Expression Profiles of VEGF-A, CD31 and GRP78 in Non-Small Cell Lung Cancer.

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Abstract

Background

Angiogenesis is mandatory for tumor growth and progression. The modest response to the anti-angiogenic therapies in non-small cell lung cancer reflects the presence of confounding molecular factors. The aims of this study were to investigate the expression levels of VEGF-A, CD31 and GRP78 and test for significant correlations between them.

Methods

Paraffin-embedded NSCLC tissue samples (71 adenocarcinoma and 23 squamous cell carcinoma) were retrospectively collected from 94 patients who underwent surgical resection between 2008 and 2015; and did not receive chemotherapy or radiotherapy prior to surgery. The expressions of VEGF-A, CD31 and GRP78 were determined by immunohistochemistry.

Results

High expression levels of VEGF-A, CD31 and GRP78 were observed in 15, 36 and 74 cases, respectively. Adenocarcinomas expressed higher levels of the aforementioned proteins as compared with squamous cell carcinomas (p-value < 0.05). Moreover, a statistically significant association was found between VEGF-A and CD31 expression levels (p-value = 0.006).

Conclusions

Our study was the first to investigate the associations between GRP78 and the angiogenesis markers CD31 and VEGF-A in NSCLC patients. High GRP78 expression was revealed in the majority of the investigated samples. Nevertheless, no relationship was found between GRP78 and VEGF-A or CD31 which could be attributed to small sample size. On the other hand, the positive association between VEGF-A and CD31 expression levels suggests that VEGF-A may cooperate with CD31 to promote angiogenesis in NSCLC.

Introduction

Despite the critical progress in the diagnosis and treatment, lung cancer is still the second most common cancer in both sexes accounted for about 25% of all cancer cases in 2019 [1]. Moreover, it is the most common cause of cancer-related mortality responsible for about one fourth of cancer deaths [1]. NSCLC a devastating subtype of lung cancer that represents the majority of the cases [2], is a heterogeneous disease in terms of histology and is classified into three major groups; squamous cell carcinoma, adenocarcinoma and large-cell carcinoma [3, 4].

Angiogenesis, or neovascularization, is the process of proliferation, migration, and sprouting of endothelial cells resulting in remodeling of blood vessels. This is considered a viable step in the process

of carcinogenesis to provide the blood supply necessary for the growth and metastasis of solid tumors. Vascular endothelial growth factor A (VEGF-A) is one of the most important proangiogenic factors and is an angiogenesis marker that was shown to be overexpressed in NSCLC [5, 6]. It has driven the advent of bevacizumab, a humanized recombinant monoclonal antibody to VEGF-A which is approved for treatment of non-squamous subtypes of lung cancer [7].

CD31, also called platelet endothelial cell adhesion molecule-1 (PECAM-1), is another angiogenesis marker and is used as a direct measure of the intratumoral microvessel density (MVD) [8]. It is a 130-kd cell membrane protein that belongs to the immunoglobulin superfamily and is expressed on endothelial cells and stimulates vascular development and migration of tumor and endothelial cells [9].

Due to the modest response to standard chemotherapy, the treatment of NSCLC has moved from only recruiting traditional anticancer agents to the use of targeted biological drugs such as antiangiogenic agents. Despite the many trials that extensively evaluated the efficacy of antiangiogenic agents, only the addition of bevacizumab to the platinum-based chemotherapy in treatment of advanced NSCLC has resulted in a moderate increase in patients' overall survival [10, 11]. This was mainly attributed to the development of resistance and the unavailability of predictive biomarkers for antiangiogenic therapy response. Thus, more focus should be directed toward better understanding of the angiogenesis pathways and their biological components.

GRP78, also referred to as binding immunoglobulin protein (BiP), is an endoplasmic reticulum stress marker. The correlation between GRP78 and angiogenesis was extensively studied *in vitro* and *in vivo*, and GRP78 was found to be a key regulator of tumor angiogenesis [12-14]. Knockdown of GRP78 in endothelial cells of mammary tumors in mice was associated with reduced angiogenesis in the tumor tissue and not in the normal tissues, and this was attributed to inhibiting VEGF receptor 2 and VEGF-induced angiogenesis [12-14]. However, up to our knowledge, there are no studies reporting the relationship between GRP78 and neovascularization in NSCLC. The aims of this study were to investigate the expression levels of VEGF-A, CD31 and GRP78 and test for significant correlations between them.

Methodology

Patients and tissue samples

This is a retrospective analysis of a total of 94 paraffin-embedded NSCLC tissue samples (71 adenocarcinoma and 23 squamous cell carcinoma) provided by the department of pathology of King Abdulla University Hospital (KAUH) in Irbid, Jordan. The samples represented patients who underwent surgical resection between 2008 and 2015; and did not receive chemotherapy or radiotherapy prior to surgery.

Immunohistochemical (IHC) staining

IHC staining was performed using the Dual Link System HRP Kit, K8000 (Dako, Glostrub, Denmark) according to the manufacturer's instructions. Briefly 4 μm sections of paraffin-embedded NSCLC tissues were performed to analyze the expression of GRP78, VEGF-A, and CD31 proteins. The sections were deparaffinized and dehydrated with xylene and graded ethanol (100%, 95%, 70%, 50%, and 30%) then rehydrated with distilled water. Antigen retrieval was performed in a PT-link Instrument (Dako Glostrup, Denmark) using high pH citrate buffer for 20 minutes. Endogenous peroxidases activity were blocked by incubation with 2.5% hydrogen peroxide for 10 minutes. Slides were then washed with phosphate buffer saline twice. Next, the slides were incubated for 45 minutes at room temperature with the primary antibodies against GRP78, 1:200; VEGF-A, 1:200; and CD31, 1:100 (ab21685, ab46154, ab28364 respectively; rabbit anti-human polyclonal immunoglobulins; Abcam Biotech Company, Cambridge, UK).

Slides were then washed twice with PBS and then signal visualization was carried out using the Envision Dual Link System HRP Kit , K8000 (Dako, Glostrup, Denmark). Slides were then washed with distilled water and finally counterstained with Mayer's hematoxylin.

Staining evaluation

The stained sections were reviewed by two experienced pathologists who did not have access to the clinical data of patient cohorts, and then reevaluated by another independent pathologist.

GRP78 and VEGF expression score

Immunoreactivity of GRP78 and VEGF-A was evaluated in a semiquantitative fashion where we considered both the intensity of staining and the proportion of cells stained. The intensity scores (IS) were; 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The proportion of cells stained ranged from 1%-100%, then proportion scores (PS) were given as follows: 0 for staining <5%; 1 for staining 5%-25%; 2for staining 26%-50%; 3for staining 51%-80%, and 4 for staining >80%. Then a total score (TS) was calculated by multiplying the IS and the PS with a range from 0-12. For statistical analysis, we divided the patients into three groups, samples with a TS of 0 were considered negative, a TS of $\geq$7 were considered high, while those with a TS of $\leq$6 were considered low.

CD31 expression score

CD31 was scored as a percentage of CD31 positive vessels in the tumor field with the highest vessel density as; high (high vessel tumors), vessels are 50% or more of tumor surface area; low (low vessel tumors), vessels are less than 50% of tumor surface area

Statistical analysis

Patients' data were presented as mean ± standard deviation (continuous variables) or as numbers and percentages (categorical variables). The predictors for GRP78 expression level (CD31, VEGF-A expression, age, gender and histological type) were examined using Student t-test, Chi-square (χ^2) test or Fisher's exact test as appropriate. All tests were two-sided and statistical significance was considered as $p<0.05$. All analyses was carried out using the Statistical Package for Social Sciences (SPSS) version 19.

Results

This study was performed on 94 retrospective cases of NSCLC received at the pathology department of King Abdulla University Hospital from 2008 to 2015.

Clinical characteristics

The mean age of patients was 63.15 years old (SD ± 11.88) and 73.4% of them were males (n=69). Approximately, three quarters of the patients had adenocarcinoma (n=71, 75.5%) compared to about a quarter of them who had squamous cell carcinoma (n=23, 24.5%).

Pathology results

VEGF-A was expressed in the cytoplasm of the cancer cells as shown in (**Figure 1**). 80 cases (85.1%) demonstrated positively stained tumor cells, of which only 15 cases (18.8%) had high VEGF-A level (**Table 1**).

Table 1. VEGF immunoreactivity in human NSCLC.

	Immunonegative, n (%)	Immunopositive cases, n (%)	
Histological subtype		Low	High
Adenocarcinoma	5 (7)	51 (71.8)	15 (21.2)
Squamous cell carcinoma	9 (39.1)	14 (60.9)	0
p-value <0.001, adenocarcinoma versus squamous cell carcinoma (χ^2-test)			

Regarding CD31 expression, the protein was expressed on the membrane of the endothelial cells (**Figure 2**), and 36 specimens expressed high levels of the protein (**Table 2**).

Table 2. CD31 immunoreactivity in human NSCLC

	Immunopositive cases, n (%)	
Histological subtype	Low	High
Adenocarcinoma	37 (52.1)	34 (47.9)
Squamous cell carcinoma	21 (91.3)	2 (8.7)
p-value = 0.001, adenocarcinoma versus squamous cell carcinoma (χ^2-test)		

82 specimens (87.2%) demonstrated GRP78-positively stained tumor cells, the remaining 12 samples revealed negative staining. Staining was cytoplasmic (**Figure 3**) and of the positively stained samples, 74 cases exhibited high GRP78 expression and 20 cases exhibited weak expression (**Table 3**).

Table 3. GRP78 immunoreactivity in human NSCLC

	Immunonegative, n (%)	Immunopositive cases, n (%)	
Histological subtype		Low	High
Adenocarcinoma	6 (8.5)	5 (7)	60 (84.5)
Squamous cell carcinoma	6 (26.1)	3 (13.1)	14 (60.8)
p-value = 0.045, adenocarcinoma versus squamous cell carcinoma (χ^2-test)			

Expression of VEGF-A, CD31 and GRP78 is associated with tumor histological subtype

Statistically significant associations were found between histological subtype and the expression levels of VEGF-A, CD31 and GRP78.

VEGF-A was positively expressed in 66 cases (93%) of the adenocarcinoma and 14 cases (60.9%) of the squamous cell carcinoma. The frequency of VEGF-A immunopositivity with high expression was significantly greater in the adenocarcinoma subtype than the squamous cell carcinoma (p-value < 0.001, **Table 1**).

CD31 high expression was observed in 34 cases (47.9%) of the adenocarcinoma and only 2 cases (8.7%) of the squamous cell carcinoma. The frequency of CD31 high expression was significantly higher in the adenocarcinoma compared to squamous cell carcinoma (p-value = 0.001, **Table 2**). GRP78 immunopositive expression was observed in 65 cases (91.5%) of adenocarcinoma and 17 cases (73.9%) of squamous cell carcinoma. The frequency of highly positive GRP78 expression was significantly greater in the adenocarcinoma compared to the squamous cell carcinoma tissues (p-value = 0.045, **Table 3**).

VEGF expression is associated with CD31 expression

A statistically significant association between VEGF-A and CD31 was observed. 60.4% of low-CD31 expressions show low VEGF-A expressions and 16.7% of the high-CD31 expressions exhibited high VEGF-A expressions (p-value = 0.006, **Table 4**).

Table 4. Association between VEGF and CD31

	CD31, n (%)		p-value
VEGF	Low	High	
Negative	14 (24.1)	0	**0.006**
Low	35 (60.4)	30 (83.3)	
High	9 (15.5)	6 (16.7)	

GRP78 expression is not associated with VEGF-A or CD31 expression

As shown in **Table 5**, there were no significant associations between GRP78 expression and VEGF-A or CD31 expressions.

Table 5. Association between GRP78 and VEGF

	GRP78, n (%)			p-value
VEGF	Negative	Low	High	
Negative	2 (16.7)	0	12 (16.2)	0.419
Low	8 (66.7)	8 (100)	49 (66.2)	
High	2 (16.6)	0	13 (17.6)	
CD31				
Low	8 (66.7)	4 (50)	46 (62.2)	0.743
High	4 (33.3)	4 (50)	28 (37.8)	

Discussion

It is well known that angiogenesis is mandatory for tumor growth and progression which prompted the innovation of antiangiogenic therapies. Nevertheless, their usefulness in the treatment of NSCLC is limited by the occurrence of resistance and the unavailability of reliable predictive biomarkers. This

highlights the urgent need for the identification of novel biomarkers that aid in the selection of the appropriate antiangiogenic agent to achieve the best therapeutic outcomes in patients with NSCLC. Furthermore, these biomarkers may represent new therapeutic target for new improved antiangiogenic agents.

VEGF-A, a proangiogenic growth factor, was first reported as a vascular permeability factor then as an endothelial-specific mitogen responsible for the induction of angiogenesis via promoting endothelial cell migration, proliferation, differentiation and survival [15, 16]. It was overexpressed in several types of cancers such as breast cancer [17], pancreatic cancer [18], and NSCLC [5, 6], among others [19, 20]. However, a positive correlation between VEGF-A and MVD could not be proved in many studies [21-23]. Thus indicating that other molecular factors play a role in the angiogenesis process.

GRP78, a key regulator of the endoplasmic reticulum, promotes cancer cell proliferation, invasion and migration and different studies revealed its proangiogenic role in cancer [24-26]. One study revealed that the expression of GRP78 is necessary for the angiogenesis required for the progression of the tumor xenograft [27]. Moreover, GRP78 was overexpressed in many types of tumors and it was related to a more aggressive behavior and poor prognosis [28-31]. Our data demonstrate that GRP78 was highly expressed in about 79% of the NSCLC cases.

Our statistical analysis showed higher staining scores of VEGF-A, CD31 and GRP78 in adenocarcinoma compared with squamous cell carcinoma (p-value < 0.001, p-value = 0.001, and p-value = 0.045, respectively). The positive correlation between VEGF-A expression and adenocarcinoma was previously revealed in many studies [32-35]. On the other hand, other researchers could not come out with the same conclusion neither for CD31 [8, 35-38].

Controversy exists in literature regarding the association between GRP78 expression and histological type of NSCLC. In parallel with our result, a study by Kwon, et al also reported higher expression of GRP78 in adenocarcinoma than squamous cell carcinoma [39]. However, an opposite finding was shown by Imai et al [40]. Nevertheless, no association was revealed by other studies [41-44].

Interestingly, we found a significant association between VEGF-A and CD31 (p = 0.006). This result is in keeping with that reported by El-Gohary et al. in prostatic adenocarcinoma tissues [45]. However, several other studies on different types of cancer showed no association between the two proteins [8, 35, 46, 47]. Our result indicates a possible contribution of VEGF-A in microvessel formation, but further studies with larger sample size are required to determine the type of the relationship between VEGF-A and CD31 in NSCLC in order to have a clearer insight into the related angiogenesis pathway.

In the current study, there were few limitations that may have affected the results. First, this was a retrospective study which may have introduced bias into our results. Second, the sample size was small and the number of adenocarcinoma versus squamous cell carcinoma cases was disproportionate. Third, we focused on adenocarcinoma and squamous cell carcinoma and did not investigate other histological

subtypes. Fourth, other clinicopathological information such as TNM staging were missing so we were not able to identify the prognostic value of VEGF-A, CD31 or GRP78.

Conclusions

In conclusion, we found that adenocarcinomas have higher expression of VEGF-A, CD31 and GRP78 as compared with squamous cell carcinomas. Up to our knowledge, our findings are the first to identify a positive correlation between VEGF-A and CD31 in lung cancer suggesting that VEGF-A may cooperate with CD31 to promote angiogenesis in NSCLC.

Our study was the first to investigate the associations between GRP78 and the angiogenesis markers CD31 and VEGF-A in NSCLC patients. High GRP78 expression was revealed in the majority of the investigated samples. Nevertheless, no relationship was found between GRP78 and VEGF-A or CD31 which could be attributed to small sample size.

Abbreviations

Non-small cell lung cancer (NSCLC); 78-kDa glucose regulated protein (GRP78); Vascular endothelial growth factor type A (VEGF-A); platelet endothelial cell adhesion molecule-1 (PECAM-1); microvessel density (MVD); immunohistochemistry (IHC).

Declarations

Ethics approval and informed consent

This study was approved by the Institutional Review Board at Jordan University of Science and Technology. Informed consent was not required for the study due to the anonymity of the data used.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

There are no conflicts of interest to declare.

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Authors' contributions

M.S. A conceived of the presented idea and developed the theory, M. A. A., M. M. A. and H. W. A. performed the pathological analysis of the tumor tissues. B. A. A. collected the patients' information. M. S. A. and B. A. A. performed the statistical analysis of the data and wrote the manuscript. All authors contributed in manuscript revision.

Disclosure

The abstract of this paper was only published online in an American Society of Clinical Oncology (ASCO) journal. The abstract was published in the Journal of Clinical Oncology with DOI: 10.1200/JCO.2019.37.15_suppl.e20500

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Figures

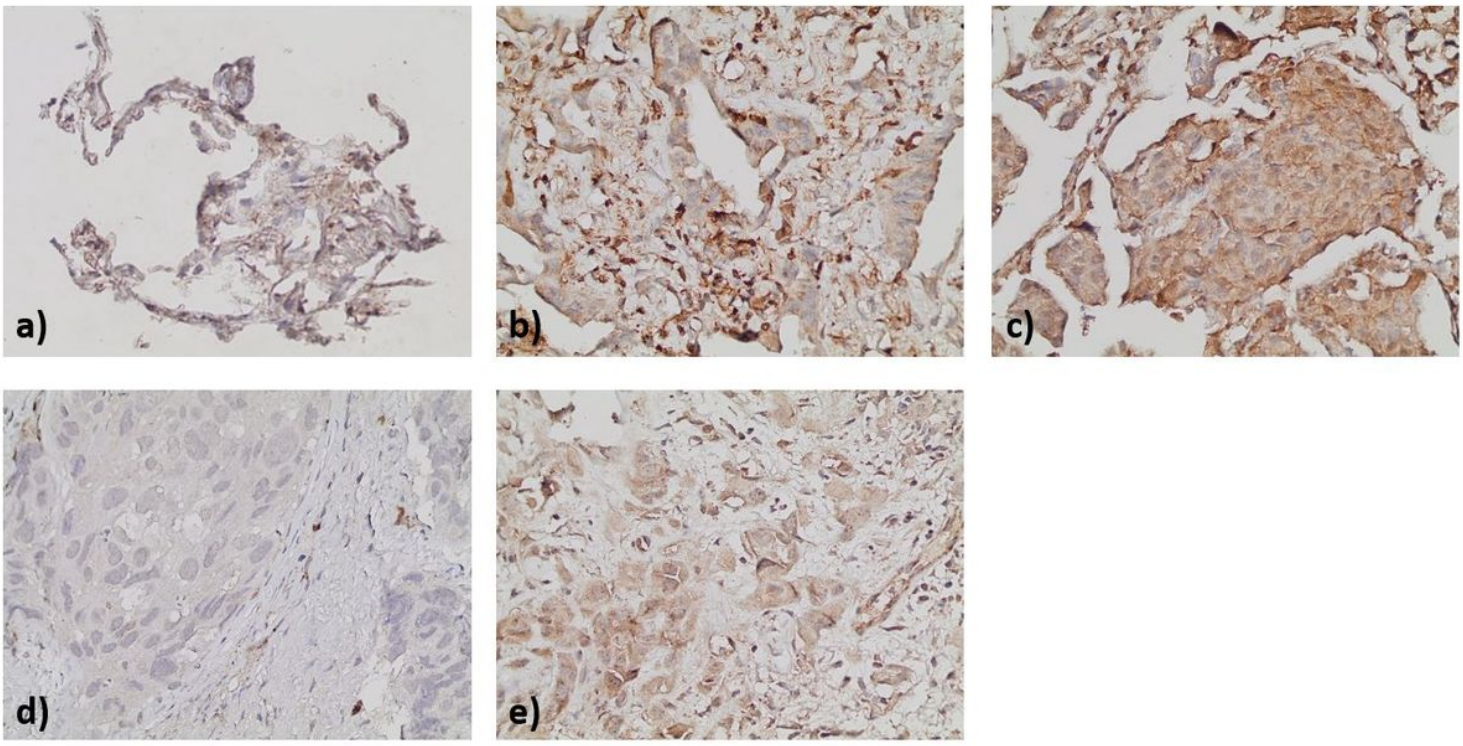
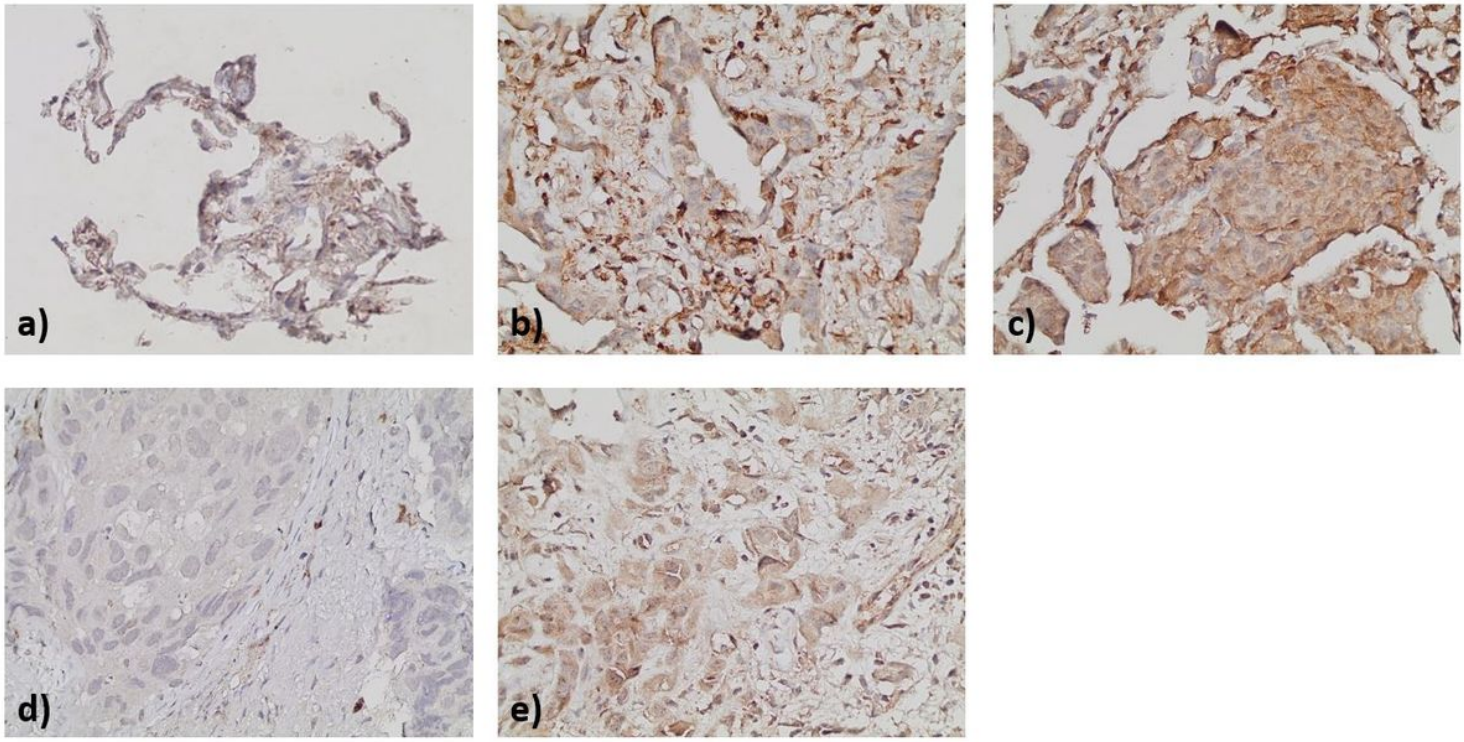


Figure 1

Immunohistochemical staining results of VEGF-A expression in lung tumor cells. Negative, low and high VEGF-A immunoreactivity was present in the cytoplasm of cancer cells. Adenocarcinoma tissues had three categories; negative expression (TS = 0), low expression (TS ≤ 6), and high expression (TS ≥ 7) (a-c, respectively) and squamous cell carcinoma had two categories (negative and low) (d & e, respectively).



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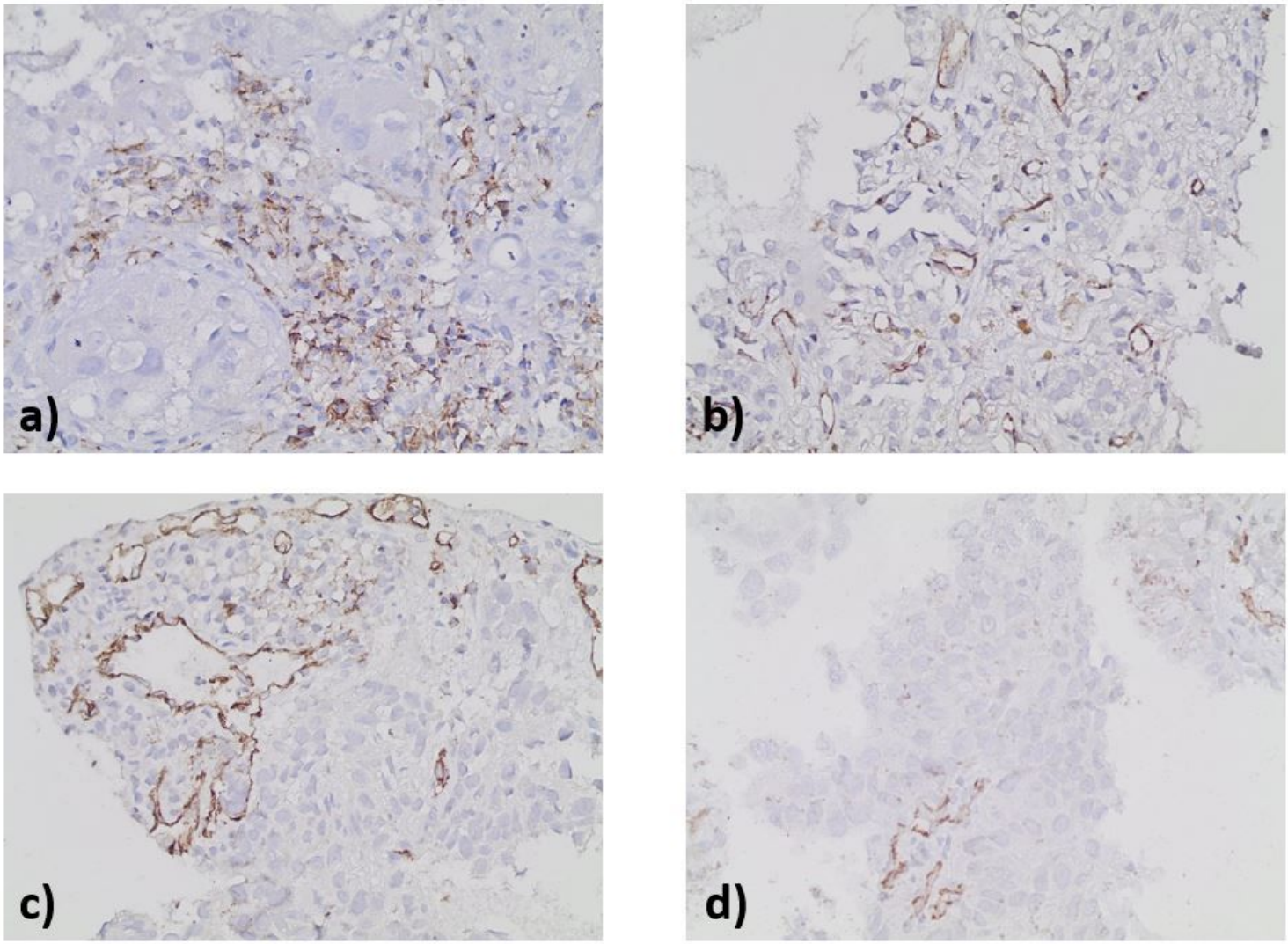
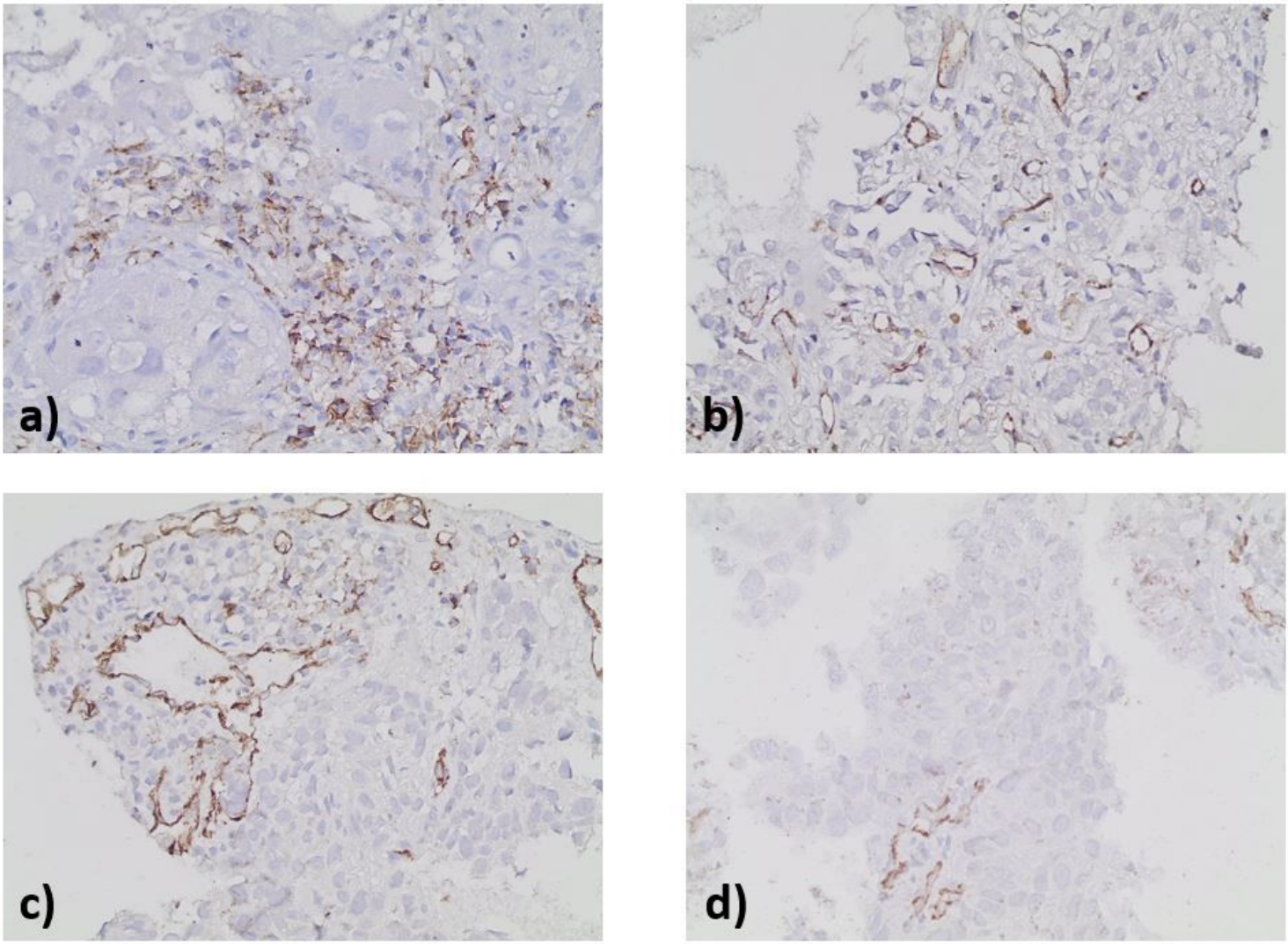


Figure 2

Immunohistochemical staining results of CD31 expression in lung tumor cells. Low and high CD31 immunoreactivity was present on the membrane of the endothelial cells. Adenocarcinoma and squamous cell carcinoma tissues had two categories; low (a & c, respectively) and high expression (b & d, respectively)



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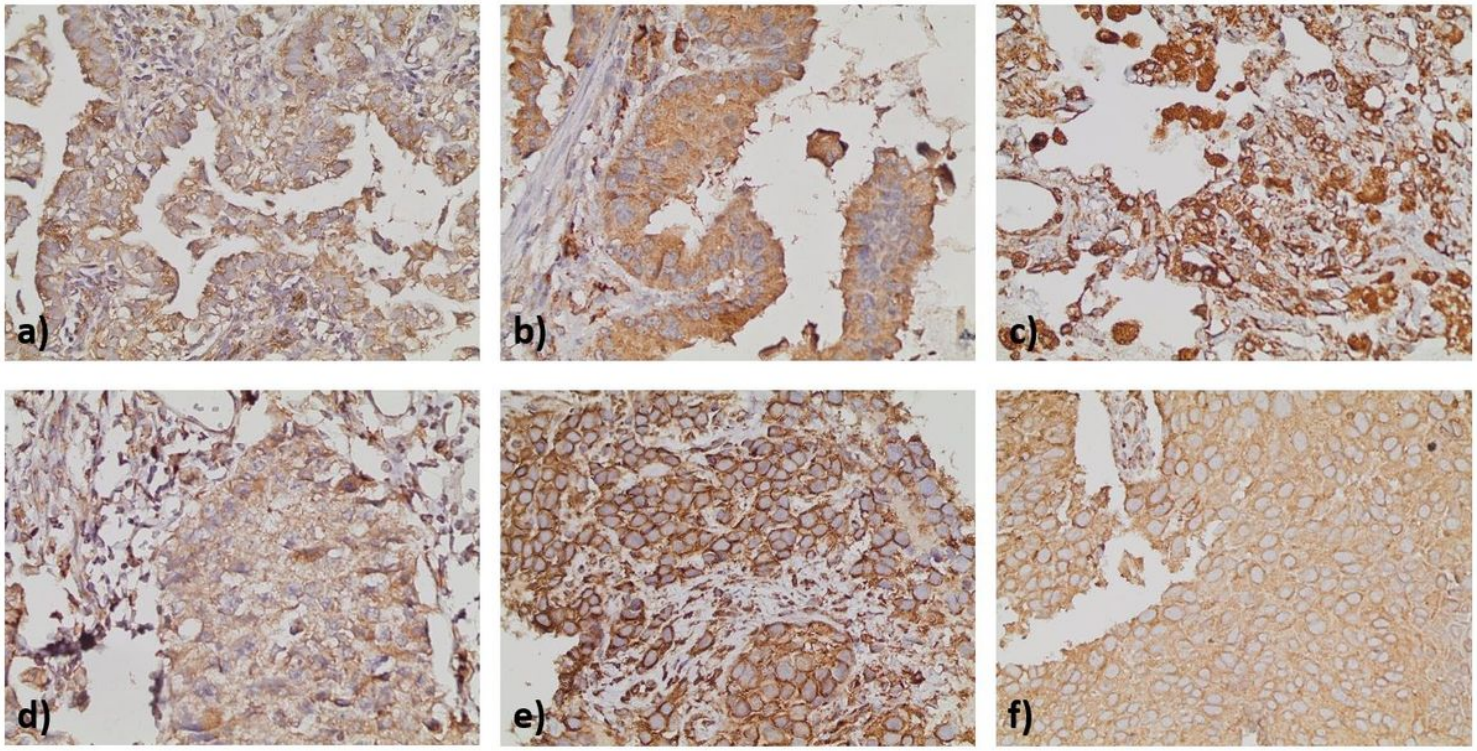
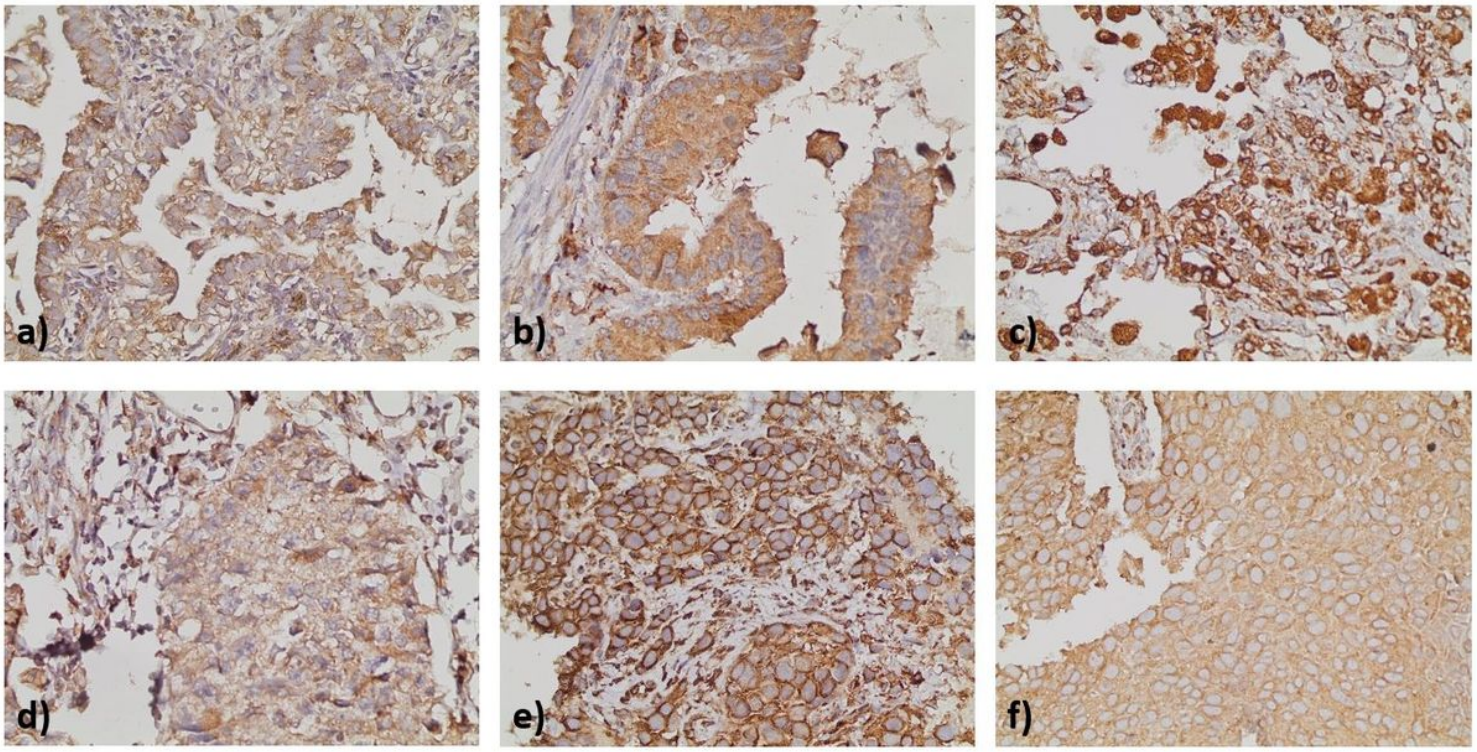


Figure 3

Immunohistochemical staining results of GRP78 expression in lung tumor cells. Negative, low and high GRP78 immunoreactivity was present in the cytoplasm of cancer cells. Adenocarcinoma tissues had three categories; negative expression (TS = 0), low expression (TS ≤ 6), and high expression (TS ≥ 7) (a-c, respectively) and squamous cell carcinoma had three categories (negative, low and high) (d-f, respectively).



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