval process for 15 minutes at 95°C using EnVision Flex Target Retrieval Solution, High pH (9.0) (Abcam, UK). The endogenous peroxidases were blocked using EnVision Flex Peroxidase Blocking reagent for five minutes. After that, the slides were washed with Tris-Buffer Solution (TBS) for two minutes. Then, they were incubated with primary antibody rabbit polyclonal anti-human endocan/ ESM-1 (bs-3615R, Bioss, USA dilution 1:300) for one hour at room temperature.

The slides were then washed twice in TBS for two minutes and then allowed to react with the corresponding secondary antibody solution for 30 minutes at room temperature. After two washes using TBS for two minutes each time, the slides were visualized using 0.05% diaminobenzidine tetrahydrochloride (DAB) before being washed again using the running tap water for five minutes. Following that, the slides were counterstained with haematoxylin and washed in the running tap water and dehydrated with increasing alcohol concentration (70%, 80%, 95%, and twice 100%) for two minutes each. Finally, the tissue slides were cleared with xylene for two minutes and applied with a coverslip using dibutylphthalate polystyrene xylene (DPX) mounting solution (Merck, USA).

Endocan-MVD assessment

The MVD was assessed according to the Weidner counting method (Weidner, 2008; El Behery et al., 2013). The criteria to judge countable vessels were any highlighted microvessels (capillaries, arterioles, and venules) regardless of the presence or absence of the lumens. A single countable microvessel was characterized using brown stained endothelial cells or endothelial cells-cluster, clearly separated from adjacent microvessels, tumor cells, and other connective tissue elements. The large or thick-walled blood vessels were excluded. The immunostained sections were scanned at a low power field (40x) to identify the three most vascularised areas with the highest density of distinctly highlighted microvessels within the tumor (hot spots). The individual microvessels were counted manually in the three hot spots at 200x magnification (Olympus BX51 optical microscope, with wide-field eyepiece number 22, providing a histological field of 0.950 mm² at 200x magnification) (Olympus, Japan).

The number of microvessels were recorded as a total number per unit of area. The mean microvessel count

was then calculated for each specimen and recorded as 'endocan-MVD' in microvessels per 200x field. As a validated cut-off point had yet to be established for endocan-MVD in epithelial ovarian cancer, the value was categorized using the mean value as a separating point (Ibrahim et al., 2015). The value of endocan-MVD was categorized using the mean value as a separating point. High level of endocan-MVD was defined as a value ≥ 21.63 (the mean value), while low-endocan MVD was defined as a value of < 21.63.

Statistical analysis

The data was analyzed using IBM SPSS Statistics for Windows, Version 26.0 2019. (Armonk, NY: IBM Corp). The clinicopathological data were analyzed using descriptive analysis. The data frequency and percentage were presented. The association of clinicopathological characteristics with the endocan-MVD level was analyzed using the Pearson chi-square test or Fischer's exact test (p-value< 0.05).

Ethics consideration

The study protocol was approved by Human Research Ethical Committee of the School of Medical Sciences, USM (JEPeM) (USM/JEPeM/18120783).

Results

Clinicopathological data

A total number of 89 primary epithelial ovarian cancer cases were enrolled. The clinicopathological characteristics of the analyzed primary epithelial ovarian cancer were summarized in Table 1. The patients' age ranged between 12 and 78 years old and the mean ± standard deviation (SD) age was 51.1 ± 13.20 years old at diagnosis. The majority of patients had Type II cancer (53.9%), high grade (66.3%), and late stages (58.4%).

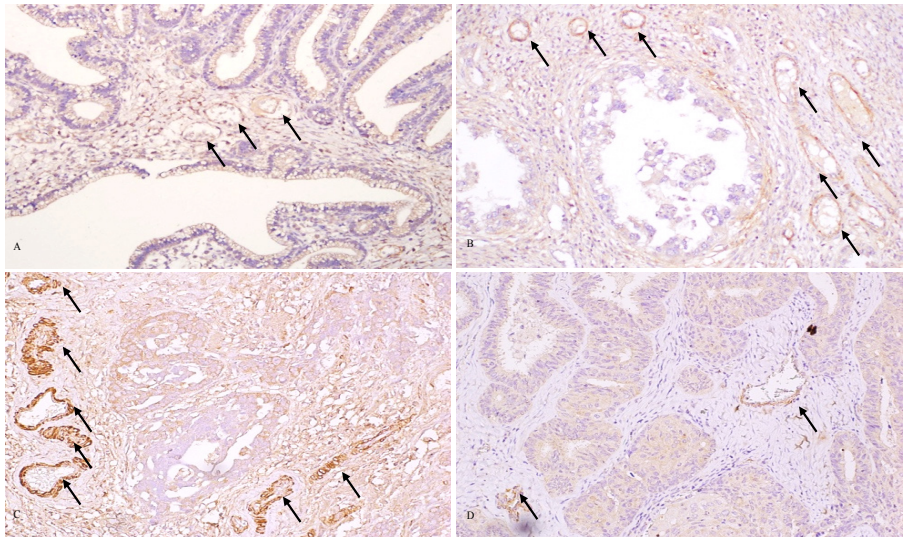

Figure 1. Scoring for Endocan-MVD. Low power view showing representative fields of epithelial ovarian cancer of intratumoural and peritumoural microvessels stained by endocan immunohistochemistry: A, B, C & D. Some of the representative microvessels are indicated by the arrow. Endocan immunohistochemical stain, magnification 200x.

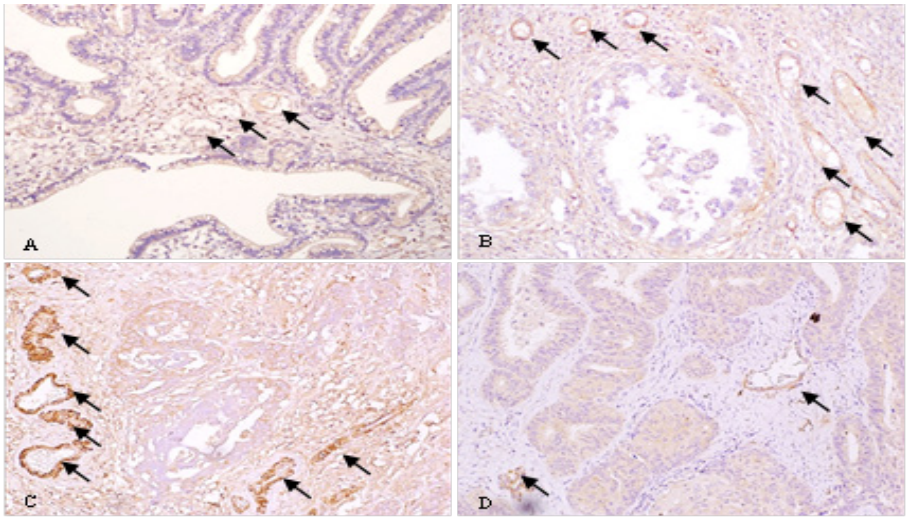

Figure 2. Representative Views from Endocan Stained Endothelial Cells in Different Histological Subtypes of Epithelial Cancer. A, Mucinous carcinoma; B, Clear cell carcinoma; C, High-grade serous carcinoma; D, Endometrioid carcinoma. Some of the representative microvessels are indicated by the arrow. Endocan immunohistochemical stain, magnification 200x.

Table 1. Clinicopathological Characteristics of the Primary Epithelial Ovarian Cancer Patients (n= 89)

Variables		Frequency (%)
Age (years)	Less than 45	23 (25.8)
	More than 45	66 (74.2)
Race	Malay	84 (94.4)
	Others	5 (5.6)
Serum CA-125 (U/mL)	< 35	7 (7.9)
	≥ 35	81 (91.0)
	Unavailable	1 (1.1)
Tumour size (mm)	< 100	29 (32.6)
	≥ 100	60 (67.4)
Histological type	Type I	41 (46.1)
	Type II	48 (53.9)
Subtype	Low-grade serous carcinoma	2 (2.2)
	Low-grade endometrioid carcinoma	5 (5.6)
	Clear cell carcinoma	11 (12.4)
	Mucinous carcinoma	22 (24.7)
	Malignant Brenner tumour	1 (1.1)
	High-grade serous carcinoma	41 (46.1)
	High-grade endometrioid carcinoma	7 (7.9)
Tumour grade	Low (Grade 1 or 2)	30 (33.7)
	High (Grade 3)	59 (66.3)
Lymph nodes metastasis	N0 – No	72 (80.9)
	N1 – Yes	17 (19.1)
Distant metastasis	M0 – No	78 (87.6)
	M1 – Yes	11 (12.4)
FIGO stages	Early (Stage I or II)	37 (41.6)
	Late (Stage III or IV)	52 (58.4)
Diabetes Mellitus	No	74 (83.1)
	Yes	15 (16.9)
Family history of malignancy	No	75 (84.3)
	Yes	14 (15.7)
Endometriosis	No	76 (85.4)
	Yes	13 (14.6)
Laterality	Right	30 (33.7)
	Left	22 (24.7)
	Bilateral	37 (41.6)
Recurrence	No	62 (69.7)
	Yes	27 (30.3)

The endocan-MVD level

The endocan positively stained the endothelial cells' cytoplasm in both intratumoral and peritumoral areas (Figures 1 and 2). All cases of epithelial ovarian cancer were positive in terms of endocan immunohistochemistry staining. The endocan-MVD level ranged from 2.67 to 68.67 microvessels per 200x field. The mean ± SD value was 21.6 ± 14.60 microvessels per 200x field. Fifty-three cases (59.6%) had low endocan-MVD values and 36 cases (40.4%) had high endocan-MVD values.

Endocan-MVD level was significantly associated with the older age group (p-value=0.009), smaller tumor size (p-value<0.001), type II tumor (p-value<0.001), high-grade tumor (p-value<0.001), advanced FIGO stage (p-value=0.002), and presence of tumor recurrence (p-value=0.017) among primary epithelial ovarian cancer (Table 2). However, there was no significant association between endocan-MVD with the other clinicopathological characteristics such as race, pre-operative serum CA-125 level, diabetes mellitus, endometriosis, lymph node involvement, distant metastasis, and family history of malignancy.

Table 2. Factors Influenced Endocan-Microvascular Density among Patients with Primary Epithelial Ovarian Cancer (n=89)

Variables		Endocan-MVD		p-value
		Low	High	
		n (%)	n (%)	
Age (years)	< 45	19 (82.6)	4 (17.4)	0.009[a]
	≥ 45	34 (51.5)	32 (48.5)	
Race	Malay	52 (61.9)	32 (38.1)	0.153[b]
	Others	1 (20.0)	4 (80.0)	
Serum CA-125 (U/mL)	< 35	6 (85.7)	1 (14.3)	0.233[b]
	≥ 35	46 (56.8)	35 (43.2)	
Tumour size	< 100 mm	8 (27.6)	21 (72.4)	<0.001[a]
	≥ 100 mm	45 (75.0)	15 (25.0)	
Histological type	Type I	34 (82.9)	7 (17.1)	<0.001[a]
	Type II	19 (39.6)	29 (60.4)	
Tumour grade	Low grade	27 (90.0)	3 (10)	<0.001[a]
	High grade	26 (44.1)	33 (55.9)	
Lymph nodes involvement	N0 – No	44 (61.1)	28 (38.9)	0.537[a]
	N1 – Yes	9 (52.9)	8 (47.1)	
Distant metastasis	M0 – No	46 (59.0)	32 (41.0)	>0.950[b]
	M1 – Yes	7 (63.6)	4 (36.4)	
FIGO stages	Early stage	29 (78.4)	8 (21.6)	0.002[a]
	Late stage	24 (46.2)	28 (53.8)	
Diabetes Mellitus	No	43 (58.1)	31 (41.9)	0.538[a]
	Yes	10 (66.7)	5 (33.3)	
Family history of malignancy	No	47 (62.7)	28 (37.3)	0.166[a]
	Yes	6 (42.9)	8 (57.1)	
Endometriosis	No	46 (60.5)	30 (39.5)	0.650[a]
	Yes	7 (53.8)	6 (46.2)	
Recurrence	No	42 (67.7)	20 (32.2)	0.017[a]
	Yes	11 (40.7)	16 (59.3)	

[a], Pearson Chi-square test, [b], Fisher's exact test

Discussion

The MVD associated biomarkers have been utilized as biomarkers to examine neovascularization in tissues. These markers (CD31 and CD34) are utilized to stain for micro vessels associated with tumors. CD31 stain are used extensively for the assessment of MVD though with low specificity. Similar to CD31, endocan expression also correlates with CD34 expression due to the presence of specific endocan staining in vascular endothelial cells expressing CD34 (Leroy et al., 2010; Cornelius et al., 2012; Matano et al., 2014).

This is the first local study concerning endocan-MVD in epithelial ovarian cancer. The results showed that the mean ± SD of endocan-MVD in ovarian cancer

was equal to 21.6 ± 14.60. Other studies have used other MVD markers, CD105-MVD, and CD31-MVD in ovarian carcinoma and reported results consistent with our endocan-MVD results (28.78 ± 22.20 and 28.69 ± 18.57 per 200x field, respectively) (Taskiran et al., 2006). Our result however is lower than the endocan-MVD values reported by El-Behery et al., (2013) at 73.5 ± 9.10. The discrepancy between the data might be due to experimental conditions; so that El-Behery et al., (2013) utilized an average of three 200x field hot spots without referencing the exclusion of large and thick-walled blood vessels and the size of the field area used in their study.

All cases of epithelial ovarian carcinoma in this study were positive for endocan expression. The presence of endocan in the cytoplasm of the vascular endothelial cells of the primary epithelial ovarian carcinoma were also detected in both intra-tumoral and peri-tumoral regions. The authors did not evaluate the endocan immunoreactivity within the normal endothelial cells or the normal ovarian tissue due to study limitations. However, negative endocan endothelial cell immunoreactivity within normal ovarian tissue has been previously reported (El Behery et al., 2013).

The results also showed that high endocan-MVD level in epithelial ovarian cancer was significantly associated with older age group. The expression of endocan-MVD increased in women within the age 45 years and above. El Behery et al. (2013) used a cut-off point of 50 years old in their research and found no association between the age group and endocan-MVD (El Behery et al., 2013). Published data by studies based on other MVD markers in ovarian cancer and other forms of cancer also shown discrepancies as to the association between age and MVD. A previously published study on ovarian cancer has shown that levels of CD34-MVD in women 45 years old and older had a lower CD34-MVD compared to women in younger age group (Chan et al., 2004). The association of endocan-MVD with age in other types of the tumor was also studied. For example, a research on 142 gastric patients found no association of endocan-MVD with the age of patients using 60 years old as a cut-off point (Chang et al., 2016). Another study on colorectal cancer tissues found that older patients (aged more than 56 years old) had a higher CD31-MVD as compared to the younger age group, although the association was not statistically significant (p-value=0.062) (Hutajulu et al., 2018). On the other hand, another research found a significant association with increased MVD and older age (more than 60 years) in 152 Duke's B colorectal cancer tissues (Sundov et al., 2013).

Moreoer, high endocan-MVD level was also associated with advanced FIGO stage in ovarian carcinoma suggesting the ideal utility of endocan as biomarkers for ovarian cancer assessment. Studies using other MVD markers have shown the inconsistencies as to the association between MVD densities and FIGO stage. Elevated microvessel density stained with CD31 was associated with advanced stage of epithelial ovarian cancer in a study involving 202 patients (Stone et al., 2003). In contrast, Ferrero et al. (2011) investigated 350 patients with epithelial ovarian cancer and found no significant relationship between CD34-MVD with the FIGO stage (Ferrero et al., 2011). Furthermore, Rubatt et al., (2009) examined 106 cases of advanced epithelial ovarian cancer and found no correlation between CD31-MVD or CD105-MVD with FIGO stage III and IV (Rubatt et al., 2009).

In this study, it was discovered that small tumors have higher endocan-MVD values. It was hypothesized that this could be due to highly aggressive tumors like HGSC that yielded high MVD, which were usually smaller in size than the type I tumors (Tanaka et al., 2016). However, this finding was in contrast with the previous study by El Behery et al. (2013), who found that larger ovarian carcinomas had higher endocan-MVD value than the smaller tumors (El Behery et al., 2013). The current result shared similarity with the other types of tumors that also reported considerably higher mean MVD in smaller tumors. Marion-Audibert et al., (2003) studied 82 pancreatic endocrine tumors and found that mean CD34-MVD was significantly higher in tumors measuring less than 2 cm in diameter compared to the tumors measuring 2 to 5 cm and more than 5 cm groups (Marion-Audibert et al., 2003). Messerini et al., (2004) and Imao et al., (2004) found an inverse correlation of CD34-MVD with the tumor size in hepatocellular carcinoma and renal cell carcinoma respectively.

The current study also found a significant association between high endocan-MVD and recurrence. A meta-analysis on MVD in ovarian cancer using various markers failed to evaluate the influence of MVD in recurrence of ovarian cancer due to the limited studies on that association (He et al., 2015). The association of endocan with recurrence was studied in other tumors. Ziol et al., (2013) evaluated the prognostic value of biomarkers on 150 patients with compensated cirrhosis. They treated the early-stage uninodular hepatocellular carcinoma with radiofrequency ablation (RFA) and discovered that endocan expression by stromal endothelial cells had an independent predictive value for early recurrence after the RFA (Ziol et al., 2013). In addition, Kim et al., (2012) found that endocan tissue expression was an independent prognostic factor for recurrence in a study involving 143 colorectal carcinoma tissues (Kim et al., 2012).

Several studies have found a significant association between endocan-MVD and tumor grade in various neoplasms. A study on 58 cases of oral squamous cell carcinoma found that a higher endocan-MVD count was seen in an intermediate and high grade of oral squamous cell carcinoma as compared to the low-grade tumor (Irani and Amiri, 2019). A study on gastric cancer also discovered a significant association of high endocan-MVD with poorly differentiated carcinomas (Chang et al., 2016). Maurage et al. studied 82 cases of gliomas and discovered endocan was preferably expressed in hyperplastic endothelial cells of high-grade gliomas (glioblastoma) while most of endothelial cells of the low-grade gliomas were endocan negative (Maurage et al., 2009). Studies on MVD associated with tumor grade in ovarian cancer, assessed by endothelial markers other than endocan, also showed a significant association (Lal et al., 2017; Liu et al., 2012; Priya et al., 2017). As expected, a high level of endocan-MVD was significantly associated with type II

ovarian carcinomas, which were composed of high-grade tumors of which the vast majority were HGSC.

In conclusion, endocan has emerged as a very interesting protein due to its elevated expression in tumor vessels. A significant association was found between endocan-MVD and age, tumor size, tumor type, histological grade, FIGO stage, and recurrence of epithelial ovarian carcinoma. This shows that endocan-MVD has the potential to be a predictive tissue biomarker for the response of anti-angiogenesis therapy for the epithelial ovarian carcinoma in the future.

Author Contribution Statement

F. Baba: conceptualization, study design, literature search, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing, manuscript review. AI. Yajid: conceptualization, study design, data acquisition, manuscript review. SAA. Hamid: conceptualization, study design, statistical analysis, manuscript preparation, manuscript review. WNAW. Adnan: manuscript preparation, manuscript editing, manuscript review. NAC. Che Jalil NA: conceptualization, study design, data acquisition, data analysis, manuscript review.

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Conflict of interest

The authors declare no conflict of interest.

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