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ORIGINAL ARTICLE

Angiopoietin 2 levels in serum and bronchial lavage fluids and their relationship with cancer stages in lung cancer patients

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Abstract

Background: Angiopoietin 2 (Ang-2) has an important role in tumor angiogenesis. In this study, Ang-2 levels of serum and bronchioloalveolar lavage fluids (BALF) in patients with lung cancer were measured and correlated with clinical and biochemical parameters.

Methods: Thirty-five cases newly diagnosed with lung cancer and 18 controls with non-cancerous lung diseases were included in the study. Tumor histology, staging, metastasis, tumor markers, biochemical and clinical parameters were all recorded.

Results: Serum Ang-2 levels were significantly higher in the lung cancer group compared to the control (lung cancer median: 2.42 ng/mL [2.19–2.98], control 0.67 [0.31–1.10]; $P < 0.001$), whereas Ang-2 levels in BALF were lower in the lung cancer group compared to the control (lung cancer median 0.41 ng/mL [0.22–0.79], control 0.67 [0.46–1.03]; $P = 0.02$). In the cancer group, higher serum Ang-2 levels ($r = 0.52$, $P < 0.001$) were associated with the stage of cancer. No significant correlation was observed between BALF Ang-2 levels and non-small cell lung cancer stages and small-cell lung cancer advanced stage ($P = 0.793$, $r = 0.07$). Serum Ang-2 levels were significantly higher in distant metastasis (M1) versus no distant metastasis (M0) (M1: 2.57 ng/mL [2.38–2.87], M0: 2.22 [1.49–2.40], $P = 0.01$). No significant correlation was observed between BALF Ang-2 levels and M1 ($r = 0.11$, $P = 0.53$).

Conclusions: Serum Ang-2 levels were significantly higher in lung cancer patients and positive correlations were observed between serum Ang-2, tumor stage, and metastasis.

Introduction

Lung cancer is the leading cause of death among cancer patients, despite treatment choices and new therapy instruments.[1] Advances in molecular pathology have enabled us to learn more about tumor biology, including tumor growth, metastasis, and angiogenesis. For example, it is now understood that tumors can grow to a determined volume without angiogenesis.

Angiogenesis is a physiological process controlled by angiogenic and antiangiogenic factors. When this equilibrium is changed on the side of angiogenic factors, pathological angiogenesis occurs. This situation can be observed in some ocular, chronic inflammatory, pulmonary, and cardiovascular diseases, and also in tumor growth and metastasis.[2]

Angiopoietins are glycoprotein molecules that are the members of the growth factor family affecting the vascular endothelium and consisting of an angiopoietin specific area of 46 kDa molecular weight. Angiopoietin-1, 2, 3 and 4 (Ang-1,2,3,4) are four different types that have been discovered.[3]

Ang-1 is mainly secreted from perivascular or mural cells, such as pericyte or smooth muscle cells.[4] The pericyte originated signaling system, Ang-1, provides vessel stabilization by strengthening the supportive tissues surrounding (smooth

muscles and extra cellular matrix) and inside of endothelial cells.[5] Ang-1 inhibits secretion of adhesion molecules and diminishes vascular endothelial growth factor (VEGF) associated inflammation.[6]

Ang-2 is a competitive inhibitor of Ang-1 and is produced by endothelial cells, stored in Weibel-Palade bodies before they are secreted. The secretion of Ang-2 is stimulated by endothelial cells when they are in stress, VEGF, fibroblast growth factor and hypoxia. Adhesion of Ang-2 to Tie 2 receptors inhibits the effects of Ang-1. Endothelium is made sensitive to inflammatory agents (vessel destabilization) and VEGF associated angiogenesis occurs easily.[7,8]

The role of Ang-2 in angiogenesis is associated with VEGF-A, a protein that in humans is encoded by the VEGF-A gene and functions as a glycosylated mitogen that particularly acts on endothelial cells and has various effects including angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration and inhibiting apoptosis. When VEGF-A is present in the environment, Ang-2 causes vascular destabilization and supports vascular budding. A lack of VEGF-A results in increased vascular regression.[9]

Ang-2 secretion is stimulated by various factors produced by tumors and near tissues in tumor angiogenesis. The most important is VEGF elevation from tumor origin. Other factors, which stimulate Ang-2, are insulin like growth factor, platelet derived growth factor (PDGF), and hypoxia.

The relationship between Ang-2 and VEGF is basically within tumor angiogenesis. The secretion of Ang-1 is more likely found in normal tissue, while Ang-2 is found in tumor tissue. This situation is thought to be the main step in tumor angiogenesis.[10] Ang-2 secreted from tumor tissue has effects on the endothelium, which enhances tumor growth and vascularization. High levels of Ang-2 levels in the system decrease tumor growth and cause vessel regression. This development is associated with VEGF-A existence in the environment. Where VEGF-A is found to exist in tumor tissue, Ang-2 causes vascular destabilization in the tumor tissue and enhances the effect of VEGF-A via the formation of new vessels.[11]

There are recent studies about tumor angiogenesis in cancer treatment. Ang-2 is one of the molecules studied in target therapy. In our study we measured serum and bronchioloalveolar lavage fluid (BALF) Ang-2 levels in lung cancer patients and evaluated its association between stage, type of tumor, clinical and biochemical parameters.

Methods

Study population

This single center case control study was performed at the department of pulmonary diseases at Gulhane Military Medical Academy Haydarpasa Training Hospital, Istanbul over the period of October 2009 and June 2010.

Thirty-five cases diagnosed with lung cancer and 18 control cases of other non-cancerous lung diseases were included in the study. The exclusion criteria included patients <18 years of age, with a preexisting history of malignancy, and contraindication for bronchoscopy. Detailed medical history was taken from all patients. Complaints at admission to hospital, coexisting diseases, smoking history, demographics, physical examination, complete blood count (CBC), erythrocyte sedimentation rate (ESR) and tumor markers (carcinoembryonic antigen [CEA], alpha-fetoprotein [AFP], cancer antigen 125 [CA-125], cancer antigen 19-9 [CA-19-9]) were recorded. Staging of non-small cell lung cancer (NSCLC) patients was performed with the tumor, node and metastasis classification of the International Association for the Study of Lung Cancer (IASLC TNM). The Veterans Administration Lung Cancer Group (VALG) classification was used in small cell lung cancer (SCLC) patients. Tumor size and involvement was diagnosed with computed tomography (CT), magnetic resonance imaging (MRI) and bronchoscopy. Regional lymph node involvement was investigated with CT, positron emission tomography (PET-CT), mediastinoscopy and distant metastasis with ultrasonography, CT, scintigraphy, and PET-CT respectively.

The institutional review board (IRB) of GATA Haydarpaşa Training Hospital approved the study protocol.

Bronchoscopy

Fiber-optic bronchoscopy (FOB) was performed in all cases. BALF was taken from the bronchial segment where a tumor lesion was observed in the lung cancer group. In the control group, BALF was taken from an unaffected lung diagnosed using chest CT. BALF was aspirated before diagnostic procedures like bronchial biopsy, brushing etc.

Angiopoietin-2 measurement

Blood samples were taken for biochemical analysis simultaneously with bronchoscopy. Blood and BALF samples were centrifuged at 2000 rpm for 10 minutes and kept at −80°C for analysis. The Human Ang-2 Enzyme-linked immunosorbent assay (ELISA) Kit (RayBiotech, New York, USA) was used to measure Ang-2 levels in the serum and BALF samples.

Statistical analysis

All statistical analyses were performed using SPSS for Windows Version 14.0.0 (SPSS, Inc., Chicago, IL). Qualitative measurements were defined in reel numbers and percentages. Descriptive analyses were presented using means and standard deviation (SD) (min-max) for normally distributed

variables or median values and Interquartile Range (IQR) for the non-normal distributed and ordinal variables.

Nonparametric Pearson's correlation coefficient was conducted between BAL Ang-2 and serum Ang-2. Serum and BALF Ang-2 level and association of tumor stage, size or extent of the primary tumor (T), number of involved lymph nodes (N), metastases (M), histology of tumor as SCLC or NSCLC, and size of tumor in diameters, were evaluated. Kruskall-Wallis tests were used to compare median levels of serum and BALF Angiopoietin 2 levels among TNM classification. Statistical significance was at $P < 0.05$, two-tailed.

Results

Patient demographics

In the lung cancer patient group, 30 (85.7%) were male and five (14.3%) were female in gender. In the control group, 11 (61.1%) were male, and seven (38.9%) were female ($P = 0.04$). The mean age was 66 ± 9 (min:49–max:86) in the lung cancer group. In the control group, the mean age was 60 ± 17 (min:31–max:90) ($P = 0.93$).

There were 32 (91.4%) smokers in study group and 10 (55.6%) in the control group ($P = 0.004$). The median smoking amount was 45 (IQR:3–60) pack-years in study group versus 7.5 (IQR:0–31.25) pack-years in the control group ($P < 0.001$) (Table 1).

Disease characteristics

Twenty-nine of the lung cancer patients (82.8%) were diagnosed with NSCLC and six (17.2%) with SCLC. In the NSCLC group 16 (45.7%) had squamous cell carcinoma, eight (22.8%) had adenocarcinoma, one (2.85%) had neuroendocrine carcinoma, one (2.85%) had carcinoid tumor, and three (8.5%) had undifferentiated type lung cancer.

The majority of NSCLC cases (17 cases, 58.6%) were in advanced stages (Stage IIIB or IV). All of the SCLC cases had advanced disease. Endobronchial lesion was observed in 23 cases (65.7%). Twenty-three of the cases (65.7%) were diagnosed by bronchoscopy, 10 (28.5%) by transthoracic fine needle biopsy or tru-cut, and two (5.8%) with open lung biopsy.

Nineteen (54.29%) patients had metastasis. Five cases (27.7%) had bone metastasis, four cases (22.2%) liver, four cases (22.2%) opposite lung, two cases (11.1%) surrenal, two cases (11.1%) pleural fluid, two cases (11.1%) brain, one case (5.5%) pericardial fluid, one case had both liver and bone metastasis, and one had both opposite lung and bone.

The control group consisted of patients without lung cancer who underwent FOB for other respiratory reasons.

Ang-2 levels in serum and bronchoalveolar lavage fluids

Serum Ang-2 levels were significantly higher in cancer patients compared to the control group (median [IQR] serum Ang-2 [ng/mL]: lung cancer median: 2.42 [ng/mL]: [2.19–2.98], control 0.67 [0.31–1.10]; $P < 0.001$) (Fig 1a). BALF Ang-2 levels were significantly lower in patients with cancer compared to the control group (median [IQR] BAL Ang-2 [ng/mL]: lung cancer 0.41 [0.22–0.79], control 0.67 [0.46–1.03]; $P = 0.02$) (Fig 1b).

Serum Ang-2 levels in SCLC patients were significantly higher than NSCLC patients (serum median [IQR] Ang-2 [ng/mL], SCLC patients: 37.41 [20.05–58.70], NSCLC: 2.38 [2.12–2.86], $P < 0.001$). BALF Ang-2 levels were not statistically significant in patients with SCLC compared to NSCLC (median [IQR] BALF Ang-2 [ng/mL], SCLC patients: 0.79 [0.25–1.07], NSCLC: 0.38 [0.22–0.72], $P = 0.33$). Serum and BALF Ang-2 levels and histologic type of tumor in NSCLC were nonsignificant ($P = 0.36$).

When non-small cell lung cancer stages and Ang-2 levels were evaluated, we found that significantly higher amounts of serum Ang-2 levels were observed as the stage the tumor was increasing ($r = 0.52$, $P = 0.03$). No significant correlation was observed between BALF Ang-2 levels and NSCLC stages ($P = 0.793$, $r = 0.07$) (Table 2). Serum and BALF Ang-2 levels and the longest diameter of tumor, tumor size and involvement (T) and nodal involvement (N) were not correlated.

Table 1 Demographic features in patients with lung cancer and control group

	Study group	Control	P-value
n	35	18	
Age			0.93
Mean age (min, max)	66 ± 9 (49–86)	60 ± 17 (31–90)	
Sex			0.04
Male (%)	30 (85.7%)	11 (61.1%)	
Female (%)	5 (14.3%)	7 (38.9%)	
Smoking			0.004
Smokers (%)	32 (91.4%)	10 (55.6%)	
Nonsmokers (%)	3 (8.6%)	8 (44.4%)	

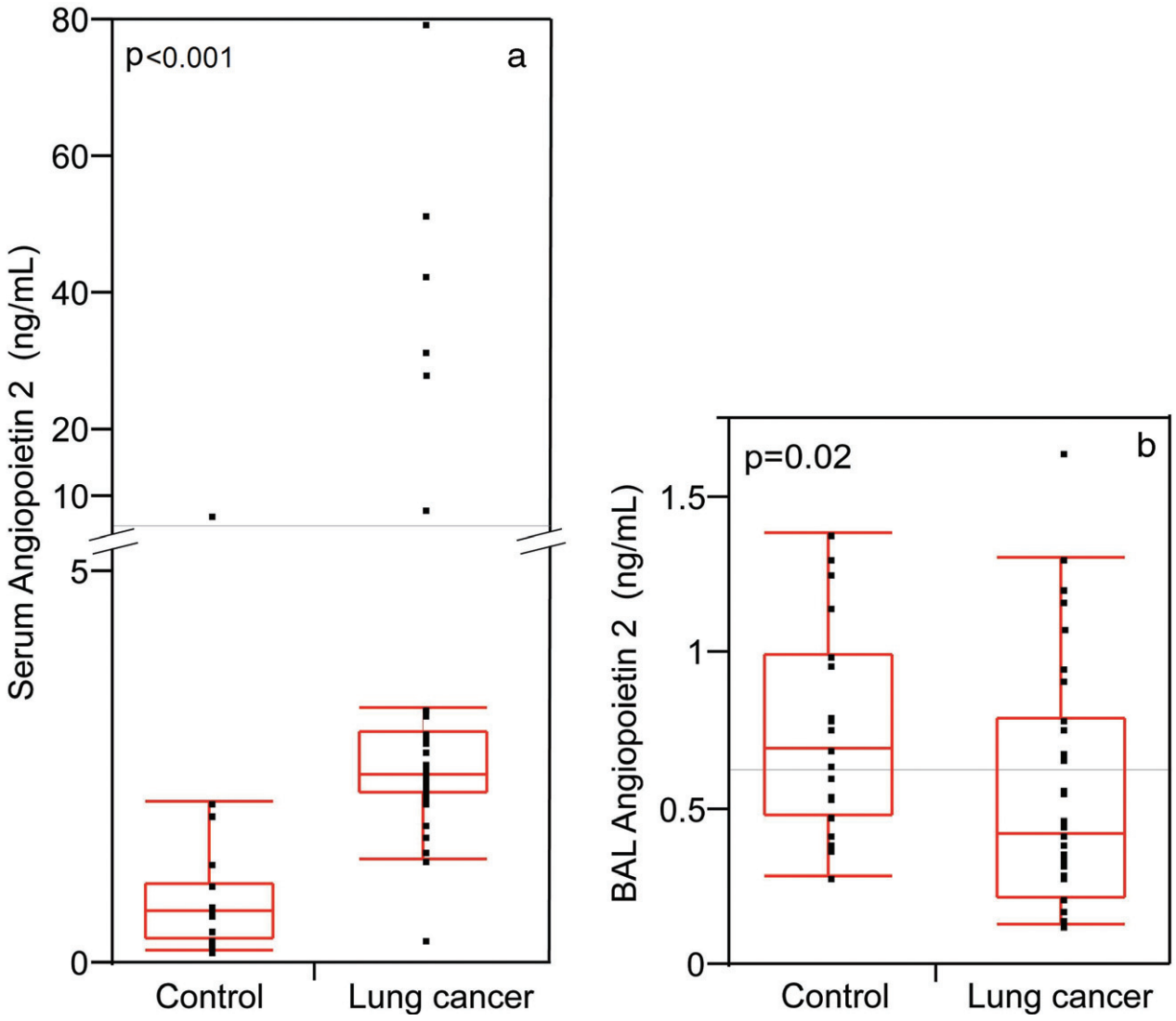

Figure 1 (**a**) Serum Angiopoietin 2 levels in the patients with lung cancer compared to control group. (**b**) Bronchial lavage fluids Angiopoietin 2 levels in the patients with lung cancer compared to control group.

Patients with distant metastasis (M1) in the NSCLC group had significantly higher serum Ang-2 levels than (M0) patients with no distant metastasis (Median [IQR] Serum Ang-2 [ng/mL], M1: 2.57 [2.38–2.87], M0: 2.22 [1.49–2.40], P = 0.01). As all patients in the SCLC group were in advanced stages, M0 and M1 differences were not evaluated. No correlation was observed between BALF Ang-2 levels and M1 (r = 0.11, P = 0.53).

Table 2 Angiopoietin-2 levels in serum and bronchoalveolar lavage in patients with non-small cell lung cancer according to tumor, node, metastasis (TNM) classifications

	Stage 1 (n = 1)	Stage 2 (n = 4)	Stage 3 (n = 11)	Stage 4 (n = 13)	p Value
Serum Ang-2 (ng/ml)†	1.32 (1.32–1.32)	1.80 (0.61–2.34)	2.27 (1.78–2.92)	2.57 (2.38–2.87)	0.03
BALF Ang-2 (ng/ml)‡	0.91 (0.91–0.91)	0.33 (0.17–0.66)	0.34 (0.22–0.65)	0.45 (0.19–0.67)	0.79

†Serum Angiopoietin-2 levels. ‡Bronchoalveolar lavage fluid Angiopoietin-2 levels.

Association of Ang-2 levels with disease and laboratory parameters

Serum Ang-2 levels were associated with serum sedimentation rate ($P = 0.02$, $r = 0.31$), urea ($r = 0.44$, $P < 0.001$), lactate dehydrogenase (LDH) ($r = 0.78$, $P < 0.001$), alkaline phosphatase (ALP) ($r = 0.60$, $P < 0.0001$) alanine aminotransferase (ALT) ($r = 0.38$, $P < 0.001$), and aspartate aminotransferase (AST) ($r = 0.56$, $P < 0.001$), CA 19-9 ($r = 0.45$, $P < 0.001$).

No association was observed between BALF Ang-2 levels and other laboratory parameters. When we evaluated the association of laboratory parameters and TNM characteristics, we only found a significant correlation with ESR and the longest diameter of tumor ($r = 0.20$, $P = 0.01$) and nodal involvement in all the subjects of the investigated group ($r = 0.30$, $P = 0.003$).

ESR in the cancer group was significantly higher compared to the control group (61.09 ± 33.69 vs. 37.05 ± 29.15, $P = 0.01$).

Discussion

Angiopoietins are members of the growth factor family, discovered within the last decade when VEGF family studies were performed. Their role on angiogenesis is not completely understood. Their effects on the steps of vascular maturation and stabilization have been shown in some studies.[4,12,13]

In our study, we measured serum and bronchial lavage levels of Ang-2 in lung cancer patients in the diagnosis period and compared these to non-cancerous patients. We tried to define the relation between Ang-2 levels and tumor characteristics.

Park *et al*. observed significantly high levels of serum Ang-2 in NSCLC patients, in contrast to healthy subjects. In their study, serum Ang-2 levels were in higher amounts related to patients in advanced stages and in patients with distant metastasis, but no correlation was observed in Ang-2 levels of SCLC patients and the control group.[5]

In our study we observed significantly higher levels of Ang-2 in the serum of SCLC and NSCLC patients. Similar to Park *et al*.'s findings, we observed an association between higher levels of Ang-2 and advanced stages of cancer in NSCLC patients. NSCLC patients with metastasis had significantly higher levels of Ang-2 than non-metastatic NSCLC patients. There was no correlation between the serum Ang-2 level and the diameter of tumor size. Although this situation seems confusing, tumoral enlargement doesn't only depend on angiogenesis, but a multifactorial period. In this period, tumoral cell growth kinetics, heterogeneity of the tumor and tumor angiogenesis play a role. According to all of these factors Ang-2, a marker for angiogenesis, may not reflect tumoral growth by itself. The extension marker of a tumor is stage. Angiogenesis is a step, which provides for the extension of a tumor. Thus, the association of Ang-2 levels with advanced stage and metastasis is meaningful.

In contrast to the findings of Park *et al*. we observed significantly higher levels of serum Ang-2 in SCLC than in both the control and NSCLC. We were unable to find any data in the literature regarding high levels of serum Ang-2 in SCLC patients. The high Ang-2 levels of SCLC patients in our study can be related to high metastasis potential, poor prognosis and shorter doubling time characteristics of this type of cancer.

Ang-2 secretion has been shown in various body fluids. Kalomenidis *et al*. reported significantly higher levels of Ang-2 in exudative pleural fluid than in transudative fluids.[14] In this study, a positive correlation was observed between pleural fluid Ang-2 levels and inflammation markers. Kanazawa *et al*. observed high Ang-2 levels in the sputum of asthma patients and revealed the relationship between Ang-2 and the vascular permeability index. In this study, sputum Ang-2 levels were diminished after montelukast treatment.[15]

There are existing studies in literature exploring tissue, serum, sputum, and pleural fluid Ang-2 levels. Our study is the first study measuring bronchial fluid Ang-2 levels in lung cancer patients. We found a study about IPF and bronchial Ang-2 levels. Margaritopoulos *et al*. reported an increased expression of Ang-2 protein in the BALF of patients with IPF.[16]

Ohta *et al*. studied VEGF levels in BALF of NSCLC patients and reported a relationship between tumor stage and histological type. In this study, the levels of VEGF in bronchial lavage levels were significantly higher than VEGF serum levels of the patient and the control group and the levels of bronchial lavage VEGF. They reported no correlation between bronchial lavage VEGF levels and tumor size, histological type, and stage of tumor, but they reported a negative correlation between VEGF levels and nodal involvement and metastasis.[17]

We found significantly higher levels of bronchial fluid Ang-2 levels in the control group compared to the patient group. There was no correlation between bronchial fluid Ang-2 levels and the stage of tumor, histologic type, and distant metastasis. In some studies a high Ang-2 secretion in tumoral tissue of NSCLC patients has been reported.[18,19]

ESR can increase in infectious, inflammatory, tissue destructive diseases and tumors. When the inflammation is limited, this increase may not be observed. Zhang *et al*. reported higher ESR in lung cancer patients than those found in other chronic lung diseases like COPD, asthma etc. No correlation was observed between ESR and the stage and type of tumor.[20] In our study, ESR in the cancer group was significantly higher compared to the control group. There was a positive correlation between the serum Ang-2 level and ESR. There was also a positive correlation between ESR and nodal involvement (N) and the largest diameter of the tumor.

LDH is secreted from the heart, liver, muscle, erythrocyte, and kidneys in high amounts, but is also secreted in low amounts from the lungs and brain. Ozdemir *et al*. found that high levels of LDH in NSCLC patients were associated with shorter survival rates than the controls (6.5 months vs. 12.9 months).[21] We found a correlation between serum Ang-2 and LDH levels in our study. Kalomenidis *et al*. reported a correlation between pleural fluid Ang-2 and LDH levels.[14] O'Connell et al. found that NSCLC patients with high LDH levels had a low survival rate which related a to low chemotherapy and remission success rates.[22] Park *et al*. reported significantly less survival rates in lung cancer patients with high serum Ang-2 levels, but the reason for this has not yet been demonstrated.[5] The mechanisms for Ang-2 and LDH correlations in serum and body fluids still need to be investigated.

Serum Ang-2 levels were significantly associated with ALT, AST and ALP levels in our study. Salcedo *et al*. reported that serum Ang-2 levels decreased in chronic hepatitis C patients after antiviral treatment, thus, serum Ang-2 levels were associated with a decrease in the serum ALT level.[23] LDH, ALT, AST and ALP levels generally increase in diseases with tissue destruction. It is well known that lung cancer causes tissue destruction. Interestingly, in our study we observed parallelism between tissue destruction and angiogenesis.

We only found a statistically significant correlation between serum Ang-2 levels and serum CA 19-9 levels; other tumor markers (CEA, CA 125, AFP, CA 15-5) had no significant correlation. CA 19-9 is a well-known tumor marker used in the diagnosis and follow up of pancreas cancer patients. There is no routine usage in lung cancer patients at this point. In the literature, we found studies reporting increased levels of CA 19-9 in both benign lung diseases (pulmonary fibrosis, bronchiectasis, idiopathic interstitial pneumonia etc.) and lung cancer.[24,25] Oshio *et al*. reported 59% of patients with malign epithelial tumor of the lung had increased levels of CA 19-9.[26]

CA 19-9, also secreted from bronchial epithelium, has been found in the BALFs of patients with lung fibrosis. In the literature, we were unable to find any study reporting a correlation between Ang-2 and CA 19-9 levels in lung cancer patients. However, a decrease in serum CA 19-9 levels with anti-VEGF treatment in patients with metastatic colon cancer has been reported. In this study there is no comment about CA 19-9 levels and angiogenesis, CA 19-9 has been reported as an important tumor marker after anti-VEGF treatment in follow up.[27]

Since Ang-2 is a VEGF dependent molecule, we had thought that there might be an indirect connection between CA 19-9 and Ang-2. We therefore suggest specific studies be conducted in future to investigate the correlation between these molecules.

Conclusion

Angiogenesis is necessary for expansion, maturation, and metastasis of tumor tissue. Angiopoietin 2, expressed from lung tissue, has an important role in tumor angiogenesis and is one of the molecules studied in target therapy. In our study, we measured serum and bronchial lavage fluid Ang-2 levels in lung cancer patients and evaluated its association between stage, type of tumor, clinical and biochemical parameters. We found that serum Ang-2 levels were significantly higher in lung cancer patients and positive correlations were observed between serum Ang-2, tumor stage, and metastasis. Biochemical parameters, such as sedimentation, LDH, AST, ALT, urea and ALP were correlated with serum Ang-2. Mechanisms associated with angiogenesis, tumor enlargement, metastasis, and destruction of lung tissue and the role of Ang-2 in this process still need to be investigated.

Disclosure

No authors report any conflict of interest.

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