Research Article

Angiogenesis as a prognostic factor of pathological stage IA lung adenocarcinoma: Developing an angiogenic score

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Abstract

Purpose: This work was designed to correlate the expression of markers of tumoral angiogenesis in lung adenocarcinoma as a prognostic factor and create a predictive model with these factors.

Patients and methods: A clinical, observational and analytical research study was undertaken. This work sought to describe and compare prognosis value of angiogenic markers of 119 resected patients, classified as pathological stage IA. The tumour angiogenesis analysis was performed, the survival and predictive value of microvascular density (MVD) expression with tumoral relapse and survival were evaluated. Finally, an accurate score, "Angioscore", was calculated by combining different markers.

Results: Low MVD-CD34 is associated with a worse disease-free survival (DFS) and cancer specific survival (CSS). High MVD-CD31 is significantly related to reduce DFS and CSS. High MVC-CD105 is associated with worse clinical outcomes. The predictive capacity of these angiogenic markers independently it is not exact. The "Angioscore" is able to provide better information about patient prognosis (74.8% and 73.7%). Two groups were obtained with the risk value obtained by "Angioscore". A significant difference in the prognosis of both groups was accomplished (p < 0.001). Being in the multivariate analysis the only independent prognostic factor in patients with lung ADC in stage IA of this series.

Conclusion: Tumour angiogenesis is a prognostic factor in early stage lung adenocarcinoma, the analysis of this factor being more accurate using a score, with great predictive value for the prognosis of patients.

Introduction

Tumours, like normal tissues, need a constant supply of nutrients and O_2, as well as the elimination of metabolic residue and CO_2, which is why they induce angiogenesis. Former research works have demonstrated that the degree of intratumoural vascularisation is a factor with a significant independent prognostic value for the recurrence and survival of some tumour-related diseases, including lung cancer (LC) [1-3].

According to world data that have recently become available, LC is the most frequently diagnosed neoplasia that causes most deaths worldwide. Despite recent advances, this disease still has a bad prognosis with survival below 19% [1,4,5].

Adenocarcinoma (ADC) is the most frequent histological subtype among non-small lung cancers (NSLC) and is also the most variable and heterogeneous form of LC. This seems to be one of the determining reasons why different clinical behaviours appear in patients with the same tumour stage. Thus, it has become a relevant focal point to investigate different prognostic factors to classic TNM in an attempt to improve directed therapies and survival [6].

Prognostic factors aim to predict the probability of an event occurring in cancer, mortality or relapse. However, a single factor is not usually helpful for this purpose. Therefore, it is necessary to create predictive models or risk scores that include a combination of prognostic factors, and to more precisely approach the probability of the event under study.

This work was designed to investigate and correlate the expression of markers of tumoural angiogenesis in lung ADC as a prognostic factor in resected patients classified as pathological stage IA according to the 8th edition of the TNM classification to establish risk groups, and to create a new score that better stratifies patients with stage IA lung ADC.

Materials and method

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. They had undergone surgical resection in the first hospital between 1 January 1990 and 31 December 2007, and during the period from 1 November 2008 to 31 January 2012 in the second hospital.

After revising samples, the patients whose pathological anatomy differed from ADC, those 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 started with surgery and continued until the patient relapsed or died. It continued until the study ended on 1 January 2018 for those who survived. 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 and national legal regulations. The protocol was approved by our Hospital's Ethical Research Committee (EC).

Tumour tissue samples were analysed by ruling out those areas morphologically altered by atelectasis or lung emphysema. Conventional histological techniques, previously published by our group, were used for the morphological study [7].

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

All preparations were scanned and microphotograghs by a Pannoramic SCAN 150 1.17® processor (3DHISTECH Ltd, Hungary) and the Pannoramic Viewer® software 1.15.3, respectively. Six areas from each preparation were randomly selected and microphotographed at the 20X objective. The included area was 0.32 mm² to obtain a total of 1.92 mm². Samples were analysed by an experienced pathologist. The Pro-Plus 6.1 Media- Cybernetics® (USA) imaging system was used to study tumour angiogenesis by counting microvessels per mm² (MVD). The study variables were age, gender, smoking, surgical excision extension. The presence (from 5%) or absence of each histological component was evaluated, as were tumour size, invasion size and pathologic TNM. Vascular (IV) and lymphatic (IL) microscopic invasions were defined as absent or present. Finally, the nuclear grade was analysed according to the criteria of Barletta *et al.* [8].

Tumour recurrence was evaluated according to the patients' outcomes. Its location was recorded as local-regional recurrence, this being the presence of tumoral recurrence in the primary tumour location, and was even taken as mediastinal adenopathies being present, distant relapse, presence of systemic metastases. The patients' condition was designated as living, exitus by LC and exitus due to others cause than LC..

Statistical analysis

The statistical analysis was performed with version 22 of SPSS Windows®. The Kaplan-Meier method was used to calculate disease-free survival (DFS) and cancer-specific survival (CSS). The log-rank test was applied to compare the survival curves according to the different prognostic factors. The continuous variables were previously dichotomised by seeking the optimum cut-off point. Determining the predictive value of microvascular density (MVD) expression when tumoural relapse or death by cancer appeared was done by using the functions curves with receptor operation characteristics (ROC).

A score was calculated by combining different markers to obtain the most significant results. The angiogenesis markers were included in a Cox proportional hazard model for the multivariate analyses, which was utilised to obtain the regression coefficient. It was finally used to create the score, named the *"Angioscore"*. The predictive *"Angioscore"* value was verified with the ROC curve for tumoural relapse and for death by ADC. The ROC curves for each tumoural relapse type were also analysed. An optimum cut-off point was sought, like the other variables, and two groups were created: low *"Angioscore"* and high *"Angioscore"*. The survival curves were analysed by the Kaplan-Meier method and were compared to the log-rank method.

The clinical and pathological variables that were significant in a previous study were analysed in a multivariate forward stepwise Cox proportional hazard model, along with the *"Angioscore"*, to establish the independent prognostic factors. The hazard ratio (HR) was reported with a 95% confidence interval (95%CI). Statistical significance was defined when the p value was ≤ 0.05.

Results

The clinical and pathological characteristics are shown in Table 1. CD34 and CD31 were positive in the whole sample. When 115 lung ADC samples were analysed, CD105 was found to be positive in 87.8% of the cases (101).

MVD was categorised at an optimum cut-off point of three markers, with 192 vessels/mm² for CD34, and 339 vessels/mm² for CD31 which coincided with the mean. CD105 also coincided with the mean of 243 vessels/mm². A survival analysis was done, and a comparison was made of the curves between the "low MVD" and the "high MVD" of each marker, which were compared by the log-rank method (Figure 1).

To evaluate the predictive capacity of each angiogenesis marker for prognosis purposes, the area under the curve (AUC) was analysed by a ROC analysis for tumoural relapse and death by cancer. This predictive capacity was significant only for the MVD with CD105, but it was not very exact (Figure 2).

The "*Angioscore*" was created using the following equation with the regression coefficients shown in Table 2. ANGIOSCORE = (CD34 * −1. 256) + (CD31 * 1. 705) + (CD105 * 1. 300)

When the *"Angioscore"* score for each patient was calculated, the risk median was set at 0.45 with a minimum value of -1.26 and a maximum one of 3.01. Its predictive value was analysed with the ROC curve. The results are presented in Figures 3A and 3B, with a statistically significant predictive value higher than that obtained separately with each marker. Table 3 also shows the ROC curve analysis of the *"Angioscore"* as a predictor of brain- (Figure 3C), lung- (Figure 3D), and loco-regional type relapses, and of other less frequent metastases, which were grouped. Except in these last cases, the results were significant.

Two groups were obtained with the risk value obtained by the *"Angioscore"*: "low *Angioscore*" and "high *Angioscore*". In both these groups, the Kaplan-Meier method was applied to analyse DFS and CSS.
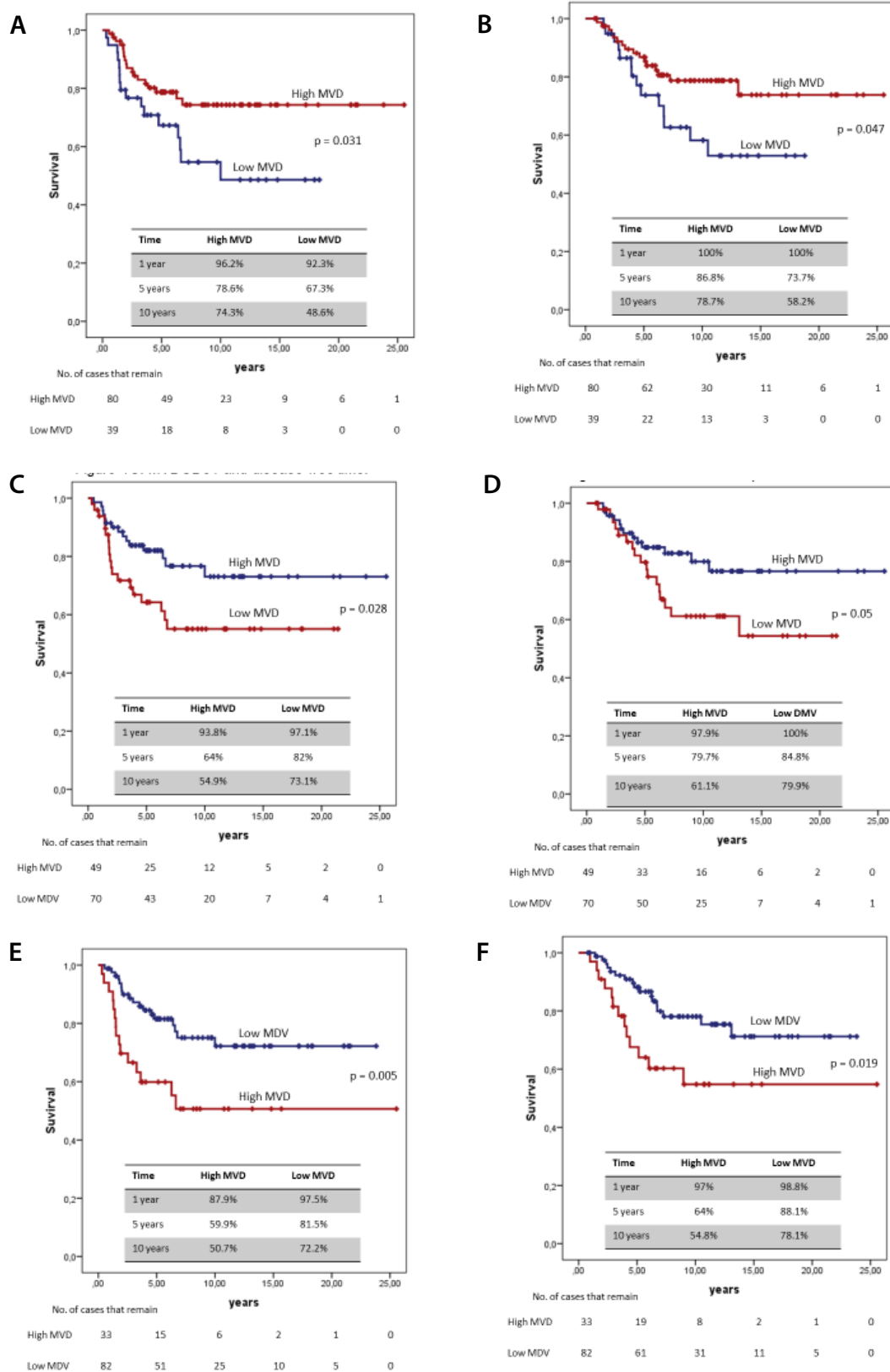
Figure 1. Survival curves. (A) MVDCD34 and disease-free time (B) MDVCD34 and survival specific cancer (C) MVDCD31 and disease-free time (D) MVDCD31 and cancer specific survival (E) MDVCD105 and disease-free time (F) MDVCD105 and specific cancer survival
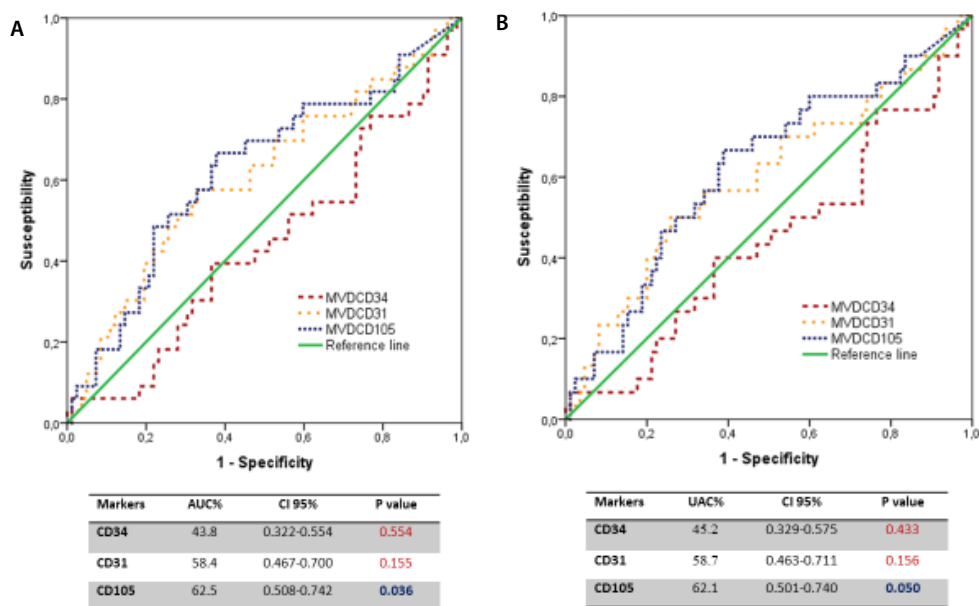
Markers	AUC%	CI 95%	P value
CD34	43.8	0.322-0.554	0.554
CD31	58.4	0.467-0.700	0.155
CD105	62.5	0.508-0.742	0.036

Markers	UAC%	CI 95%	P value
CD34	45.2	0.329-0.575	0.433
CD31	58.7	0.463-0.711	0.156
CD105	62.1	0.501-0.740	0.050

Figure 2. (A) ROC curves. Angiogenic markers and tumour recurrence (B) Angiogenic markers and cancer mortality

Markers	AUC%	CI 95%	P value
Angioscore	**74.8%**	0.638 – 0.857	0.000

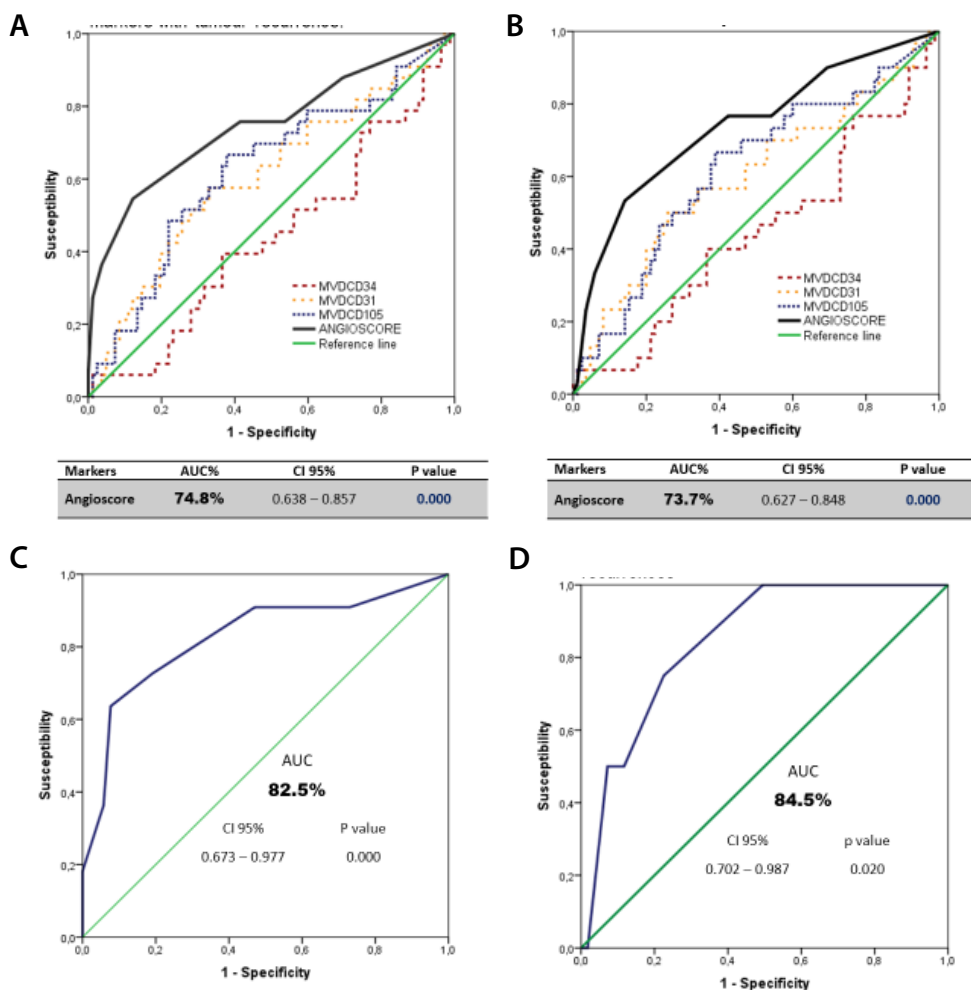| Markers	AUC%	CI 95%	P value
Angioscore	**73.7%**	0.627 – 0.848	0.000

Figure 3. ROC curve. (A) "Angioscore" and endothelial markers with tumour recurrence (B) "Angioscore" and endothelial markers with cancer mortality (C) "Angioscore" with brain metastases (D) "Angioscore" with pulmonary recurrences

Table 1. Clinical and pathological characteristics of the series

		N (119)	%
Gender	Women	27	22.7
	Men	92	77.3
Years	Mean (SD)	61.6 (8.5)	
Smoking habit	No smoker	20	16.8
	Smoker	66	55.5
	Former smoker	33	27.7
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
Histological pattern	Lepidic	53	44.5
	Acinar	101	84.9
	Papillary	18	15.1
	Micropapillary	12	10.1
	Solid	69	58
Nuclear degree	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
Tumoural necrosis		62	52.1
Recurrence		34	28.6
	Local -regional	*7*	*5.9*
	Metastasis	*27*	*22.6*
Status	Alive	43	36.1
	Cancer mortality	30	25.2
	Mortality by other causes	37	31.1
	Second primary tumour	9	7.6

SD: Standard deviation, ADC: Adenocarcinoma, AIS: Adenocarcinoma *In Situ,* MIA: Minimally invasive ADC

Table 2. Multivariate analysis of CD34, CD31 and CD105 angiogenic markers

	β	HR	CI 95%	p value
CD34	-1.256	0.285	0.131-0.620	0.002
CD31	1.705	5.502	2.392-12.657	0.000
CD105	1.300	3.671	1.753-7.687	0.001

Table 3. ROC analysis of the "Angioscore" and type of tumour recurrence (AUC: Area under the curve)

Recurrence	AUC%	95%CI	p value
Pulmonary	84.5	0.702-0.987	0.020
Brain Metastasis	82.5	0.673-0.977	0.000
Local- Regional	78.2	0.591-0.973	0.020
Others	46.8	0.309-0.633	0.721

Curves were compared with the log-rank test. A significant difference in the prognosis of both groups was found (Figure 4).

The clinical and pathological variables and *"Angioscore"* were analysed as prognosis factors in a forward stepwise Cox multivariate regression model for DFS and CSS. The "*Angioscore*" was the only factor to enter the regression as an independent prognosis factor for DFS and SCS, with HR 5.748 (95%CI: 5.878-11.481, p<0.001) and HR 5.169 (95%CI: 2.507-10.657, p<0.001), respectively (Table 4).

Discussion

Lung cancer is a serious health problem with a very strong impact from both the healthcare and socio-economic points of view. The relapse rate in stage I NSCLC lies between 10% and 30% according to consulted authors [9-11]. In our series, 28.6% of the patients suffered relapse, of whom 70.6% had distance relapse, mainly in the brain, and 25.2% of them died from this disease.

In the present-day, a huge challenge lies in managing patients with pulmonary resection by NSCLC, that of developing new markers that can help to identify subjects at higher risk of recurrence and mortality.

This last point was the reason for this research, which included a series of 119 patients diagnosed with lung ADC classified in stage IA. The impact of tumour angiogenesis on DFS and CSS was analysed, along with other morphological factors, and a score was created. CD34 is a highly glycosylated transmembrane glycoprotein, [12] and the role of this marker as a prognosis factor in LC is controversial [1,3,12-14]. This work found an association between a low MVD-CD34 expression and worse results in these patients. Despite very few studies agreeing with these data, Pomme *et al.* [15] evaluated the expression of several
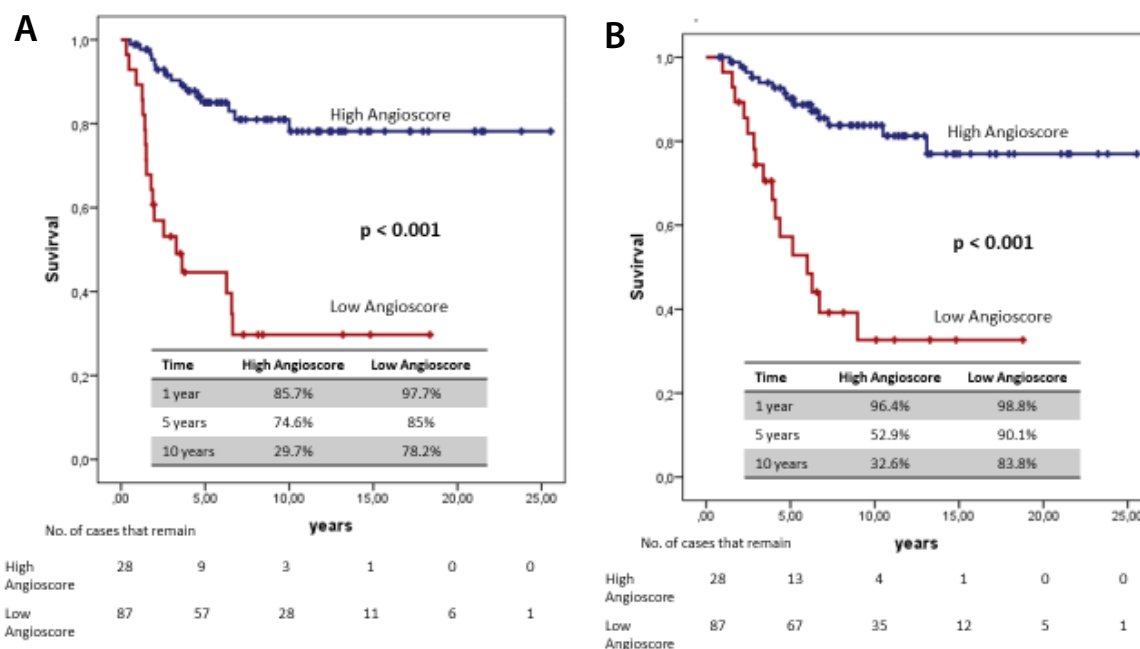
Figure 4. Survival curves (A) "Angioscore" and disease-free time (B) "Angioscore" and survival specific cancer

Table 4. Multivariate analysis between clinicopathological characteristics and angio score with DFS and CSS of the series

Variable	DFS			CSS		
	HR	CI 95%	p value	HR	CI 95%	p value
"Angioscore"	5.748	2.878-11.481	0.000	5.169	2.507-10.657	0.000
Variables that are not in the equation						
	score		p value	score		p value
Micropapillary patterns	2.737		**0.098**	2.701		0.100
Necrosis	2.131		0.144	2-014		0.156

markers related to tumoural angiogenesis in NSCLC. These authors found that MVD-CD34 < at 65/mm2 in the centre of the tumour was related to the worst prognosis. In a recent study, Xing *et al.* [16] related MVD-CD34 and blood flow with ultrasound (US) in lung tumour grafts in rabbits, and found a relation between higher MVD-CD34 and better survival. These contradictions have also been observed in clear cell renal carcinoma by Yildiz *et al.* [17] who found that high MVD-CD34 was related to "non-metastatic status, lack of microscopic vascular invasion, small tumour size and early tumour stage".

The reason why low MVD-CD34 expression is related to worse prognosis is not clear. Some authors have previously described how the lung vascular architecture is unique by demonstrating that bronchial carcinoma employs existing blood vessels [1]. Others have suggested a "non-angiogenic" growth pattern in LC [18], as in the liver, lymph glands [19] and lung metastases [20]. Moreover, as MVD in the lung is normally high, hypoxic tumour regions are rare and, therefore, the oxygen concentration needed to induce proangiogenic factors does not seem enough to stimulate the formation of new vessels, at least not in initial tumour stages [1].

CD31 is a cell surface molecule that has been proposed as a standard Immunohistochemistry marker to evaluate angiogenesis according to an international consensus about methodological criteria to assess MVD [2]. Contradictory results have been found in the literature, which are mentioned later [12,21-23].

In their large study conducted with 515 NSCLC patients, Pastorino *et al.* [21] found no relation to MVD-CD31 and these patients' prognosis. These authors pointed out that the angiogenic or "non-angiogenic" pattern was more significant than studying MVD. Nor did Duarte *et al.* [22] find an increase in MVD, but CD31 was associated with a worse prognosis. However, Koukourakis *et al.* [24] evaluated MVD-CD31 in tumour tissue and evidenced that high MVD was related to survival. Giatromanolaki *et al.* [25] conducted a study with 107 patients classified as T1-2N0-1M0, and related high MVD to a worse prognosis. This finding coincided with *Kuang et al.* [23] and Bacic *et al.* [4], who found that prognosis significance was more evident in those patients in advanced ADC stages.

The present study evidenced a marked relation between a worse prognosis of this disease and increased MVD-CD31. Nevertheless, an increase in MVD in lungs does not indicate greater angiogenic activity [24]. Hence the present study evaluated the expression of CD105, which is a marker of activated endothelial cells, a membrane glycoprotein-type that binds to TGFβ 1 and 3 [3]. It has also been studied as a marker of angiogenesis and a prognostic factor in many tumour types [3,26,27].

A significant relation was found between high MVD-CD105 expression and a worse prognosis, with a higher relapse rate for this group of patients.

Mineo *et al.* [14] and Pomme *et al.* [15] also reported worse survival for patients with high MVDCD105. However, the multivariate analysis

did not enter regression as an independent variable. MVDCD105 has been related to a bad prognosis factor in breast cancer [26,28], cancers of the digestion system, head/neck neoplasias [28] and LC [3,27,28].

In general terms, several research works have attempted to evaluate which markers better express a tumour's vascular status independently and its relation to patient outcomes. By means of a ROC curve, the present study evaluated in each angiogenesis marker the capacity to discriminate patients according to their outcomes. We found that these markers were not very exact at discerning the prognosis of patients with stage IA lung ADC for both relapse and specific cancer mortality. The AUCs for MVD-CD34 and MVD-CD31 were not statistically significant. However, AUC showed a significant level for MVD-CD105, but it was not very exact.

The weakness of these markers is basically due to tumoural vascularisation being much more complex and dynamic, where several proangiogenic factors participate, it is essentially where neoangiogenesis outbreaks appear, and it is not the only type of vascularisation used by tumours. Other widely studied vascularisation types in LC are the cooptation of host vessels accompanied by their vascular remodelling by apoptosis [18]. Thus, tumoural vasculogenesis has been demonstrated in the studies by Maeda et al. [13]. Finally, there is the vasculogenic mimicry observed by researchers like Kaessmeyer *et al.* [29]. Based on not only the poor exactness of the separately analysed endothelial markers to discriminate patients' prognoses, but also on the search for a better evaluation of a tumour's vascular status and its relation to patient evolution, a decision was made to create a scoring system called *"Angioscore"*.

Former studies have used the combination of certain proangiogenic markers as a prognosis factor. Sanmartin *et al.* [30] found an association between a high VEGFA mRNA expression, a low VEGFB expression and D mRNA with worse patient evolution. Zhang *et al.* [31] identified that combining the mRNA VEGFA/FLT1/KDR expression was highly consistent as a prognosis factor in NSCLC, colon and brain cancer. However, the study populations in these works were highly heterogeneous in terms of clinical, pathological characteristics, treatment, and results.

The present work found that the ability of the "*Angioscore*" to discriminate between a patient with or without tumour relapse, and between those who had died or not by ADC, was 74.8% and 73.7%, respectively. These values are much more accurate than when the analysis was done with each marker separately. Likewise, its discriminating capacity was estimated according to relapse type, with 84.5% for lung-related relapses and 82.5% for brain-related relapses.

The *"Angioscore"* allowed two patient groups to be obtained according to risk, with a statistically difference between them in DFS and CSS.

A Cox multivariate analysis was run to evaluate the *"Angioscore"* jointly with the morphological variables of ADC of our series. In this new analysis, the *"Angioscore"* was the only independent variable in patient evolution; that is, those patients with a high *"Angioscore"* were at an almost 5.75-fold higher risk of tumoural relapse and one of 5.17- fold of dying from this tumour type than those with a low *"Angioscore"*.

Therefore, the data obtained with this study suggest that the *"Angioscore"* could be a useful tool because it acts as a prognosis factor in stage IA lung ADC at the expense of its clinical evaluation.

Conclusion

By way of conclusion, low MVD-CD34 is associated with a worse DFS and CSS but offers no predictive value for relapse and mortality. High MVD-CD31 is significantly related to low DFS and CSS, but the marker was not predictive of tumoural progression. High MVD-CD105 is associated with worse clinical outcomes. Its predictive value is significant, but it acts as an inaccurate marker. Grouping endothelial cells surface markers by the *"Angioscore"* is a useful tool for evaluating the vascular status of patients with stage IA lung ADC. The *"Angioscore"* represents a more exact predictive value for relapses and death because it allows high- and low-risk groups to be classified. Finally, the *"Angioscore"* was the only independent prognosis factor of patients with stage IA lung ADC in our series.

Acknowledgment

Conflict of interests

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

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