RESEARCH ARTICLE

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Determining the Vasohibin-1 Levels of the Serum and Broncoalveolar Lavage Fluid in the Patients with Lung Cancer

ABSTRACT

Objective: Lung cancer constitutes 17% of all cancer cases and accounts for 23% of the deaths caused by cancer all over the world. Vasohibin-1 (VASH-1) is an angiogenesis-inhibiting factor synthesized by endothelial cells (ECs). This study aimed to examine the VASH-1 levels of the bronchoalveolar lavage (BAL) and serum in the patients with lung cancer.

Methods: A total of 82 patients participated in this study. 39 and 43 of them had a benign lung disease and lung cancer, respectively. The VASH-1 levels of serum and BAL were measured using the Enzyme-Linked Immunosorbent Assay (ELISA).

Results: The BAL VASH-1 levels of the patients in the lung cancer group were found to be statistically significantly lower than those of the patients in the benign lung disease group (p=0.032). No statistically significant difference was found between the individuals with lung cancer and benign lung disease in terms of the serum VASH-1 concentration (p=0.206). A statistically significantly moderate positive correlation was found between the serum and BAL VASH-1 levels in the benign and malignant cases (benign r=0.442, p=0.005; malignant r=0.364, p=0.016). When the lung cancer patients were categorized into pathological stages and histological types, no significant difference was found between the stages and histological types in terms of the serum and BAL fluid VASH-1 concentrations.

Conclusions: BAL VASH-1 concentrations decreased in the lung cancer patients compared to the individuals having a benign lung disease. Considering the results reached in this study, it was thought that the BAL VASH-1 concentrations might be beneficial in distinguishing between the benign and malignant lung diseases.

Keywords: Angiogenesis, Broncoalveolar Lavage Fluid, Lung Cancer, Metastasis, Vasohibin-1.

Akciğer Kanserli Hastalarda Serum ve Bronkoalveolar Lavaj Sıvısında Vasohibin-1 Düzeylerinin Belirlenmesi

ÖZET

Amaç: Akciğer kanseri tüm dünyada kanser olgularının %17'sinden, kanser ölümlerinin ise %23'ünden sorumludur. Vasohibin 1 (VASH-1) endotel hücreleri tarafından sentezlenen bir anjiyogenez inhibe edici faktördür. Bu çalışmada akciğer kanserli hastalarda, bronkoalveolar lavaj (BAL) ve serum VASH-1 düzeylerinin incelenmesi amaçlandı.

Gereç ve Yöntem: Bu çalışmaya toplam 82 hasta katıldı. Bunlardan 39'u benign akciğer hastalığı ve 43'ü malign akciğer kanserine sahipti. Serum ve BAL VASH-1 düzeyleri, enzim bağlı immünosorbent analizi (ELISA) kullanılarak ölçüldü.

Bulgular: Akciğer kanseri grubunda, BAL VASH1 düzeyleri, benign akciğer hastalığı olan gruptan anlamlı derecede düşüktü (p=0,032). Ancak serum VASH-1 düzeyleri açısından her iki grup arasında anlamlı bir fark yoktu (p=0,206). Malign ve benign vakalarda serum VASH-1 ile BAL VASH-1 düzeyleri arasında istatistiksel olarak anlamlı, orta dereceli, pozitif bir korelasyon vardı (benign r= r=0.442, p=0.005; malign r=0.364, p=0.016). Akciğer kanserli hastalar patolojik 'stage' lerine ve histolojik tiplerine göre ayrıldığında, evreler ve histolojik tipler arasında serum ve BAL sıvısı VASH1 konsantrasyonları bakımından anlamlı bir farklılık yoktu.

Sonuç: BAL VASH-1 konsantrasyonları, akciğer kanseri hastalarında benign akciğer hastalığı olan bireylere kıyasla azaldı. Elde edilen sonuçlar ışığında, BAL VASH-1 konsantrasyonlarının benign ve malign akciğer hastalıklarını ayırt etmede faydalı olabileceği düşünülmüştür.

Anahtar Kelimeler: Akciğer Kanseri, Anjiogenez, Bronkoalveolar Lavaj Sıvısı, Metastaz, Vazohibin-1.

INTRODUCTION

Lung cancer constitutes 17% of all cancer cases and accounts for 23% of the deaths caused by cancer all over the world (1). At the time of diagnosis, 86.7% of the cases had locally advanced or advanced stage diseases (2). Although it depends on the stage of the disease, the average 5-year survival rates were found to be 11% between 2000 and 2008 (3). Various genetic and carcinogenic factors have a key role in the etiology. It was reported that the risk for lung cancer increased 24-36 times in the smokers compared to the nonsmokers (4).

The methods used to pathologically diagnose the lung cancer can be applied to the primary tumor and metastasis site. Bronchoscopy is a common method used to diagnose the lung cancer. It has a high diagnostic value especially for the central tumors. On the other hand, the transbronchial needle aspiration and biopsy are widely used in the diagnosis of peripheral lesions (5).

The bronchoscopy, needle aspiration, and biopsy carried out for the diagnostic purposes are expensive and invasive techniques with a high morbidity and they disturb the patient's comfort. Therefore, there is a need for the simple, low-cost, and non-invasive new molecular markers in the diagnosis and follow-up of lung cancer. The purpose of the biochemical studies is to identify the new tumor markers to be used to predict the structural features and prognosis of tumors. VASH-1 is one of the molecules that have been investigated for this purpose.

The VASH-1 is a protein that is composed of a mature protein core of 365 aa, produced at 44 kDa, and known to increase the resistance of endothelial cells to the stress and to stabilize the vessels as well as to terminate the angiogenesis in the terminal region (6,7).

Vasohibin-1 (VASH-1) is a protein that has an anti-angiogenic activity and that is induced by the vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF2), which are some of the angiogenic factors expressed from the activated endothelial cells (8,9). It has been found that the VASH-1 protein expression in the blood vessel endothelial cells of tumors is associated with various cancer types like the breast cancer, hepatocellular carcinoma, non-small-cell lung cancer, prostate cancer, renal cell carcinoma, and the upper urinary tract urothelial carcinoma (10-18).

This study aimed to measure the VASH-1 levels in serum and BAL fluid in the lung cancer patients and to compare the serum and BAL VASH-1 concentrations in the individuals having a benign lung disease. Furthermore, we also examined whether the serum and BAL fluid VASH-1 concentrations were correlated with the lung cancer histological types and the habit of cigarette smoking.

MATERIAL AND METHODS

This study was conducted after receiving the approval from the Ethics Committee of XXX University Faculty of Medicine (20.03.2014, meeting no: 4, decision no: 6). In this study, the 1st group consisted of 43 patients who were admitted to the Chest Diseases Clinic between March 2014 and March 2015 with the complaints such as cough, shortness of breath, and hemoptysis and who were diagnosed with lung cancer by the radiological and biochemical examinations as well as the histopathological evaluation of the samples taken after the bronchoscopy and biopsy (if necessary). The 2nd group consisted of 39 patients who had the same demographic characteristics and diagnosed with non-cancerous lung disease. Of the cases with benign diseases, 7 had been diagnosed with sarcoidosis, 11 tuberculosis, 10 pneumonia, 4 hemoptysis, and 7 chronic obstructive pulmonary disease (COPD). All subjects in the study groups were informed about the study and included in the study after they signed the consent forms.

The inclusion criteria were to be 30-85 years old and newly diagnosed with lung cancer or non-cancer lung disease. The criteria for the presence of non-cancer lung disease were as follows: being admitted with the symptoms suggestive of the lung disease such as cough, shortness of breath, and hemoptysis; and having one of the diseases affecting the interstitial tissue of the lungs such as sarcoidosis, tuberculosis, pneumonia, hemoptysis, and COPD.

The exclusion criteria of the study were as follows: the presence of any malignancy other than the lung cancer; actively getting chemotherapy, immunotherapy, or radiation therapy; and having a surgery for the lungs in the last three months.

Blood Samples: Before starting any medication or carrying out a surgical intervention, the blood samples taken for the routine biochemistry tests were kept in the tube in an upright position for 10-20 min. and then centrifuged at 4000 rpm for 15 min. at +4°C. The serum samples obtained were aliquoted and kept at -80°C until the day of analysis.

Bronchoalveolar Lavage Samples: Before starting any medication or carrying out a surgery and after the bronchoscopy, with the purpose of diagnosing, the brushing was applied to the patients or some serum was administered to a lung segment bronchus which was determined physiologically (provided that biopsy was not carried out). The fluid administered was back-aspirated with a defined pressure. The lavage samples obtained were aliquoted and then kept at -80 °C until the day of analysis.

Methods for the Determination of Analytes: The measurements of the serum and BAL VASH-1 levels were made on the same day to avoid the inter-day variation. The VASH-1 levels in the serum and BAL samples were measured through the

ELISA method using "Human Vasohibin-1 ELISA Kit (Lot:30211832) in line with the instructions of the manufacturer. The values were expressed in ng/mL.

Statistical Evaluation: SPSS v20.0 (Windows, SPSS Inc, IL, US) was used for the statistical evaluations and recording the data. The descriptive statistics of the data obtained were expressed in numbers and % for the categorical variables and in average ± standard deviation for the numerical variables. Kolmogorov Smirnov Test and the histogram graphing method were used for analyzing the data's fitness to the normal distribution. For the data not meeting the normality requirements, Mann-Whitney U test was used to compare two independent groups and Kruskal-Wallis Test was used for the multiple comparisons. Spearman's rho correlation method was used to examine the relationship between the numeric non-normally distributed data. The statistical significance level was set at $p<0.05$.

RESULTS

Information on gender, age and diagnosis of lung cancer type and stage is provided in Table 1. A significant difference was observed between the groups in terms of age (p=0.009). Therefore, in order to determine whether the difference between the cancer patients and the benign patients was due to the age, the weighting was carried out.

Table 1. Clinical characteristics of lung cancer and benign lung disease study populations

Samples	Characteristics	Lung Cancer	Benign lung disease
Total (n)		43	39
Gender (n,%)	Females	7 (16.3%)	17 (43.6%)
	Males	36 (83.7%)	22 (56.4%)
Age (years)	Mean± sd	63.6 ± 10.9	55.6 ± 15.8
Histology (n)	squamous cell carcinoma	20	
	adenocarcinoma	7	
	small cell carcinoma	16	
Stage (n)	II	7	
	III	7	
	IV	29	

The serum VASH-1 levels were below the detection limit in 7 patients in the lung cancer group and in 1 patient in the benign lung disease group. The serum VASH-1 levels in both groups are given in the Table 2. No statistically significant difference was found between the groups in terms of the serum VASH-1 levels (p=0.206) (Figure 1).

Table 2. Serum and BAL fluid VASH-1 Concentrations

Groups	Group 1	Group 2	P
Serum VASH-1 (ng/mL)	0.30±0.25	0.38±0.31	0.206
BAL VASH-1 (ng/mL)	0.10±0.12	0.19±0.18	**0.032**

Values are expressed as mean ± standard deviation

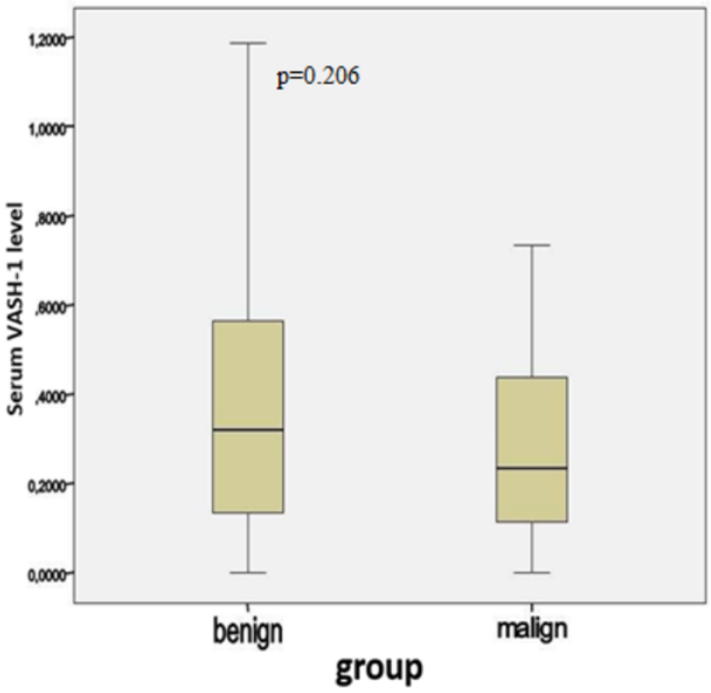

Figure 1. Intergroup comparison of the serum VASH-1 levels

When the BAL fluid VASH-1 levels were evaluated, they were found to be below the detection limit in 8 patients in the lung cancer group and in 5 patients in the benign disease group. The BAL fluid VASH-1 levels in both groups are given in the Table 2. There was a statistically significant difference between the two groups in terms of the BAL fluid VASH-1 results (p=0.032) (Figure 2). There was a highly significant difference in both groups between the means of the BAL VASH-1, which were evaluated separately in the benign and malignant patients after weighting the cases based on the age ($p<0.001$).

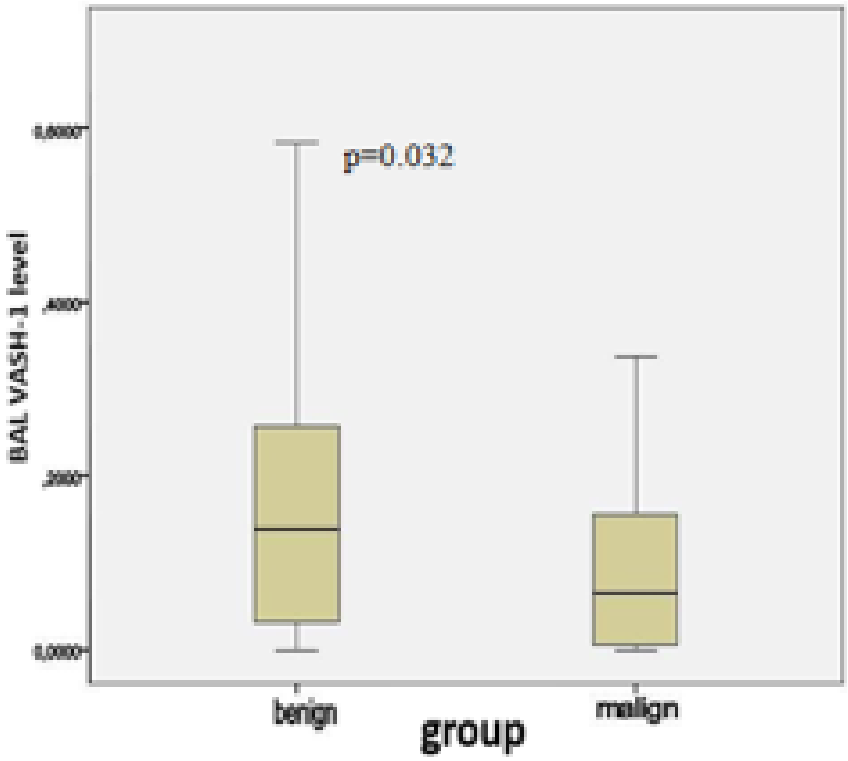

Figure 2. Intergroup comparison of the BAL VASH-1 levels

There was a statistically significantly moderate positive relationship between the serum and BAL fluid VASH-1 levels in the benign and malignant cases (r=0.442, p=0.005 for the benign; r= 0.364, p=0.016 for the malignant).

The lung cancer patients were typed and staged based on the pathology results (Table 1). The result of the comparison of the malignant cases in terms of their stages, the difference between the groups was found to be not significant in terms of the serum (χ^2 = 0.199, p=0.905) and BAL (χ^2 = 1.535, p=0.464) VASH-1 means. Also, no statistically significant difference was observed between the histological types in terms of the serum (χ^2 = 1.608, p=0.448) and BAL (χ^2 = 0.208, p=0.901) VASH-1 means. Even when 27 patients were classified as NSCLC and 16 patients were classified as SCLC, it was observed that there was no significant difference between the groups in terms of the serum (p = 1.000) and BAL (p = 0.308) VASH-1 means.

There was not a significant difference between the serum VASH-1 means of the smokers and the non-smokers (p=0.359). A significant difference was observed between the means of the BAL fluid VASH-1 in the non-smokers and the smokers (p = 0.04). A significant, positive, and high relationship (r=0.365, p=0.002) was found between the serum and BAL VASH-1 levels in the smokers; a significant, positive, and moderate relationship (r=0.571, p=0.026) was also found in the non-smokers. There was a highly significant difference in both groups between the means of the serum and BAL VASH-1, which were evaluated separately in the smokers and non-smokers after weighting the cases based on the age (p<0.001).

In the analysis carried out separately in terms of the means of the serum and BAL fluid VASH-1 between the benign diseases, no significant difference was observed between the benign diseases in terms of the means of the serum VASH-1 (p=0.448) and BAL VASH-1 (p=0.901).

DISCUSSION

Lung cancer is one of the commonest cancers that are highly morbid and mortal. The mortality rate of the lung cancers is high due to the difficulties experienced in early diagnosis, close follow-up, and the right treatment if deemed necessary. On the other hand, the diagnostic value of the noninvasive tests such as cytology and tumor marker in determining the early diagnosis, presence of recurrence, invasion, and metastasis in lung cancer is still not at the desired level. The need for the follow-up at close intervals led the researchers to find the noninvasive biochemical markers instead of the invasive interventions with high morbidity. The promising use of the proteins such as VASH-1, which is a candidate for being a tumor marker, in revealing the presence of tumor, recurrence, invasion, and metastasis paved the way for the researchers to carry out more and more studies on this subject.

As the expression of molecules in the vascular endothelium increases due to various reasons, their levels in the general circulation will also increase. In case the expression of molecules increases in the vascular endothelium in the wall of

lung bronchioles, it is also possible to detect the molecules in the BAL fluid obtained by the washing procedure after the bronchoscopy. Moreover, the contact of the bronchial basal epithelial cells with this fluid can also be a reason for being able to detect these molecules in the BAL fluid. Therefore, the level of some markers in the BAL fluid as well as the serum is examined in the lung cancer cases (19).

The local invasion and metastasis of the tumor cell primarily require the destruction of the basement membrane and the neovascularization. For the neovascularization, various biomolecules stimulating the angiogenesis in the existing vascular endothelial cells need to be synthesized and secreted. VEGF is one of the most important molecules known to activate the angiogenesis (20). With the increase of the synthesis of VEGF, the synthesis of the VASH-1 molecule, which has a key role in the maturation of new vessels and their termination where needed, also increases (8). In various studies carried out on the VASH-1 molecule in recent years, it has been revealed that this molecule is related with the tumor invasion, metastasis, and poor prognosis (21).

In our study, the serum and BAL fluid samples of the individuals with lung cancer and benign lung disease were measured using the ELISA technique and the groups were compared based on these results. Since it was impossible to use the bronchoalveolar lavage samples as well as the serum sample and to take this sample from the healthy individuals, the cancer patients were compared with the patients having a benign lung disease rather than the healthy individuals. While in the lung cancer group, the BAL fluid VASH-1 levels were lower than those having a benign disease; there was no significant difference between the malignant and benign patients in terms of the serum VASH-1 levels. However, a statistically significantly positive correlation was found between the serum and BAL fluid VASH-1 levels in the patients with both benign and malignant disease. The lower VASH-1 levels in the BAL fluid samples of the cancer patients compared to the benign patients explained the fact that the tumor tissue grew faster than the normal tissue and there was more vascular structure which lacked the mural cells and thus was immature.

Furthermore, in the malignant patients, no significant relationship was found between the serum and BAL fluid VASH-1 levels, which were compared based on the classification in terms of the stage and histological type of the tumor. Moreover, the VASH-1 level in the BAL fluid samples in the smokers were found to be lower than that in the non-smokers. These results suggested that the decrease in the VASH-1 levels might be an indicator for the increased cancer susceptibility in the smokers.

In the literature review we carried out, it was found that there were few studies examining the expression of VASH-1 in the tissue samples of the lung cancer patients. In one of these studies, Zhang et al. (16) used the immunohistochemical method to measure the expression of the VASH-1 protein in the biopsy specimens taken from the patients with non-small-cell lung cancer. They detected VASH-1 in the cytoplasm of vascular endothelial cells of both cancer tissue and normal tissue. When the number of samples with high VASH-1 expression was compared with the normal tissue samples, it was found that they were higher in the lung cancer patients. Furthermore, they revealed that the high VASH-1 expression was correlated with the TNM staging, but the high VASH-1 expression in the tissues of the patients with cancer had no significant correlation with the age, gender, smoking, histologic type, and tumor size. The VASH-1 expression was shown to increase as the stage of the disease increased. Also, many studies show that significant correlation between the degree of malignancy (tumor grade and stage) and poor prognosis and VASH1-positive vessel density (18,22,23).

In a study carried out by Watanabe et al. (24) it was found that the preoperative high plasma VASH-1 concentration was related with a better prognosis in the individuals having non-small cell lung carcinoma (24).

In another study carried out on the lung cancer patients, Hosaka et al. (10) examined the lung cancer tissue samples of 44 patients with NSCLC using the immunohistochemical method and inoculated Lewis lung carcinoma (LLC) cells into the VASH1 -/- and wild-type (WT) mice to better explain the function of VASH-1. As a result of the examination carried out on the tissue samples, they showed that the VASH-1 protein was expressed in the tumor stroma more prominently than in the non-cancerous resection sites. In the same study, it was also revealed that as a result of the inoculation of LLC cells into VASH-1 -/- mice, compared to WT mice, the tumor diameter was much larger, its growth was faster, the vascular area was wider, and the vessels were immature and lacked the mural cells. It was also shown that, when the exogenous VASH-1 was administered to the VASH-1 -/- mice, the tumor size in mice was inhibited, the vascular area was reduced, and the tumor vessels become more mature. In conclusion, the study in question suggested that the endogenous VASH-1 protein may be a new test for the diagnosis and treatment of the lung cancer because it inhibited the tumor angiogenesis (10). Our study may be important in terms of the idea that the VASH-1 protein, which is found in low levels in the BAL fluid, can be used for the therapeutic purposes apart from giving information about the tumor density.

CONCLUSION

All in all, the studies have revealed that the VASH-1 protein has a complex inhibitory role in the malignant tumor behavior. It has been asserted in the studies that the low level of VASH-1 can be the indicator for the metastasis, deep invasion, and the poor prognosis. Almost all animal studies in the literature have suggested that the VASH-1 protein is a new treatment modality that can be used to inhibit the angiogenesis in cancer and other diseases. In our study, the VASH-1 level in BAL fluid of the malignant patients was found to be lower than that of the benign patients. This supported the fact that

the angiogenesis was faster and the immature vascular density was higher in the patients with cancer and, therefore, the metastasis and invasion could occur, and the prognosis was worse than the benign patients. Considering the fact found in our study that the VASH-1 levels of BAL fluid were lower in the patients with malignant disease, it was thought that this difference could be used in distinguishing the benign malignant diseases.

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