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Research Article

Immunoreactivity of Angiogenesis Markers in Stage IA of Lung Adenocarcinoma

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Abstract

Purpose: This work aimed to determine microvascular density through morphometry in tumor tissue sections with angiogenic markers.

Methods: A clinical, observational and analytical research study was undertaken by collecting the retrospective data patients. This work sought to describe and compare its relation with the clinic-pathological features of 119 resected patients, classified as pathological stage IA adenocarcinoma. The tumor angiogenesis analysis was performed, the average number of microvessels, the accumulated stained area/mm2 and diameter of the vessels were evaluated.

Results: CD34 was positive in 100% of the samples. Lung samples with no tumor pathology, there was even more microvascular density than in tumor samples. Lower CD34 microvascular density was observed in the patients with micropapillary and solid subtypes Adenocarcinoma, and in T1c tumors, tumor differentiation III, nuclear grade 3, tumors with more than six mitoses and tumors with necrosis.

CD31 expressed as 100% of the sample. A statistical association was found between the tumor differentiation and CD31-microvascular density, which was lower in grade III.

CD105 marker, were positive in 87.8% of the cases. Higher CD105-microvascular density was found in the patients who relapsed and was in those who died due to Adenocarcinoma.

Conclusion: CD34 and CD31 expressions were higher in the normal lung than in lung Adenocarcinoma. Lower CD34 expression was associated with pathological features in relation to poor prognosis. Lower CD31 expression was related only to tumor differentiation GIII. Higher CD105 was associated with worse clinical results.

Keywords: *Lung Adenocarcinoma; CD34; CD31; CD105*

Introduction

According to the most recent data, lung cancer (LC) is the most frequently diagnosed neoplasm and one of the leading causes of death from cancer in the world [1].

Adenocarcinoma (ADC) is the most frequent histological type, is also the most variable and heterogeneous form of LC. This appears to be one of the responsible reasons for finding different clinical behaviors among patients in the same tumor stage [2].

New research lines based on the environment surrounding tumor cells have emerged. The study of tumor angiogenesis represents a research field that might be of significant clinical application [3]. Although the literature is limited, some authors have shown that the expression of markers related to angiogenesis differently behaves depending on the ADC histological subtype [4].

CD34 is a highly glycosylated transmembrane protein. It is a panendothelial marker that has been widely studied as a prognostic factor in many tumor types [5,6].

CD31 is a cell surface molecule [7,8]. An international consensus on methodological criteria has been proposed to evaluate microvascular density (MVD) as a standard immunohistochemistry (IHC) marker for angiogenesis assessments [8].

CD105, or endoglin, is a membrane glycoprotein expressed in activated EC that binds to TGFβ (transforming growth factor) 1 and 3 [9]. It has been studied in many tumor types as a angiogenesis marker and a prognostic factor [9-12].

Aim of the Study

Therefore, this work aimed to determine MVD through morphometry in tumor tissue sections with IHC markers, to describe and compare its relation with the clinic-pathological features of resected patients, classified as pathological stage IA.

Methodology

A retrospective clinical, analytical and observational research project was carried out with cases using medical records from two hospitals.

The included patients had been diagnosed with pathologic stage IA lung ADC according to the 8th edition of the TNM classification and had undergone surgical resection in the first hospital between January 1, 1990 and December 31, 2007, and in the second hospital during the period from November 1, 2008 to January 31, 2012.

After revising samples, those patients whose pathological anatomy differed from ADC, the patients who were on some form of neoadjuvant therapy and any patient with previous malignant neoplasia were excluded from the study. The final study cohort included 119 patients.

The follow-up period went from surgery until the patient relapsed or died, or for those who survived, continued until the study ended on January 1, 2018. Follow-up was carried out during the external consultations held in both hospitals by means of anamnesis and an image scan.

The study was conducted in accordance with the principles of the Declaration of Helsinki. This study was approved by the Hospitals' Ethical Committee (EC).

Tumor tissue samples were analyzed by ruling out those areas morphologically altered by atelectasis or lung emphysema. For the morphological study, conventional histological techniques were used as reported in the article published by our group [13].

In order to evaluate angiogenesis, endothelial markers CD31, CD34 and CD105 (monoclonal antibody, DAKO®, Glostrup, Denmark) were used.

All the slides were scanned by the Pannoramic SCAN 150 1.17® processor (3DHISTECH Ltd, Hungary) and photos were taken with the Pannoramic Viewer version 1.15.3® software. In all cases, six photos were taken of each staining with an increase of 20X.

Each field comprised an area of 0.32 mm^2 by obtaining a total of 1.92 mm^2 for the analysis in each case and staining. Samples were analyzed by an experienced pathologist, and the morphological and tumor angiogenesis characteristics discussed below were studied.

The study variables were: age, gender, smoking, surgical excision extension, morphological classification according to WHO 2015 [2]. In this study is not included ADC variants. Tumor of differentiation according to the predominant growth pattern, grade I for ADC type *in situ* (AIS), ADC minimally invasive (MIA) and invasive ADC predominantly lepidic non mucinous, grade II for ADC predominantly papillary or acinar, and grade III for invasive ADC predominantly solid or micropapillary. The presence (from 5%) or absence of each histological (lepidic, acinar, papillary, micropapillary and solid) component was assessed. Tumor size, invasion size and pathologic TNM were also evaluated. Microscopic vascular and lymphatic invasion was defined as absent or present. If vascular invasion (VI) or lymphatic invasion (LI) with H-E was no conclusive. The tumoral necrosis catalogued as absent or present was evaluated. Therefore, it was included as minimal necrosis, until large amount of necrosis. Consequently, the number of mitoses was also evaluated. Finally, the nuclear grade was analyzed according to the criteria of Barletta., *et al.* [14], identified as: G1 for the nuclei with a uniform size and morphology with no evidence for visible nucleolus; G2 for the nuclei of an intermediate size with discrete irregularity of morphology and an evident nucleolus; G3 for the nuclei of an increased size and irregular contours with enlarged nucleoli.

The tumor angiogenesis analysis was performed with an image analysis system (Pro-Plus 6.1 Media-Cybernetics®, US), to microvessels count/mm^2, such as the diameter of the vessels and the accumulated stained area/mm2 were evaluated. With CD34, six images were also taken to assess the MVD in the peritumoral area of each sample to compare it with the tumoral area.

The staining and measurements of CD34, CD31 and CD105 were performed on seven patients with no malignant lung tumor pathology to use them as a healthy control.

With the patients' results, tumor recurrence was assessed and its location was recorded as: local-regional recurrence, understood as the presence of tumoral recurrence in the primary tumor location, even as the presence of mediastinal adenopathies; distant relapse, presence of systemic metastases; a second primary tumor, according to Martini´s criteria [15].

The patient's condition was designated as live, exitus due to ADC and exitus due to another cause other than ADC.

The statistical analysis was performed with the SPSS Windows®, version 22. With the obtained information, a descriptive and analytical statistical analysis was carried out. While analyzing and comparing the means in the continuous quantitative variables, the Mann Whitney U or the Kruskall-Wallis test, was used. When two continuous variables correlated, the Spearman correlation test was applied.

The level of significance was set at $p \leq 0.05$.

Results

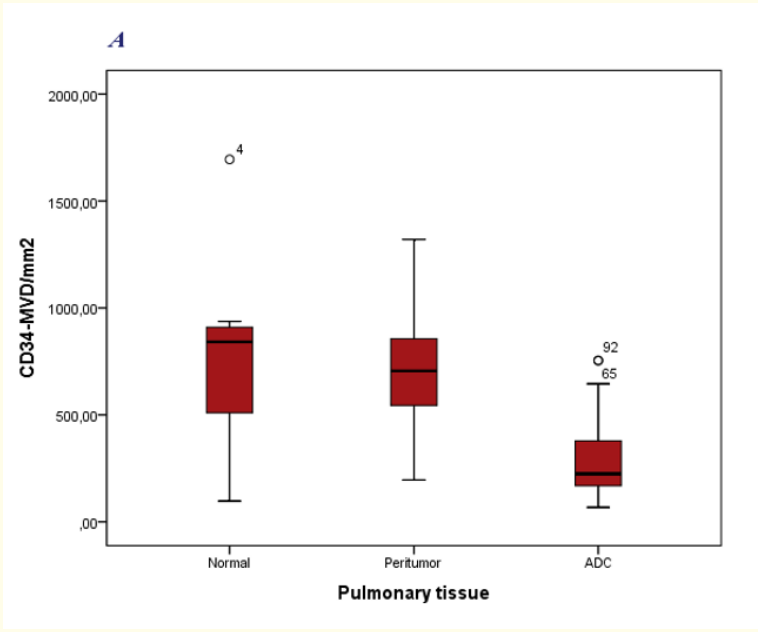
The clinic-pathological characteristics are shown in table 1.

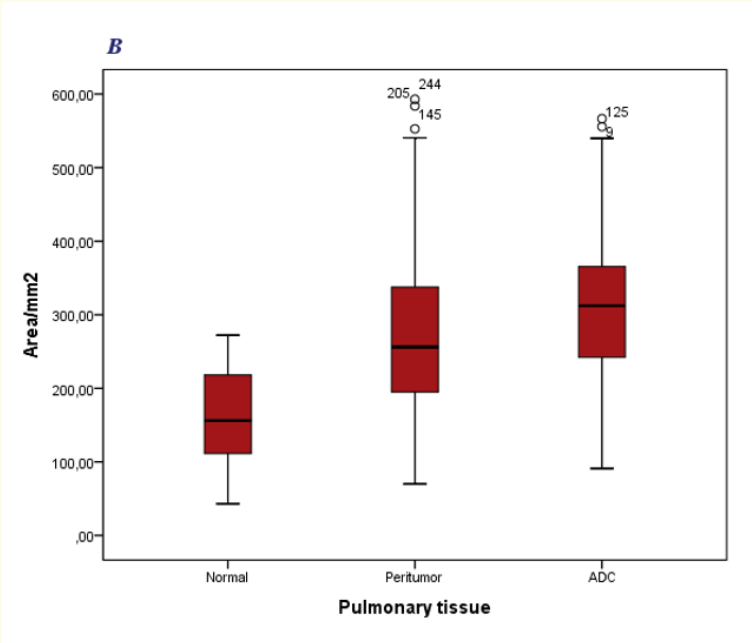
		N (119)	%
Gender	Women	27	22.7
	Men	92	77.3
Age	Mean (SD)	61.6 (8.5)	
Smoking	No Smoker	20	16.8
	Smoker	66	55.5
	Former smoker	33	27.7
Type of surgery	Anatomical segmentectomy	7	5.9
	Lobectomy	111	93.3
	Pneumonectomy	1	0.8
TNM (Stage)	Tis (0)	7	5.9
	T1a (IA1)	23	19.3
	T1b (IA2)	47	39.5
	T1c (IA3)	42	35.3
ADC subtype	AIS	7	5.9
	MIA	8	6.7
	ADC invasive	104	87.4
	Lepidic	18	15.1
	Acinar	49	41.2
	Papillary	5	4.2
	Micropapillary	3	2.5
	Solid	29	24.4
Tumor	GI	28	23.5
differentiation	GII	54	45.4
	GIII	37	31.1
Nuclear grade	1	27	22.7
	2	76	63.9
	3	16	13.4
Number of mitosis	Median (range)	6 (59)	
Lymphatic invasion		29	24.4
Vascular invasion		30	25.2
Tumoral necrosis		62	52.1
Recurrence		34	28.6
	Local regional	7	5.9
	Systemic metastases	27	22.6
Status	Live	43	36.1
	Exitus due ADC	30	25.2
	Exitus others causes	37	31.1
	Second primary tumors	9	7.6

***Table 1**: Clinical y pathological characteristics.*

SD: Standard Deviation; ADC: Adenocarcinoma; AIS: Adenocarcinoma in Situ; MIA: Minimally Invasive Adenocarcinoma.

CD34 was positive in 100% of the samples and was analyzed in the tumoral and peritumoral zones in each case. More vessels/mm^2 were found in the peritumoral area ($p < 0.001$). However, a smaller accumulated stained area/mm^2 ($p = 0.002$) was observed with a smaller diameter of vessels ($p = 0.013$). Likewise, the difference was studied with lung samples with no tumor pathology, in which there was even more MVD, and both the occupied area and the average diameter for vessels were smaller; $p < 0.001$, $p < 0.001$ and $p = 0.002$, respectively (Figure 1).





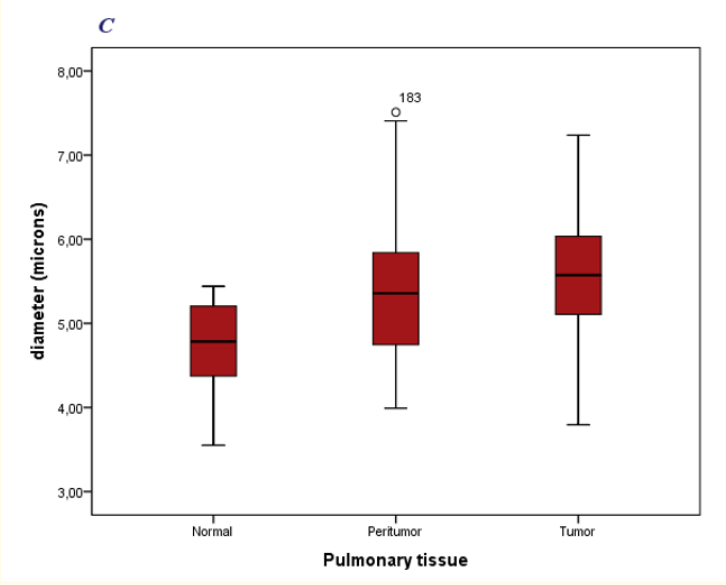


Figure 1: *Box plot shows: A) CD34-MDV in normal lung tissue, peritumor tissue and ADC tissue. B) Accumulated area of vessels with CD34 stained in normal lung tissue, peritumor tissue and ADC tissue. C) Average diameter of the vessels with CD34 stained in normal tissue, peritumor tissue and ADC tissue.*

CD34-MVD was related as a quantitative variable by nonparametric tests with the series' clinic-pathological features. Differences were observed between two groups, one formed by smokers and ex-smokers and second one with non- smokers. Lower MVD was described in the first group of smokers and ex-smokers. Minor MVD was observed in the patients with micropapillary and solid subtypes ADC, and in T1c tumors, tumor differentiation grade III, nuclear G3, tumors with more than six mitoses and tumors with necrosis. MVD was lower in the presence of LI. No significant association was found with recurrence and cancer-specific mortality (Table 2).

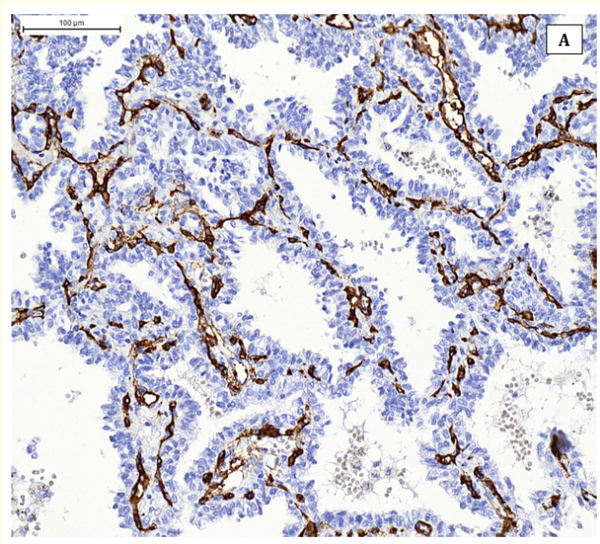
	N = 119	CD34-MVD*	p Value	CD31-MVD*	p Value	CD105-MVD*	p Value
Gender	Men	224,8 (687,7)	0,621	321 (975,8)	0,385	191,6 (1035,9)	0,163
	Women	220,4 (460,8)		260,2 (857,7)		169,3 (955,2)	
Age	≤ 62	209,7 (634,3)	0,856	288,5 (795,1)	0,815	184,5 (955,17)	0,425
	> 62	252,2 (687,7)		310,2 (975,8)		191,2 (1035,9)	
Smoking	No Smoker	284,8 (408,5)	0,053	305,8 (592)	0,782	186,4 (1035,9)	0,745
	Smoker and former	209,7 (687,7)		303,9 (975,8)		187,7 (894,6)	
ADC Subtype	AIS	290,6 (332)	0,005	310,6 (552,5)	0,921	145 (532,5)	0,855
	MIA	287,1 (320,3)		310,2 (875,4)		198,7 (762,8)	
	Lepidic	325 (402,5)		287,1 (875,4)		167,1 (890,7)	
	Acinar	252,2 (645,7)		298,1 (668,1)		188,4 (718,3)	
	Papillary	266,2 (367)		310,6 (552,5)		224,8 (1035,9)	
	Micropapillary	204,4 (189,6)		333,9 (829,2)		181,1 (289,3)	
	Solid	178,5 (347,7)		300,1 (860)		198,4 (955,2)	

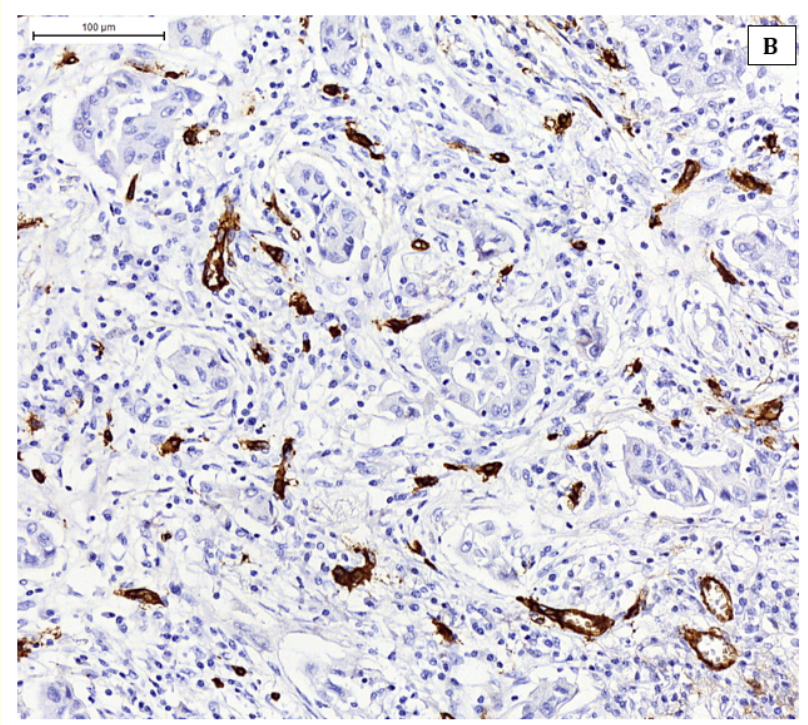
TNM	Tis	290,6 (332)	0,003	374,2 (875,4)	0,423	145 (532,5)	0,611
	T1a	295,2 (412,9)		319,3 (153,4)		175,9 (532,5)	
	T1b	261,8 (686,1)		215,8 (351,7)		184,5 (1035,9)	
	T1c	189,8 (370,6)		252 (538,6)		193,7 (894,6)	
Tumor differentiation	G I	284,8 (334)	0,000	308,3 (911,6)	0,016	145 (762,8)	0,591
	G II	273,4 (645,7)		353 (875,4)		188,1 (1035,9)	
	G III	189,9 (400,9)		250,4 (582,9)		193,9 (955,2)	
Nuclear grade	G 1	338,9 (379,3)	0,001	319,3 (911,6)	0,449	167,9 (1035,9)	0,845
	G 2	205,2 (687,7)		296,9 (921,9)		185,5 (955,2)	
	G 3	194,2 (684,3)		275,5 (526,5)		201,4 (431,8)	
Number of mitosis	≤ 6	282 (686,1)	0,006	310,4 (975,8)	0,325	188,5 (1035,9)	0,394
	> 6	201,8 (686)		273,2 (877,6)		186,6 (642)	
VI	Absent	243,6 (687,7)	0,363	310,4 (975,8)	0,410	186,6 (1035,9)	0,914
	Present	204 (350,3)		277,8 (795,1)		190 (600,5)	
LI	Absent	255,5 (686)	0,055	292,2 (975,8)	0,932	187,7 (1035,9)	0,824
	Present	198,5 (638,8)		314,4 (875,4)		186,4 (955,2)	
Necrosis	Absent	285,1 (684,3)	0,001	319,3 (911,6)	0,173	170,8 (1035,9)	0,361
	Present	197 (687,7)		271,5 (921,9)		198,4 (955,2)	
Recurrence	Absent	243,5 (686)	0,292	287,1 (975,8)	0,155	174,2 (1035,9)	0,036
	Present	207 (686)		388 (809,5)		229,9 (955,2)	
ADC mortality	No	225 (686)	0,433	287,1 (975,8)	0,156	176,6 (1035,9)	0,050
	Si	206 (686,1)		395,4 (809,5)		221,8 (955,2)	

***Table 2**: Shows relation between CD34-MVD/mm², CD31-MVD/mm² and CD105-MVD/mm² with the clinic-pathological characteristics. *In Median and Range; MVD: Microvessel Density; ADC: Adenocarcinoma; VI: Vascular Invasion; LI: Lymphatic Invasion.*

The Spearman test correlated MVD with the cellular components of ADC, and a slight, but significant, correlation was observed between the lepidic component (r = 0.342, p = 0.000) and the solid component (r = - 0.408; p = 0.000). No correlation between total tumor size and MVD was noted, but a negative and moderate correlation appeared between invasion size and MVD (r = -0.334, p = 0.000).

Figure 2 provides examples of the anti-CD34 staining of the different ADC patterns.

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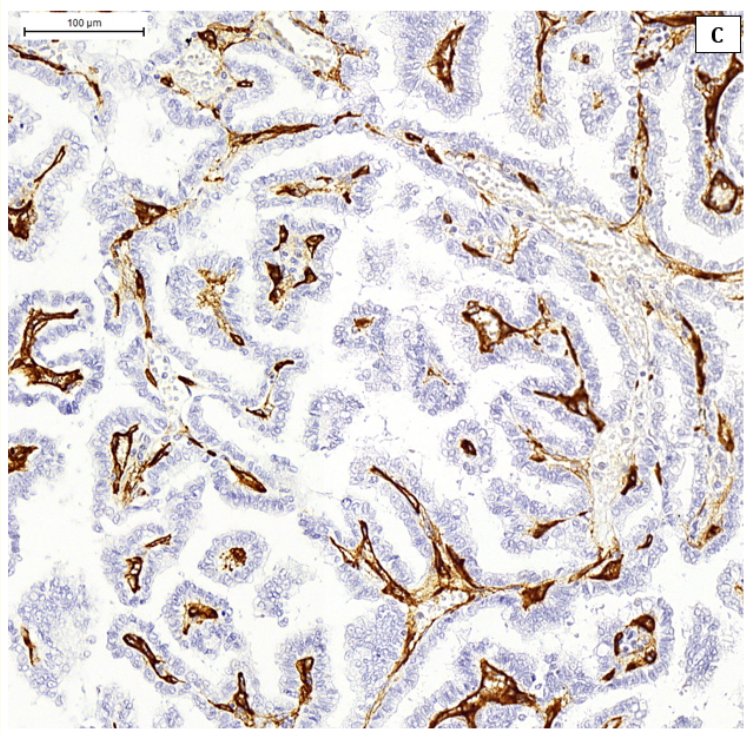
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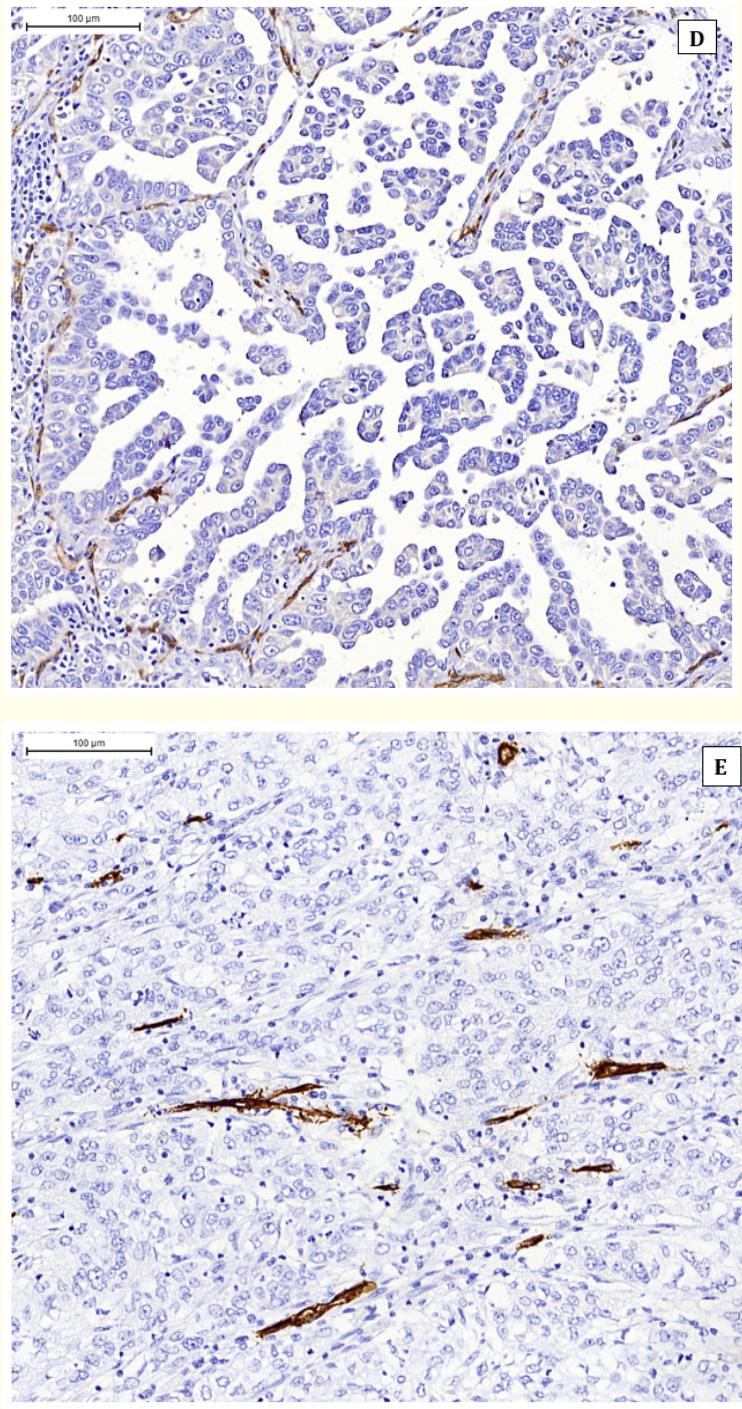

Figure 2: *Immunostaining with anti-CD34 according to ADC subtype (20X): A) lepidic pattern. B) acinar pattern. C) papillary pattern. D) micropapillary pattern. E) solid pattern.*

The CD31 analyses were performed in the tumor areas and lung samples with no tumor pathology. Expressed as 100% of the sample, significant differences were found between the MVD, accumulated stained area and diameter, similarly to CD34-MVD behavior. Higher CD31-MVD was found in the lung samples with no tumoral pathology than in the tumor samples, but the accumulated stained area and diameter were both smaller, p = 0.004, p = 0.011 and p = 0.033, respectively (Table 3).

	MVD (nº/mm²)	Area (µm/mm²)	Diameter (µm)
CD31 tumoral tissue			
Mean (SD)	339,1 (181,3)	236,5 (96,3)	5,1 (0,7)
Median (range)	302,7 (975,8)	215,4 (554,9)	5 (3,9)
CD31 normal tissue			
Mean (SD)	378,3 (333,2)	154,5 (140,9)	4,7 (1)
Median (range)	636,8 (995,4)	104,4 (399,5)	4,3 (3,1)
CD105			
Mean (SD)	243,1 (215,1)	147 (109,3)	4,7 (2)
Median (range)	187,7 (1035,9)	119,7 (463,5)	4,9 (10)

Table 3*: Shows microvessel density, accumulated stained area and diameter of vessels immunostaining with anti-CD31 and anti-CD105. MVD: Microvessel Density; SD: Standard Deviation.*

The CD31-MVD association was pursued with the other clinic-pathological variables. Association was found between the differentiation degree and CD31-MVD, which was lower in grade III. This association was statistically significant (Table 2).

The CD105 marker, or endoglin, was used to analyze 115 lung ADC samples, which were positive in 87.8% (101) of the cases. Table 3 provides the MVD/mm², the accumulated stained area and the average diameter of vessels. Like previous markers, CD105 was analyzed in the lung samples with no tumor pathology and was negative in all cases.

A statistical association of the clinic-pathological features was sought with CD105-MVD. MVD was higher found in the patients who relapsed and those who died due to ADC than for other patients. This relation was statistically significant (Table 3).

Discussion

The main objective of this study is consistent with this tendency and provides valuable information about the expression of angiogenesis markers in the earliest stage of lung ADC by considering its relation with clinic-pathological features.

Tumor angiogenesis in our series was evaluated with the expression and MVD quantification of endothelial markers such as CD34, CD31 and CD105.

In this work an intense expression of both CD34 and CD31 was observed in normal pulmonary capillaries, which agrees with Pusztaszeri., *et al*. [16] and with Muller., *et al* [17]. However, very few studies have compared between these morphological parameters. The MVD for the CD34 and CD31 markers in lung samples with no pathological tumor was higher, and MVD progressively decreased from normal lung toward the peritumoral zone until it reached the lung ADC, which was lowest. However, the accumulated stained area and the average diameter of the capillaries were bigger in ADC and smaller in the peritumoral area, and in the lung samples with no pathological tumor. We propose that these findings correspond to tumoral alterations in vessels, which are dilated and highly permeable [18,19].

Guedj., *et al*. [20], in a study that compared CD34 expression between normal lung and bronchioloalveolar carcinoma, expressed as a percentage of the stained surface, found a similar expression between both sample types, with 8% and 7% respectively. Koukourakis., *et al*. [21] studied the expression of CD31 in NSCLC and found a high MVD with this marker in the normal lung, which was lower in the tumor zone. Even between the central and peripheral areas of a tumor, these authors found that MVD significantly decreased in the central zone compared to the peripheral zone.

Unlike CD34 and CD31 expression, CD105 was null in the normal lung, while pulmonary ADC expression showed 87.8% positivity. These findings are analogous to those reported by Minhajat., *et al* [22].

Total tumor size was not associated with CD34-MVD. However, bigger invasion size showed a lower CD34-MVD expression, but this correlation was weak. Indeed CD34-MVD expression was lower in the tumors classified as T1b and T1c compared to T1a and Tis.

This study also found a relationship between the marker and the subtype of ADC with CD34-MVD, which was higher in lepidic ADC, in MIA and AIS. In the solid and micropapillary subtypes, this expression significantly decreased. When analyzing the quantitative correlation with CD34-MVD expression and the tumor cellular component, we found that the higher CD34-MVD correlated with a bigger lepidic component, while the minor CD34-MVD expression was found in the higher solid component. This difference was confirmed by the tumor differentiation, where grade III includes the solid and micropapillary ADCs subtypes. Thus, CD34-MVD expression was lower in grade III compared to grades I and II. Although the literature is not extensive, these data coincide with Mlika., *et al*. [4], who evaluated CD34 expression in the current stage I and II lung ADC subtypes. These authors found that solid ADC was less vascularized than subtypes papillary and acinar.

Significant differences were observed with nuclear grade; CD34-MVD expression decreased in the patients with nuclear grade 3 compared to nuclear grades 2 and 1. Likewise, an association was found between MVD and the number of mitosis, with a lower CD34-MVD expression in the patients with more than six mitoses. Finally, CD34-MVD expression was lower in the patients with necrosis than in those with no necrosis.

This work found a lower CD34-MVD expression in the patients with tumor recurrence and ADC mortality. Although these differences were relevant, their values were not statistically significant.

The reason that explains the low CD34-MVD expression is related to the clinic-pathological variables with a worse prognosis being unclear. Back in 1955, Thomlinson and Gray [23] described how the pulmonary vascular architecture is unique and demonstrated that bronchial carcinoma uses existing blood vessels [24]. Pezzella., *et al*. [25] suggested in 1997 a "nonangiogenic" growth pattern with no destruction of lung parenchyma to suggest co-option of septal blood vessels in a sample of 500 NSCLC patients. Passalidou., *et al.* [26] confirmed this nonangiogenic pattern in LC, as other authors did in liver [27], lymph nodes [28] and lung metastases [29]. The main prerequisite of this pattern seems to be the tumor's ability to preserve the stroma architecture of tissue and to co-optated the host vessels growing in nests between alveolar spaces [25,30]. As MVD in the lung is normally high, hypoxic tumor regions are rare and, therefore, the oxygen concentration that induces proangiogenic factors does not seem sufficient to stimulate the formation of new vessels, at least not in early disease tumor stages [24]. In their experimental study conducted with several tumor types, Holash., *et al*. [31] reported the tumor's ability to rapidly co-opted host tissue vessels to form an initially well-vascularized tumor. They hypothesized that as part of organism defense, the generalized regression of co-opted vessels might take place that leads to an avascular tumor and possible MVD decrease toward the center of the tumor, with mass loss of tumor cells. However, the remaining tumor that survives after is rescued by the angiogenesis process at the tumor periphery. Donnem., *et al*. [32] confirmed the existence of other vascularization types in LC as the aforementioned co-option of host vessels.

CD31-MVD was significantly related with the tumor differentiation degree, with MVD being lower in grade III ADC cases. No relation was found for other variables, such as TNM, ADC subtype, nuclear grade, mitosis number and tumor necrosis Other studies have found a significant relation between pathological variables and CD31-MVD [33,34].

No significant relation was observed with patients' evolution, although the increase in CD31-MVD expression was evident in the patients with tumor recurrence and those who died from LC.

Similarly to other studies [9,11,35,36], our results indicated no relation between CD105-MVD and the clinic-pathological features. However, a significant relation was found between higher CD105-MVD expression with poorer patient outcomes, and CD105-MVD expression was higher in the patients with disease recurrence and those whose die from ADC. These findings were statistically significant ($p = 0.036$ and $p = 0.050$, respectively). Therefore, CD150-MVD expression act as a potential independent marker because it was not influenced by the patients' clinic-pathological features.

Conclusion

In conclusion, CD34 and CD31 MVD expressions were higher in the normal lung than in lung ADC, unlike CD150-MVD expression. Lower CD34-MVD expression was associated with pathological features in relation to poor prognosis. A lower CD31-MVD expression was related only to tumor differentiation grade III. Finally, higher CD105-MVD was associated with worse clinical results and was significantly higher in the patients with tumor recurrence and in those who died from lung ADC. These findings denote a useful potential outlook for LC disease, since vascular markers studied support a different pathogenic mechanism for various subtypes of adenocarcinoma, showing an alternative manner of tumor progression.

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Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this article.

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