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Chaperonin (HSP60) and annexin-2 are candidate biomarkers for non-small cell lung carcinoma

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Abstract

Background: Lung cancer is responsible of 12.4% and 17.6% of all newly diagnosed cancer cases and mortality due to cancer, respectively, and 5-year survival rate despite all improved treatment options is 15%. This survival rate reaches 66% in the Stage 1 and surgically treated patients. Early diagnosis which could not be definitely and commonly achieved yet is extremely critical in obtaining high survival rate in this disease. For this reason; proteomic differences were evaluated using matrix assisted laser desorption ionization (MALDI) mass spectrometry in the subgroups of lung adenocarcinoma and squamous cell carcinoma.

Methods: Fresh tissue samples of 36 malignant cases involving 83.3% (n=30) men and 16.7% (n=6) women patients were distributed into 2 groups as early and end stage lung cancer and each group were composed of subgroups including 18 squamous cell carcinoma (9 early stage cases, 9 end stage cases) and 18 adenocarcinoma cases (9 early stage cases, 9 end stage cases). The fresh tissues obtained from the tumoral and matched normal sites after surgical intervention. The differences in protein expression levels were determined by comparing proteomic changes in each patient.

Results: In the subgroups of advanced stage adenocarcinoma; tumoral tissue revealed differences in expression of 2 proteins compared with normal parenchymal tissue. Of those; difference in protein expression in heat shock protein 60 (HSP60) was found statistically significant ($P=0.0001$). Subgroups of early and advanced stage squamos cell carcinoma have differed in certain 20 protein expression of normal tissue and diseased squamos cell carcinoma. Of those, increased protein expression level of only annexin-2 protein was found statistically significant ($P=0.002$). No significant difference was detected in early and advanced stage protein expressions of the tumoral tissues in the subgroups of adenocarcinoma and squamous cell carcinoma.

Conclusions: We conclude that with respect to early diagnosis of lung cancer that HSP60 and annexin-2 proteins are the important biomarkers in the subgroups of adenocarcinoma and squamous cell carcinoma. We also consider that these 2 proteins are molecules which may provide critical contribution in evaluation of prognosis, metastatic potential, response to treatment, and in establishment of differential diagnosis between adenocarcinoma and squamous cell carcinoma.

Abbreviations: 2D-GE = two-dimensinal gel-electrophoresis, ELISA = enzyme-linked immuno assay, HSP60 = heat shock protein 60, IHC = immunohistochemistry, MALDI = matrix assisted laser desorption ionization.

Keywords: annexin-2, cancer screening, chaperonin, lung cancer, protemic

1. Introduction

Lung cancer is the most common type of cancer with high mortality rate. It constitutes 12.4% and 17.6% of all newly diagnosed cancer cases and cancer related mortality, respectively, therefore is one of the most important public health problems.[1,2] In the treatment of lung cancer, one or several treatment options, including surgery, chemotherapy, or radiotherapy, are applied. Personalized approaches can be considered according to patient's general condition, comorbid diseases, stage, and pathologic classification of the lung cancer. Despite all the advanced treatment options in lung cancer, the 5-year-long survival is 15%.[3] This survival is 66% in patients who were in the first stage and treated with surgery.[4] However, patients who are at a stage that can be operated, constitute 15% to 20% of all diagnosed lung cancer cases. Increasing the rate of early diagnosis in lung cancer and patients who have a chance to receive surgical treatment within the diagnosed patient population is very important in order to increase the survival rate.

The screening is valuable due to high mortality and morbidity rates, and high frequency of the disease, as well as existence of long developmental time period before the disease is diagnosed, and better treatment outcomes in the early stage cases.[5,6]

Up to date, several important studies have been performed and a valuable number of approaches have been evaluated for early diagnosis of lung cancer.[7–18] Direct chest radiography and chest computed tomography, which are radiological imaging based techniques, were generally used in these studies. The protocols, with a screening purpose, were established by combining data from radiological imaging with the data obtained from studies performed on materials taken with bronchoscopic techniques.

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The desired results could not be achieved in these studies. Besides radiological and bronchoscopic methods, new approaches for lung cancer screening evolved via technological and genetic developments. With these new approaches, lung cancer screening can be done directly in population with risk as well as in some studies, radiological screening was aimed to be done in this population by identifying new risk factors.[19–22] Early diagnosis which is not 100% successful yet, is extremely critical in obtaining high survival rates for lung cancer. Assessing proteomic differences between normal and tumor tissues of patients, may help to discover new markers for early diagnosis. Therefore, in this study, we evaluated the proteomic differences between tumor and normal tissues of lung cancer patients by using matrix assisted laser desorption ionization (MALDI) and analyzed the data under the subgroups of lung adenocarcinoma and squamous cell carcinoma.

2. Materials and methods

2.1. Tissue samples

For this study, remaining tissue samples of 153 lung cancer cases were collected between January 2013 and September 2013 from pathological biopsy samples that are taken ordinarily for pathological evaluations (Fig. 1). Chemotherapy, radiotherapy, and patients with inflammatory disease that will make degradation in the protein structure and may affect our conclusion were excluded from the study. Fresh tissue samples of 36 malignant cases involving 83.3% (n = 30) men and 16.7% (n = 6) women patients were distributed into 2 groups as early and end stage lung cancer and each group were composed of subgroups including 18 squamous cell carcinoma (9 early stage cases, 9 end stage cases) and 18 adenocarcinoma cases (9 early stage cases, 9 end stage cases). Of the malignant cases, 41.7%, 7.3%, 44.4%, and 5.6% were at Stage 1, Stage 2, Stage 3, and Stage 4, respectively. 50.0% (n = 18) and 50.0% (n = 18) were classified as early and final stage cases, respectively. The fresh tissues were obtained from the tumor and its corresponding normal tissue after surgical intervention (Figs. 2 and 3). The changes in protein expression levels were analyzed by comparing the total protein profile of the tumor tissue of each patient with their corresponding normal tissue.

2.2. Protein isolation and 2-dimensional gel-electrophoresis (2D-GE):

The tissue samples were homogenized by using MediMachine and total protein isolation from the homogenized samples were isolated by lysing cells in lysis buffer (7 mol urea, 2 mol thiourea, 4% 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate solvent, 30 mM Tris–HCl, pH 8.5) and subsequent sonication (65% power, 20 seconds × 3 times). The concentrations of the protein samples were quantified by using Bradford

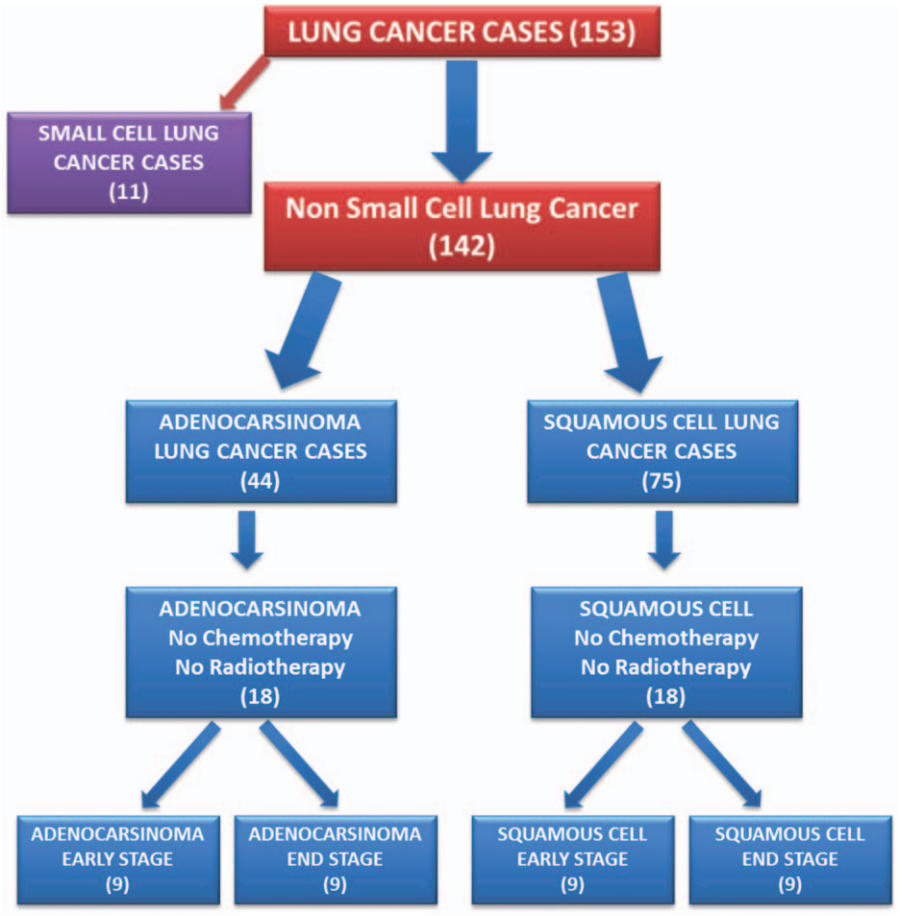

Figure 1. Tree diagram on the distribution of the patients.

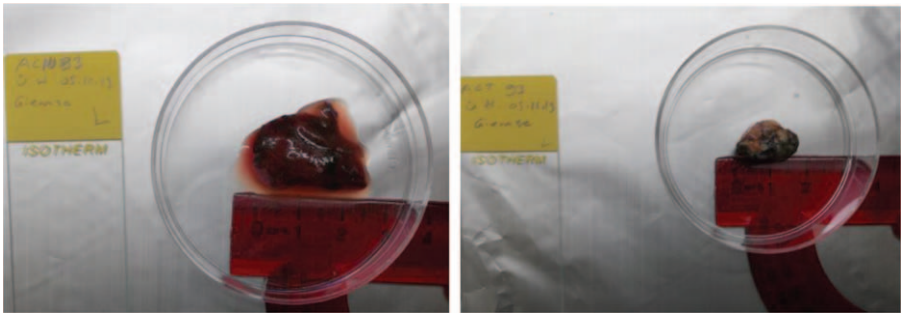

Figure 2. Adenokarcinoma macroscopic view.

assay. Two hundred fifty micrograms of total protein was used for 2-dimensional analysis. Isoelectric point separation was performed on 3 to 10 pH strips by using Protean i12 IsoElectric Focusing system (Bio-Rad) and subsequent gel electrophoresis was performed by using 12% Sodyum Dodesil Sülfat Gel. PDQuest Advanced 2-Dimensinal Analysis Software (BIO-RAD) was used for identification of differentially expressed proteins (Figs. 4 and 5).

2.3. MALDI analysis

Protein spots that were identified to be expressed differently between tumor and normal samples, were cut in dimensions of 2 to 4 mm diameter and 1 mm thickness and were placed into 1.5 mL eppendorf tubes. In-gel digestion protocol was performed the proteins were trypsin-digested at 37°C for 16 hours.

For the matrix, α-Cyano-4-Hydroxycinnamic acid, 2,5-dihydroxylbenzoic acid, or Sinapinic acid was used. After the matrixes were dissolved in the proper solution, they were mixed with the samples, and each mixture was placed in gold-plated or stainless steel MALDI target plate and left to dry at laboratory temperature. MALDI analysis results were analyzed in the Mascot search engine (v.2.2) (Matrix Science). Density differences in the protein profiles obtained in MALDI analysis results were assessed with Decodon Advanced 2-Dimensinal Gel Image Analysis system (GmbH).

2.4. Statistical analysis

Number Cruncher Statistical System 2007 and Power Analysis and Sample Size 2008 Statistical Software (NSCC, LLC) (UT) program were used for statistical analyses. In the course of assessing the study data, besides descriptive statistical methods (mean, standard deviation, median, frequency, percentage, minimum, maximum) Kruskal–Wallis test was used for the comparison of quantitative data. For the comparison of qualitative data, Fisher–Freeman–Halton test was used. Significance was assessed at $P < 0.01$ and $P < 0.05$ levels.

Non-parametric Wilcoxon and Chi-square tests were used in the comparison of patients' own normal tissues with the tumor tissues and dependent 2-group comparisons.

Our study was approved by Dokuz Eylül University Clinical Research Ethics Committee.

3. Results

The study was conducted in our Clinic with a total of 36 malignant cases, involving 83.3% (n = 30) men and 16.7% (n = 6) women. The age of patients ranged between 51 and 79 years, the mean was 61.86 ± 7.59 years. Smoking habits of the patients ranged between 0 and 50 cigarettes-years, the mean was 30.81 ± 11.34 and the median was 31 cigrattes-years. When smoking habits of the cases were examined, 1 case (2.8%) out of 36 cases was not smoking while 35 cases (97.2%) were smoking. When operation types of the cases were examined; in 8.3% of cases (n = 3) Segmentectomy, in 58.4% of cases (n = 21) Lobectomy, in 19.4% of cases (n = 7) Pneumonectomy, in 2.8% of cases (n = 1) Wedge, and in 11.1% of cases (n = 4) Bilobectomy was performed. Of the patients who were diagnosed with adenocarcinoma and were in early stage, 77.8% (n = 7) were at Stage 1 while 22.2% (n = 2) of them were at Stage 2. Of the patients who were diagnosed with adenocarcinoma and were in end stage,

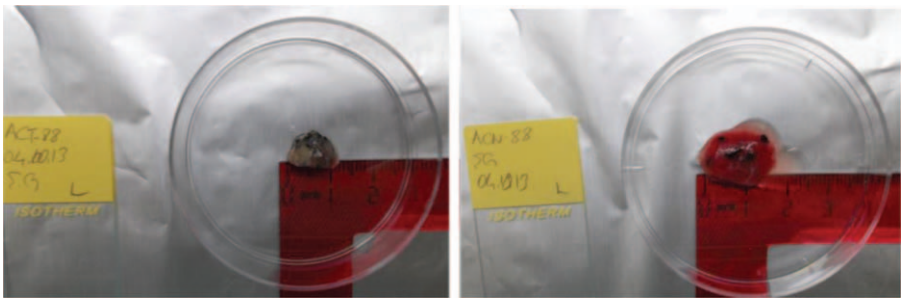

Figure 3. Squamous cell carsinoma macroscopic view.

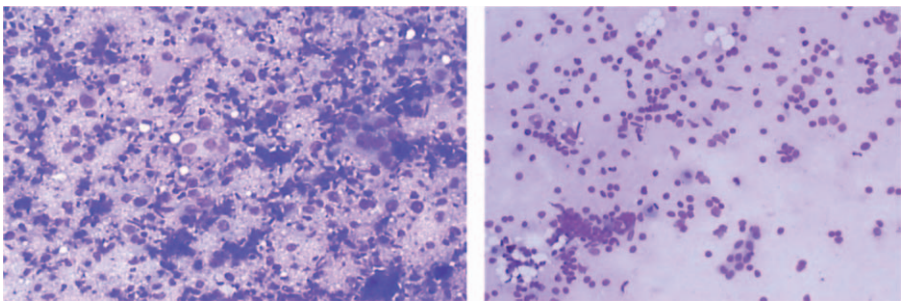

Figure 4. Adenokarcinoma microscopy view.

77.8% (n=7) were at Stage 3 whereas 22.2% (n=2) were at Stage 4. Of the patients who were diagnosed with squamous and were in early stage, 88.9% (n=8) were at Stage 1 whereas 11.1% (n=1) was at Stage 2. Of the patients who were diagnosed with squamous and were in end stage, 100% (n=9) were at Stage 3.

According to 2D GE and MALDI mass spectrometry results, in adenocarcinoma tumor and normal parenchymal tissue, 2 proteins, Beta-fibrinogen precursor, and HSP60, were found to be differentially expressed. Only the expression level of the HSP60 protein out of these 2 proteins, was significantly different in early and end stage tumor tissues compared with its normal parenchyma ($P=0.0001$) (Fig. 6, Table 1). In the course of comparing the differences among protein profiles of the early stage and end stage tumor tissues, we did not observe any statistically significant difference in early and end stage subgroups ($P=0.113$). Twenty proteins were found to be expressed differentially between squamous cell carcinoma tumor tissue and normal parenchymal tissue. Among these proteins, only annexin-2 protein expression level was found to increase significantly in early and end stage tumor tissues ($P=0.002$). In squamous cell carcinoma subgroup, comparison of early stage and end stage tumor tissue protein profiles, did not result in statistically significant difference ($P=0.181$) (Fig. 7, Table 2).

4. Discussion and conclusion

In cancer treatment, the most important step that comes after the prevention approach is early diagnosis and early treatment. In the clinical practice of lung cancer screening, diagnosis and evaluation of response to treatment are done with radiological approaches. However, new biomarkers might be useful to be used besides the radiological examinations.

Mostly enzyme-linked immuno assay (ELISA) technology has been used in cancer studies till proteomic concept was introduced in 1994. In our study, MALDI mass spectrometer which has been shown as superior to ELISA and other methods was used on the subject of assessing the proteomic differences in tumor and normal tissues. MALDI spectrometry has higher sensitivity and specificity on detecting the amino acid sequences of proteins, even on small amount of samples.[23] Our study was planned with the latest approach in this respect, and we had achieved new results compared with other studies in the literature. Limited number of MALDI spectrometry studies exists on lung cancer. These studies are performed on serum samples. Dynamic nature of the serum samples, the complex structures that they harbor, standardization problems in the serum collection, excessiveness of the protease activity in serum had been limiting the studies done on these samples. As in the case of our study, studying the proteomic differences in direct tissues will provide better knowledge and further analysis on serum samples in the light of this knowledge, is believed to provide more meaningful data. Study made on few direct tumor tissues was performed on the paraffin blocks prepared within formol by the pathology, and there is a probability of the protein structures being denatured.[24] These approaches are the most important limitation of these studies to demonstrate the accurate proteomic structure. Most of the proteins described in studies done previously are molecules related to the dynamic processes such as inflammation, cell differentiation, proliferation, and apoptosis.[25] In this regard, preserving the protein structures with a correct approach, without disruption, as in our study, is very important in terms of the accurateness of the results that will be obtained. Fresh tissues that were taken after surgery were frozen in a quick and proper way in our study. We believe that this is our most important superiority over the studies in the literature. Also while the differences in tumor tissues are worked on, as we have used the same normal parenchyma samples of the cases as a control group is valuable from the point of showing the proteins with increased expression during tumorigenesis stage and to minimize the mistakes which may be encountered while creating a control group.

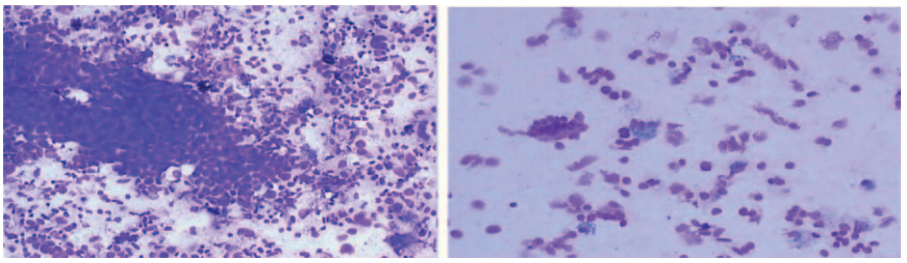

Figure 5. Squamous cell carsinoma microscopy view.

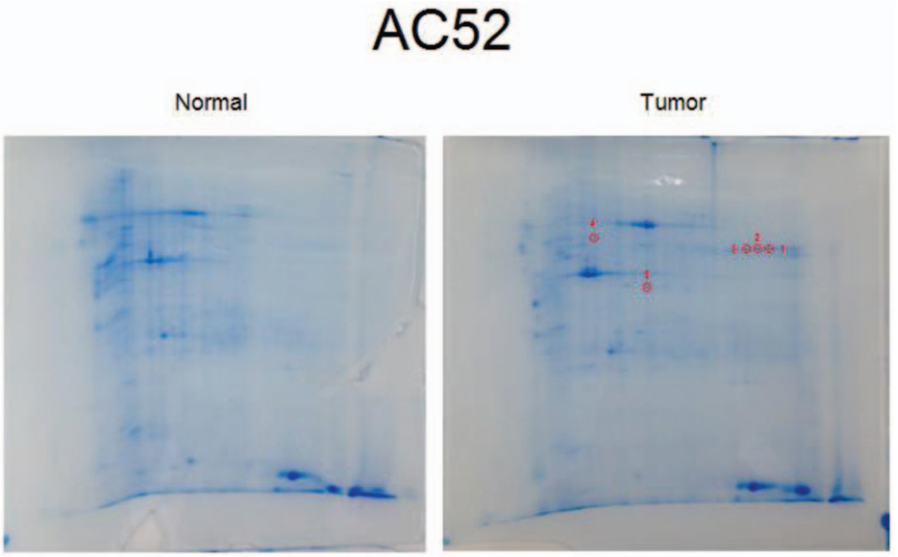

Figure 6. Adenokarcinoma spot samples in MALDI spectrometry. MALDI = matrix assisted laser desorption ionization.

By using the proteomic method for determining the proteins expressed different in normal and cancer tissue it was tried to reveal the possible biomarker candidates. Of course, to be able to say biomarker, it may be presented to the science world as a biomarker with different expressions of proteins to be shown with immunohistochemistry (IHC) determined in tissue samples including also different subgroups of lung cancer in different stages of larger patient number serials. However, the powerful part of our study is that it was shown that different proteins may take place in lung cancer by using a method like proteomic, which is not performed at each center.

In our study, for adenocarcinoma subgroup we found significant differences in 2 proteins between tumor and normal parenchyma, while for squamous cell carcinoma 20 proteins showed difference. Statistical evaluation with the Chi-square test showed difference in chaperonin (HSP60) protein ($P = 0.0001$) between early and end stage adenocarcinoma case group compared with its own normal parenchyma. HSP60 protein can be found in cell in the mitochondria, in the cytoplasm, and in the intercellular matrix. By interacting with the mitochondrial proteins, it enables them to be in the correct spatial configuration. In addition, it has been shown in the literature that it supports the tumor cells to grow and to survive. It was found that it inhibits the death of tumor cells by showing cyto-protective effect during apoptosis.[26] Xu et al[27] showed that expression increase in the HSP60 protein, which was evaluated with immunohistochemical staining of HSP60 protein in 103 cases of lung adenocarcinoma, was an independent prognostic factor on the subject of disease-free survival. It was proposed that HSP60 can be an important biomarker in clinical practice, in the cases diagnosed with lung adenocarcinoma, and on determination of prognosis. At this stage it is too early to make an assessment on the prognosis of the patients in our study. To this end, the results that will be obtained after long-term follow-up of our patients should be evaluated. Moreover, in this literature study, it was specified that in the early diagnosis of the lung cancer the HSP60 protein might be an important biomarker, and it supports the results of our study in this regard. Besides lung cancer, HSP60 protein was found to be an important biomarker in terms of prognosis and treatment response monitoring in serious ovarian carcinomas. In the study of Hjerpe et al[28] on ovarian carcinoma cases, it was determined that in the cases that express HSP60 protein, the mean survival was 31 months, while this was 60 months for the cases that do not express this protein. Furthermore, one of the important results of this study was that chemotherapy treatments that target the HSP60 protein might increase the survival. The results stated for ovarian cancer support the elevation found in the lung adenocarcinoma subgroup in our study, and suggest that the same results may be applicable in lung cancer.

In the squamous cell carcinoma lung cancer subgroup, we found that only 1 protein out of 20 proteins that are differentially expressed, was significant ($P = 0.002$). We determined the

Table 1

Poteomic Differences in Squamous Cell Lung Cancer Subgroup.

	P
1. Manganese superoxide dismutase	$P > 0.05$
2. Annexin A2 isoform 2	$P = 0.002$
3. Alpha-enolase isoform 1	$P > 0.05$
4. Muscle-specific enolase	$P > 0.05$
5. Endoplasmin precursor	$P > 0.05$
6. Heat shock protein gp96 precursor	$P > 0.05$
7. Tumor rejection antigen (gp96) 1	$P > 0.05$
8. Actin, cytoplasmic 1	$P > 0.05$
9. Chain A, human serum albumin in a complex with myristic acid and tri-iodobenzoic acid	$P > 0.05$
10. Chain A, crystal structure of lipid-free human apolipoprotein A-I	$P > 0.05$
11. Vimentin	$P > 0.05$
12. Carbonic anhydrase 1	$P > 0.05$
13. Beta-globin	$P > 0.05$
14. Myosin light chain 3	$P > 0.05$
15. Actin, alpha skeletal muscle	$P > 0.05$
16. Beta globin chain variant	$P > 0.05$
17. Phospholipase C-alpha	$P > 0.05$
18. 60 kDa heat shock protein, mitochondrial	$P > 0.05$
19. Phospholipase C-alpha	$P > 0.05$
20. 2-phosphopyruvate-hydratase alpha-enolase	$P > 0.05$

gp96 = heat shock protein 90 kDa beta member 1.

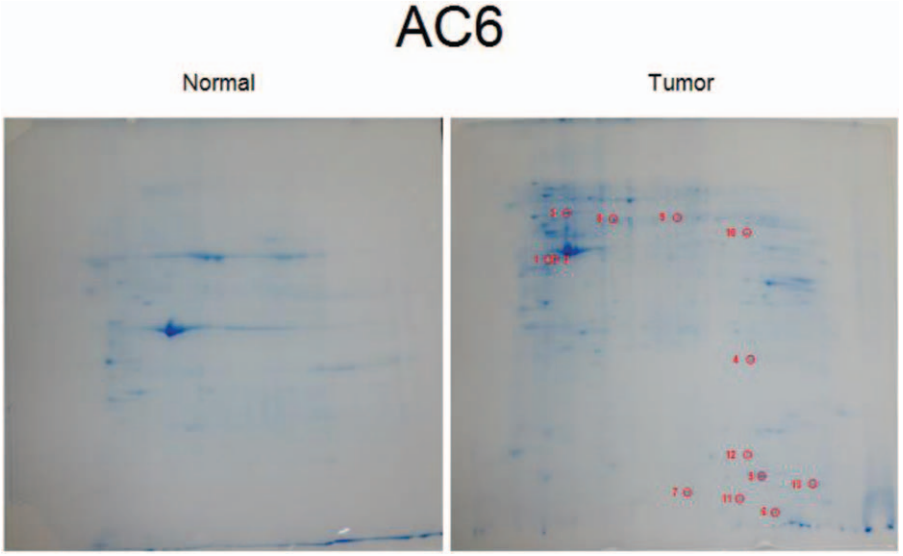

Figure 7. Squamous cell carsinoma spot samples in MALDI Spectrometry. MALDI = matrix assisted laser desorption ionization.

annexin a2 isoform-2 protein as being increased in the cases of early and end stages of squamous cell carcinomas. This protein which is also called as annexin-2 is within the group of calcium-dependent phospholipid binding proteins. It functions in cell growth and signal transduction pathways. In connection with cytoskeleton, it shows effects on formation of endocytosis, exocytosis, fibrinolysis and ion channels, and on cell motility. Annexin-2 is mainly a protein pleiotropic, and its function varies according to its location and time of expression within the cells.[29] It is considered that annexin-2 protein plays a key role in the tumorigenesis. It was shown to be associated with cell proliferation, apoptosis, morphology control, transcriptional regulations, invasion, metastasis, and angiogenesis.[30–32] According to the study conducted by Wang et al,[33] annexin-2 protein plays a part in regulating the surfactant secretion made from the lung. In a study by Qi et al[34], it was shown that, in the process of malignancy in esophagus cancer, a decrease in annexin-2 protein expression was identified, considering premalignant lesions that do not present sign of becoming malignant. Moreover, a decrease has been found in annexin-2 rates in metastasis-detected cases which were diagnosed with prostate cancer and osteosarcoma. In this cancer types it was found that unlike the decrease in the annexin-2 protein expression, annexin-2 protein levels were increased in lung carcinomas, primary neuroectodermal tumor, liver cancers, colorectal cancers, gastric cancers, pancreatic cancers, acute promyelocytic leukemia, and lip squamous cell carcinoma. Considering the literature, the effect of annexin-2 protein on different tumors displays tumor- and tissue-specific features as a form of reflecting the characteristics of pleiotropic nature as well. As a result, increase in expression was detected in cases in early and end stages in the annexin protein, which is consistent with the literature. The results we obtained in our study with regard to annexin-2 protein and their compliance with the literature indicate that this protein can be an important biomarker in early diagnosis for lung squamous cell carcinoma subgroup. It was indicated that with this protein, besides the studies that can be done for early diagnosis, in the literature potential of performing metastasis in lung cancer can be a biomarker that can be used in order to obtain information on the prognosis of the patients.[35] Additionally, it also has the potential of being a target molecule for new chemotherapy treatments, after the role in the carcinogenesis stage was presented in detail.

To have knowledge about cancer diagnosis and planning of treatment, prognosis, and also the selection of treatment it is necessary to have tissue samples from the patients as it is necessary to certainly perform staging and the other studies at tissue level. In this sense, working in tissue samples to be already taken for diagnosis and staging shall not require an additional invasive process for working on these proteins. Also, as we have shown the difference at tissue level with proteomic study and it is required to verify these proteins in tissues with IHC method in order to be a biomarker and reveal the relation of prognosis, treatment response or metastasis with clinic findings. It is required to examine whether the proteins we have revealed with this study shall be a marker or not by working on the protein levels also in the body samples obtained with non-invasive methods like blood to gain importance in order to be a biomarker.[28,29,34,35] This study is planning the examination of importance of the proteins we have revealed with our new projects in order to be a biomarker.

In the light of these results, with respect to early diagnosis of lung cancer, we conclude that, HSP60 and annexin-2 proteins can be important candidate markers in the subgroups of adenocarcinoma and squamous cell carcinoma, respectively. We also consider that these 2 proteins may provide critical contribution in evaluation of prognosis, metastatic potential, response to treatment, and in establishment of differential diagnosis between adenocarcinoma and squamous cell carcinoma.

Table 2

Poteomic Differences in Adenocarcinoma Lung Cancer Subgroup.

	P
1. Beta-fibrinogen precursor, partial	$P > 0.05$
2. Chaperonin (HSP60)	$P = 0.0001$

HSP60 = Chaperonin, heat shock protein 60.

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