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Original Article / *Özgün Makale*

Diagnostic value of signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 on serum and tissue samples in non-small cell lung cancer

Küçük hücreli dışı akciğer kanserinde signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1'in serum ve doku örnekleri üzerindeki tanısal değeri

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ABSTRACT

Background: This study aims to investigate whether there is any relationship between the type, stage and the extensiveness of lung cancer and levels of signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 in serum and lung tissues of non-small cell lung cancer patients and also whether there is any difference in signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 levels of patients with malignant or benign diseases.

Methods: The study included 55 subjects (45 males, 10 females; mean age 57.8±15.9 years; range 18 to 82 years) who were separated into three groups as 25 resectable non-small cell lung cancer patients (21 males, 4 females; mean age 64.6±9.4 years; range, 41 to 79 years) who were operated with the purpose of diagnosis and treatment (group 1), 15 unresectable non-small cell lung cancer patients (10 males, 5 females; mean age 61.8±9.6 years; range, 48 to 82 years) (group 2), and 15 patients (14 males, 1 females; mean age 42.5±19.5 years; range, 18 to 76 years) who were operated with non-cancer related reasons (group 3; control group).

Results: Preoperative serum signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 levels in groups 1 and 2 were significantly higher compared to control group (p=0.045). Serum signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 levels in group 2 were significantly higher compared to the other two groups (p=0.008). Levels of signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 in tissue samples were significantly higher in patients with non-small cell lung cancer and yielded a prognostic importance such that a 1 ng/mL rise in tissue signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 concentration caused a 1.4 fold increase in death risk (p=0.009).

Conclusion: Concentration of signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 in serum and tumor tissue may be an important biomarker in determining the diagnosis and prognosis in non-small cell lung cancer patients.

Keywords: Diagnosis; lung cancer; signal peptide-Cub-epidermal growth factor domain-containing protein 1.

ÖZ

Amaç: Bu çalışmada küçük hücreli dışı akciğer kanseri hastalarında akciğer kanserinin türü, evresi ve yaygınlığı ile serum ve akciğer dokularındaki signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 düzeyleri arasında ilişki olup olmadığı ve malign veya benign hastalıklı hastaların signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 düzeyleri arasında farklılık olup olmadığı araştırıldı.

Çalışma planı: Çalışmaya tanı veya tedavi amacıyla ameliyat edilen 25 rezektabl küçük hücreli dışı akciğer kanseri hastası (21 erkek, 4 kadın; ort. yaş 64.6±9.4 yıl; dağılım, 41-79 yıl) (grup 1), 15 unrezektabl küçük hücreli dışı akciğer kanseri hastası (10 erkek 5 kadın; ort. yaş 61.8±9.6 yıl; dağılım, 48-82 yıl) (grup 2) ve kanser dışı nedenlerle ameliyat edilen 15 hasta (14 erkek, 1 kadın; ort. yaş 42.5±19.5 yıl; dağılım, 18-76 yıl) (grup 3; kontrol grubu) olmak üzere üç gruba ayrılan 55 denek (45 erkek, 10 kadın; ort. yaş 57.8±15.9 yıl; dağılım 18-82 yıl) dahil edildi.

Bulgular: Grup 1 ve 2'de ameliyat öncesi serum signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 düzeyleri kontrol grubuna göre daha yüksek idi (p=0.045). Grup 2'de serum signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 düzeyleri diğer iki gruba göre anlamlı şekilde daha yüksek idi (p=0.008). Küçük hücreli dışı akciğer kanseri hastalarında doku örneklerindeki signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 düzeyleri anlamlı şekilde daha yüksekti ve prognostik öneme sahipti; öyle ki, doku signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 konsantrasyonundaki 1 ng/mL'lik bir artış ölüm riskinde 1.4 kat artışa neden oldu (p=0.009).

Sonuç: Serum ve tümör dokusundaki signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 konsantrasyonu küçük hücreli dışı akciğer kanseri hastalarında tanı ve prognozu belirlemede önemli bir biyobelirteç olabilir.

Anahtar sözcükler: Tanı; akciğer kanseri; signal peptide-Cub-epidermal growth factor domain-containing protein 1.

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Lung cancer is the most common cause of cancer-related deaths worldwide. Due to its natural progression, 70% of the lung cancer patients who are diagnosed during the local advanced stage or the metastatic illness are not eligible for surgery.[1] In the last decade, several important advances have been made in lung cancer treatment, particularly in target-based treatment.[2] It has been reported that angiogenesis plays an important role in the pathogenesis of non-small cell lung cancer (NSCLC). The necessary blood flow needs to be secured for tumor viability and metastasis. Increased lung tumor microvascular density is related to higher metastatic potential and reduced recovery rate.

Signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 (SCUBE1) is a member of the SCUBE family. It forms a part of the epidermal growth factor (EGF) super family and consists of several domain structures, including cysteine-rich and EGF-like repeats and Cub domain. The SCUBE family consists of three members: SCUBE1, SCUBE2, and SCUBE3.[3,4] SCUBE1 is released in the early period of embryogenesis and is present in the platelet alpha granules and endothelial cell.[5] The SCUBE gene family has been shown to be expressed in the gonads, central nervous system, and limb buds during mouse embryogenesis.[6] The SCUBE proteins perform a very important role during organogenesis and branching morphogenesis. SCUBE1 levels increase in inflammation and hypoxia, conditions resulting in angiogenesis in the osteoblasts and the bones.[7] SCUBE is associated with inflammation and hypoxia-related diseases.[8] Inflammation induces genetic changes and increases the risk of cancer.[9] Several studies have shown that SCUBE1 is related to various molecules involved in angiogenesis, such as hedgehog (HH), transforming growth factor-beta, and platelet-derived growth factor D.[7,10,11] Misregulation and mutations (PTCH1 and SMO genes) in the HH signaling pathway have been reported to lead to a range of cancers.[12] SCUBE1 was originally separated from the complementary deoxyribonucleic acid (cDNA) library of human umbilical vein endothelial cells.[10] It is released under hypoxia and inflammatory conditions from platelet alpha granules; SCUBE1 has been identified in endothelial cells and platelets.[5]

Signal peptide-Cub-epidermal growth factor domain-containing protein 1, a biomarker, is a transmembrane protein found in thrombocyte and endothelial cells. In inactive thrombocytes, SCUBE1 molecules are stored in the alpha granules, trans-blocked on the surface of the thrombocytes stimulated and activated through thrombin, secreted as small soluble pieces, and integrated into the thrombus[7] (Figure 1). SCUBE1 has been indicated to play an adhesive role in the interaction of matrix-bound or soluble forms with thrombocyte-thrombocyte or thrombocyte-matrix interaction and is a biologically important, new molecule in the cardiovascular system.[5] In the literature, the relationship between cancer and thrombosis has been investigated, and studies of stomach cancer patients have shown the presence of tumor cells within the thrombus, and increased ischemia and oxidative stress parameters.[13,14] It was reported that a clinically identifiable clotting disorder may be the first sign of the malignancy.[15]

Due to the susceptibility of malignant lung tissue to angiogenesis and thrombosis, SCUBE1 is believed to be a potential biomarker that warrants research in NSCLC patients. Therefore, in this study, we aimed to investigate whether there is any relationship between the type, stage, and the extensiveness of lung cancer and levels of SCUBE1 in serum and lung tissues of NSCLC patients and also whether there is any difference in SCUBE1 levels of patients with malignant or benign diseases. To the best of our knowledge, this is the first prospective clinical study to be conducted with these objectives.

PATIENTS AND METHODS

The study included 55 subjects (45 males, 10 females; mean age 57.8±15.9 years; range 18 to 82 years) who were separated into three groups as 25 NSCLC patients who went through complete lung resection (21 males, 4 females; mean age 64.6±9.4 years; range, 41 to 79 years) (group 1), 15 NSCLC patients who did not go

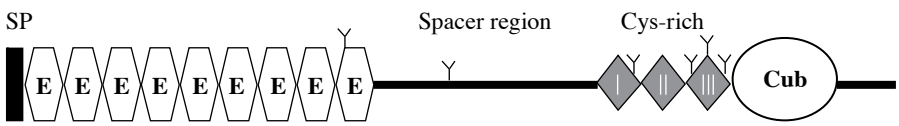

Figure 1. Molecular structure of serum SCUBE1. SCUBE1 molecule contains nine epidermal growth factor-like domains and one Cub domain at the carboxyl terminus.

SCUBE1: Signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1; SP: Signal peptide; Cys: Cysteine; E: Epidermal growth factor-like domain.

through complete resection (10 males, 5 females; mean age 61.8±9.6 years; range, 48 to 82 years) (group 2), and 15 patients (14 males, 1 females; mean age 42.5±19.5 years; range, 18 to 76 years) who were operated following an examination for a non-cancer cause (group 3; control group) at Ondokuz Mayıs University Hospital between October 2012 and July 2014. The study protocol was approved by the Clinical Research Ethics Committee of Ondokuz Mayıs University (Date: 09.12.2013, Number: B.30.2.ODM.0.20.08/731). A written informed consent was obtained from each patient. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patients who had undergone a specific treatment before the surgery, such as chemotherapy or radiotherapy, were excluded. For the working groups, patients with life-limiting conditions, lung diseases such as pneumonia, abscess or chronic liver disease, kidney failure, serious arrhythmia or ischemic heart disease, or collagen tissue diseases were excluded. In the control group, 15 patients without a previous lung or non-lung malignancy were included.

Before the surgery, venous blood was drawn from each patient in 5 mL biochemistry tubes, which were centrifuged, and the serum samples were separated after centrifugation and stored at -80ºC in the deep freezer until the time of study. Before the specimens taken during the surgery were fixed with formalin, samples of tumor-containing tissues from groups 1 and 2 and healthy lung tissues from the control group subjects were collected under the supervision of a pathologist and stored at -80ºC in the deep freezer.

Biochemical analysis

Signal peptide-Cub-epidermal growth factor domain-containing protein 1 levels in the serum and tissue were measured using a commercial kit (Cusabio Biotech Co., Catalog No. CSB-E15005h, P.R. China) and the enzyme linked immunosorbent assay method based on the instructions of the manufacturers. After the samples and reactives were brought to room temperature, serum samples were diluted 1:20 times using a dilution buffer. Tissue samples were homogenized according to the manufacturer' instructions. The tissue samples weighed at 100 mg were washed using the phosphate buffer saline (PBS) buffer and then homogenized in glass tubes to which 1 mL PBS buffer was added; thereafter, they were stored overnight at -20°C. Following two freeze-thaw applications, the samples were centrifuged at 5000 g at 2-8°C for five minutes, and the floating supernatants were used. Thereafter, 100 µL standard tissue supernatants and diluted serum samples were added to the wells indicated in the working plate and incubated at room temperature at 37°C for two hours. At the end of two hours, the fluids in the wells were removed without washing the wells. Then, 100 µL of biotin antibodies were added to each well and incubated again at 37°C for one hour, after which, the wells were washed four times using the washing buffer. Then, 100 µL of horseradish peroxidase-avidin solution was added to the wells and incubated again for one hour at 37°C. Following this, 90 µL substrate solution was added to the wells, which were washed again, and incubated in the dark for 20 minutes. To stop the reaction at the end of this time, 50 µL of stop solution was added, and the absorbents were measured using VersaMax (Molecular Devices, California, USA) microplate reader at 450 nm.

Statistical analysis

To conduct the statistical analyses of the study, IBM SPSS version 20.0 program (IBM Corp., Armonk,

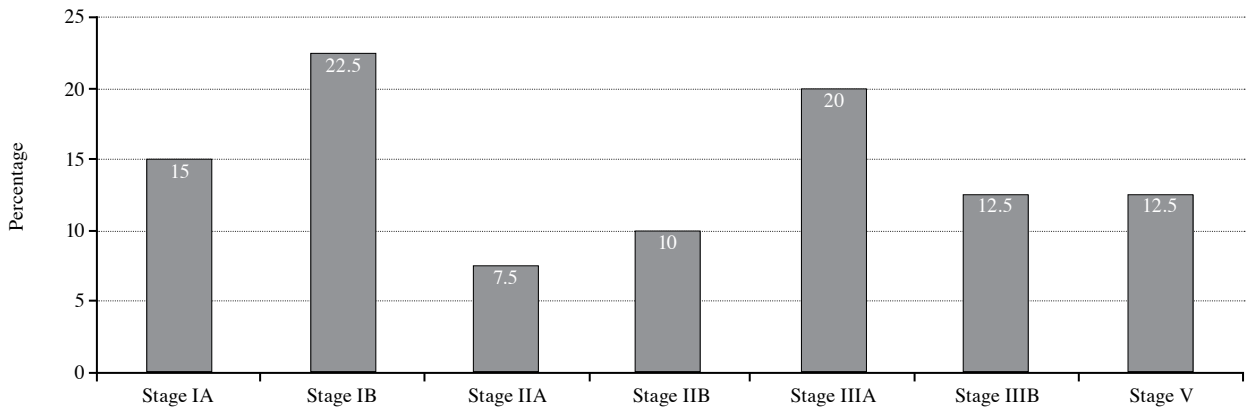

Figure 2. Stage distribution of non-small cell lung cancer patients.

Table 1. Comparison of preoperative serum and tissue SCUBE1 values between groups

	n	Mean±SD	95% CI		Min-Max	*p*
			Lower line	Upper line		
Preoperative serum SCUBE1						0.008
Group 1	25	16.7±7.9	13.47	20.00	1.73-40.84	
Group 2	15	28.9±15.6	20.29	37.56	7.19-72.66	
Group 3	15	17.0±14.4	9.07	24.97	5.29-53.68	
Total	55	20.1±13.2	16.57	23.69	1.73-72.66	
Tissue SCUBE1						0.424
Group 1	25	1.8±1.8	1.10	2.59	0.11-6.57	
Group 2	15	2.1±1.8	1.10	3.10	0.30-6.94	
Group 3	15	1.3±1.3	0.60	2.02	0.15-4.61	
Total	55	1.8±1.7	1.31	2.22	0.11-6.94	

SCUBE1: Signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1; SD: Standard deviation; CI: Confidence interval.

NY, USA) was used. The variables were checked for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. The meaningfulness between the variables in more than two categories was tested using analysis of variance) and Kruskal Wallis-H test. The relationship between the categorical variables was assessed using Chi-square test or Fisher's exact test. The median life expectancy of the two groups was tested using the log-rank test. The relevancy of the prognostic factors believed to influence survival rate was analyzed using Cox regression analysis. Furthermore, p values <0.05 were considered statistically significant.

RESULTS

Of the 55 study subjects, 81.8% (n=45) were males, while 18.2% (n=10) were females. Histopathological examination of 40 patients who had undergone surgical procedures for NSCLC showed that 22 (55%) of these patients were diagnosed with adenocarcinoma and 18 (45%) with squamous cell carcinoma. When these 40 patients were staged according to the tumor, node, metastasis system, the most commonly observed phase was stage IB (found in nine subjects [22.5%]) (Figure 2). The histopathological examination of the 15 subjects of group 3 (control group) revealed that 53.3% (n=8) had emphysema, 40% (n=6) had pulmonary bullae, and 6.7% (n=1) had interstitial lung disease. Therefore, the samples included in the control group have proven histopathology reports.

In group 1; lobectomy and mediastinal lymphadenectomy were conducted for 21 patients (84%), bilobectomy and mediastinal lymphadenectomy were performed in one patient (4%), chest wall resection with lobectomy was conducted in two patients (8%), and pneumonectomy and mediastinal lymphadenectomy were performed in one patient (4%). In group 2; cervical video-assisted mediastinoscopy was performed in 11 patients (73.3%), video-assisted thoracoscopic wedge resection in three patients (20%), and scalene lymph node biopsy in one patient (6.7%). All patients in group 3 were subjected to video-assisted thoracoscopic wedge resection for diagnosis and treatment.

Comparison of the preoperative serum and tissue SCUBE1 values between the groups revealed

Table 2. Analysis of SCUBE1 expression in groups 1 and 2 (non-small cell lung cancer) and group 3 (control)

Group	n	Median	Interquartile range	*p*
Preoperative serum SCUBE1				0.045
Non-small cell lung cancer	40	17.6	15.1	
Control	15	13.0	13.5	
Tissue SCUBE1				0.212
Non-small cell lung cancer	40	1.3	2.6	
Control	15	0.6	1.8	

SCUBE1: Signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1.

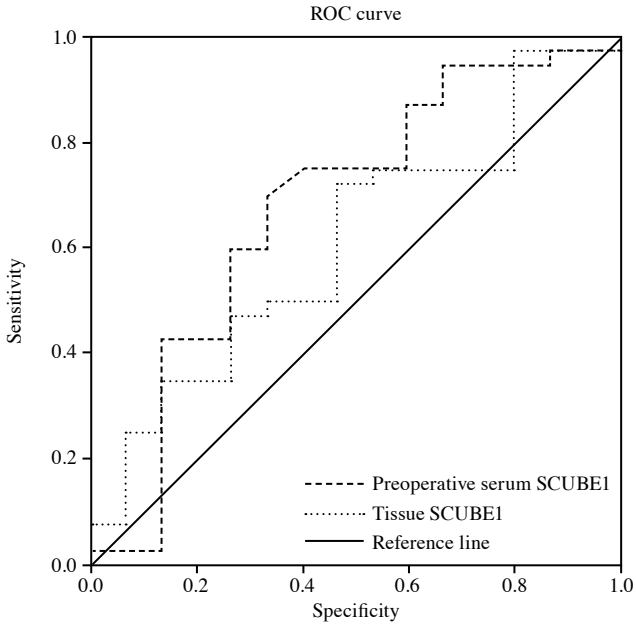

Figure 3. Receiver operating characteristic curve analysis (cut-off point: 14.1 ng/mL area under the curve: 0.677, %95 confidence interval: 0.503-0.851).

SCUBE1: Signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1.

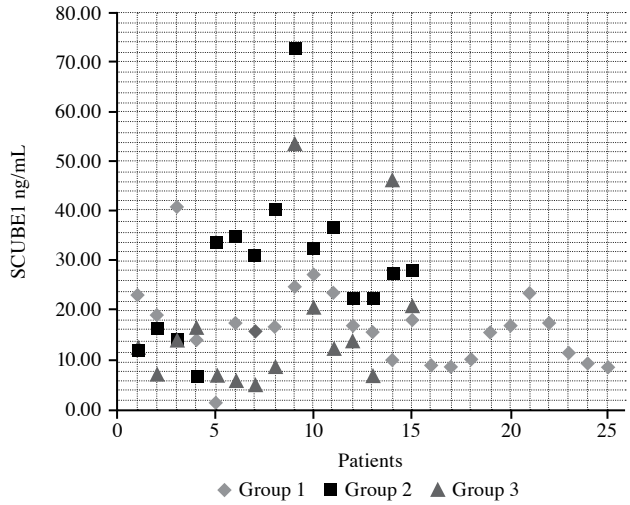

Figure 4. Distributions of preoperative serum SCUBE1 concentrations.

SCUBE1: Signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1.

that the serum SCUBE1 values in group 2 were significantly higher than those in the other two groups (average 28.92 ng/mL; minimum 7.19 ng/mL, maximum 72.66 ng/mL) (p=0.008) (Table 1). SCUBE1 levels of groups 1 and 2 were higher than those of group 3 (p=0.045) (Table 2). In the receiver operating characteristic curve analysis, the cut-off serum SCUBE1 value was identified as 14.1 ng/mL (area under the curve: 0.677, 95% confidence interval: 0.503-0.851), sensitivity as 62.3%, specificity as 83.2% (Figure 3), positive predictive value as 82.4%, and negative predictive value as 42.9%.

Dual comparisons between the groups using Tukey honest significant difference test revealed that the preoperative serum SCUBE1 expressions in group 2 were superior to those in groups 1 and 3 (p=0.01 and p=0.027, respectively) (Figure 4) (Table 3). The survival analysis of NSCLC patients showed that the average survival rate of group 1 was significantly higher than that of group 2 (p=0.001) (Figure 5).

The prognostic factors that have relevancy with survival were tested using the Cox regression analysis. In the analyses, the impact of preoperative serum SCUBE1

Table 3. Comparison of preoperative serum SCUBE1 values between group 2 and other groups

Comparison group	Mean difference	Standard error	*p*	95% CI	
				Lower line	Upper line
Preoperative serum SCUBE1					
Group 2					
Group 1	12.20	3.99	0.010	2.57	21.84
Group 3	11.90	4.46	0.027	1.13	22.67

SCUBE1: Signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1; CI: Confidence interval.

Table 4. Effect on survival of preoperative serum and tissue SCUBE1 concentrations

	β	Standard error	*p*	Relative risk
Preoperative serum SCUBE1	-0.006	0.018	0.747	0.994
Tissue SCUBE1	0.334	0.128	0.009	1.397

SCUBE1: Signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1.

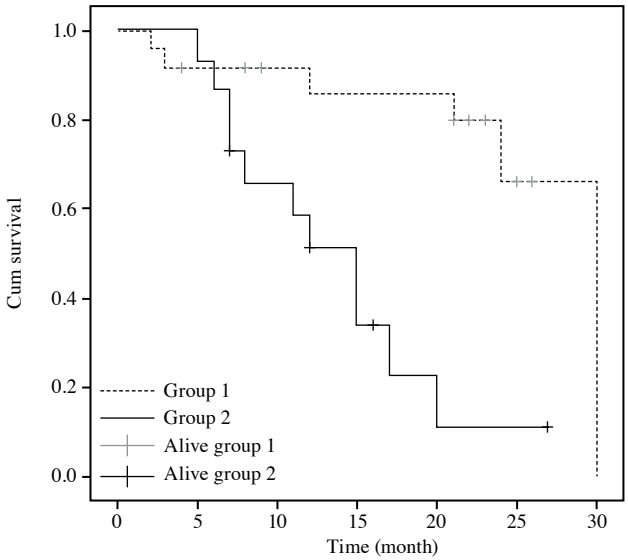

Figure 5. Survival analysis of non-small cell lung cancer patients.

and tissue SCUBE1 values on survival was evaluated. While no relationship was found between preoperative serum SCUBE1 (p=0.747) values and survival rate, the tissue SCUBE1 values (p=0.009) were found to have a significant effect on the survival rate. According to this finding, the 1 ng/mL increase in the tissue SCUBE1 concentration of NSCLC patients increased the mortality risk by almost 1.4 times (Table 4).

DISCUSSION

The most important cause of cancer-related deaths throughout the world is lung cancer. Only 15.9% of all lung cancer patients live for five years or longer following the diagnosis.[2] In the last decade, there have been significant developments, particularly in the screening and diagnosis of lung cancer as well as in the minimal invasive techniques and target-based treatments for lung cancer.[16] Epidermal growth factor is an important molecule that plays role in angiogenesis. In addition to its role in angiogenesis, the EGF/EGF receptor pathway is a primary stimulator for tumor proliferation.[17]

Signal peptide-Cub-epidermal growth factor domain-containing protein 1 is a biomarker comprising nine consecutively arranged EGF-like recurrences following an N-terminal signal peptide (22 amino acids) series, a large N glycolysis intermediary section, rich recurrent patterns of three cysteines, and a Cub section in C terminal.[18] Grimmond et al.[6] have indicated through *in situ* hybridization SCUBE1 cDNA fragment localized in 22q13 chromosome and in fibrin-rich parts inside the thrombus organized in thrombocytes. The molecular mass of SCUBE1 inside the thrombocytes was illustrated by Yang et al.[7] who used Western blot analysis and found that it is stored in alpha granules in inactive thrombocytes, secreted in small soluble pieces, and integrated into the thrombus.

Tu et al.[5] described the molecular structure of SCUBE1, identifying SCUBE1 in many tissues during rat embryogenesis, showing that it is responsible for early embryogenesis. In the same study, it was indicated that SCUBE1 has an adhesive role in thrombocyte interactions and that it plays a biologically important role in the cardiovascular system. In their research, Favre et al.[19] have shown that SCUBE1 has an impact on vascular development and angiogenesis in the lungs. In their research, Dai et al.[8] have demonstrated the excessive increase in SCUBE1 protein, dependent on the thrombocyte activation and aggregation, which occur during acute coronary syndrome and acute ischemic stroke and have indicated that this protein would be a good marker for acute thrombotic diseases. Mentese et al.[20] explored SCUBE1 levels in Crimean-Congo hemorrhagic fever (CCHF), which causes bleeding by damage to the endothelium, identifying its high levels in CCHF patients, indicating that SCUBE1 may have a role in the diagnosis and prognosis of CCHF patients.

In the series of analyses of gene expressions in the development of prostate conducted by Vanpoucke et al.,[21] SCUBE1 protein was found, and it was thought to be associated with carcinogenesis expressed in prostate cancer stromal cells. In an *in vivo* study conducted by Orr et al.,[22] SCUBE1 was used to mitigate the cancer-related fibroblast activity, and good results were obtained, proving its positive role in prostate cancer treatment. In the literature, it is determined that there is a relation between thrombosis and tumor, and tumor cells within thrombosis are shown in gastric cancer patients.[13,14] In light of this research, Mentese et al.[23] showed increased SCUBE1 concentrations in gastric cancer patients, and Topcu et al.[24] found high SCUBE1 levels in breast cancer patients.

To the best of our knowledge, our study is the first prospective clinical study to assess the possible relationship between SCUBE1 and lung cancer. SCUBE1 values in the serum and tissue specimens were significantly higher in groups 1 and 2 than in group 3, proving that serum SCUBE1 values may be elevated in NSCLC patients. In addition, the preoperative serum SCUBE1 expressions in group 2 were significantly higher than those in groups 1 and 3.

This finding indicates that the serum SCUBE1 levels of NSCLC patients have clinical prognostic significance.

In our survival analysis, we compared the average survival rates of NSCLC patients in group 1, who underwent curative surgical treatment, with those of patients in group 2, who did not undergo complete resection. The survival rate in group 1 (76%) was significantly higher than that in group 2 (26.7%), which was similar to that reported in the literature.

In the dual comparison of patients in groups 1 and 2, it was seen that the SCUBE1 concentrations in tissue samples were superior as a prognostic factor on survival, which was found statistically significant. However, we believe that our most important finding was that an increase of 1 ng/mL in the SCUBE1 concentration of tissue samples resulted in an almost 1.4 times increase in the mortality risk.

The limitation of our study was the number of samples used, which was determined using the power analysis method.

In conclusion, signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 concentrations are expressed in different amounts in different diseases. We believe that future research on this subject will help establish the disease-specific concentrations for signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 and enable its use as a more sensitive biomarker for non-small cell lung cancer. In addition, non-invasive diagnostic markers are preferred over invasive methods, such as collection of plasma, serum, and blood samples, with respect to patient comfort and suitability of the treatment. We also believe that signal peptide-Complement C1r/C1s, Uegf, and Bmp1-epidermal growth factor domain-containing protein 1 expression levels in the tumor tissues of non-small cell lung cancer patients can be used as prognostic markers for predicting survival rates.

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Declaration of conflicting interests

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