

Circulating and Local Nuclear Expression of Survivin and Fibulin-3 Genes in Discriminating Benign From Malignant Respiratory Diseases: Correlation Analysis

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Abstract

Background: Survivin is a common member of the inhibitors of the apoptosis protein (IAP) family and promoter of cell proliferation. Fibulin-3 is a matrix glycoprotein with a potential for tumor suppression or propagation. The aim of this study was to validate the expression levels of survivin and fibulin-3 in benign and malignant respiratory diseases.

Methods: This case-control study included 219 patients categorized into 5 groups. Group A included 63 patients with lung cancer, group B included 63 patients with various benign lung diseases, group D included 45 patients with malignant pleural mesothelioma (MPM) and group E included 48 patients with various benign pleural diseases. Group C included 60 healthy individuals (control group). Serum survivin and fibulin-3 levels were measured using ELISA assay kits, while their nuclear expression in the lung and pleura was assessed using western blot analysis. Data entry and data analysis are done using SPSS version 19. The Medcalc Program was used to calculate sensitivity, specificity and positive and negative predictive values with calculation of the AUC (95% CI).

Results: The overall results showed significantly higher survivin serum levels with significantly lower fibulin-3 levels among group A when compared with both patients in group B and the controls ($p < 0.001$ for all). There were statistically significant higher serum levels of survivin and fibulin-3 among group D when compared with both patients in group E and the controls ($p < 0.001$ for all). These findings were consistent with nuclear survivin and fibulin-3 expression levels. Fibulin-3 was more valid than survivin in discriminating lung cancer from MPM ($p \leq 0.05$).

Conclusions: Survivin and fibulin-3 could be helpful diagnostic markers for lung and pleural cancers. Fibulin-3 was more specific in differentiating lung cancer from MPM.

Trial registration: ClinicalTrials.gov Identifier: NCT04413292:
<https://clinicaltrials.gov/ct2/show/NCT04413292>, retrospectively registered.

Introduction

It is widely known that pathological apoptosis inhibition during homeostasis plays a significant role in the growth of cancer, progression and therapy resistance (1). Survivin is a common member of the inhibitor of the apoptosis protein (IAP) family, formed of 142-amino-acid with a molecular weight 16.5-kDa. It is encoded by a single-copy gene located on the human chromosome 17q25, with a dual role in cell proliferation and apoptosis prevention (2–4). Survivin is expressed in the nucleus and/or cytoplasm of various malignant tumor cells. In the cytoplasm, survivin has an anti-apoptotic effect however, in the nucleus; it regulates cell proliferation (5,6).

Increased expression of fibulin-3 was found to inhibit TGF- β -induced epithelial-mesenchymal transition (EMT) and endothelial permeability in breast cancer as well as cell morphology, proliferation, encroachment, adhesion and migration (7,8), whereas the loss of expression / function of fibulin-3

encouraged these TGF- β -mediated effects (9). Fibulin-3 upregulation has suppressed the invasion and migration of lung adenocarcinoma cells and suppressed the expression of N-cadherin and Snail, considered to be EMT activators (10). Fibulin-3 has also been identified to suppress extracellular signal-regulated kinase, which results in the inhibition of Wnt / β -catenin signaling (11), as well as the inhibition of the upstream modifiers of glycogen synthase kinase 3 β (GSK3 β), such as insulin-like growth factor-1 receptor and phosphoinositide 3-kinase (12). Thus, fibulin-3 appears to have the ability to act as either tumor suppressor or oncogene depending on the cellular context (13).

Since studies concerning the diagnostic efficacies of both survivin and fibulin-3 in respiratory malignancies are controversial, this study was conducted to analyze the local and circulating expression levels of both biomarkers in various benign and malignant lung and pleural diseases and to validate their utility in diagnosing and discriminating malignant from benign lesions that affect the respiratory tract.

Materials And Methods

Study design and participants

The current case-control study included 219 patients of both sexes, with recently diagnosed benign and malignant respiratory diseases recruited from Cardio-Thoracic Surgery and Oncology Departments, Qena University Hospitals, South Valley University, Egypt. The included patients were categorized into 4 groups. Group A included 63 patients with lung cancer, group B included 63 patients with various benign lung diseases, group D included 45 patients with malignant pleural mesothelioma (MPM) and group E included 48 patients with various benign pleural diseases. In addition, 60 age- and sex-matched unrelated healthy subjects served as the control group (group C). Patients with renal failure, hepatic failure, severe cardiopulmonary compromise, coagulopathy or hemodynamically unstable were excluded. The duration of the study was two years, from January 1st, 2017 to December 30th, 2019.

Data collection

Full history and thorough clinical examinations were taken for every included patient. Additionally, radiological assessments by plain chest X-rays and CT, and routine laboratory investigations [serum lactate dehydrogenase (LDH), total protein, albumin, liver and kidney functions, ESR and CBC] were performed. Multiple lung biopsies were taken and sent for histopathological examination when indicated. Thoracentesis was done for cases with proven pleural effusion. Thoracoscopy was done for patients with indecisive cytology or pleural fluid analysis, and multiple pleural biopsies were taken for histopathological examination. Malignant pleural effusion was diagnosed if pleural fluid cytology or pleural biopsy findings were positive for malignancy (14). Staging of MPM was done using van Meerbeeck et al. (15), while that of lung cancer was assessed according to Lim et al. (16).

Biochemical assays of circulating survivin and fibulin-3

A) Blood samples

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Five milliliters of venous blood was withdrawn from every included subject using serum separator gel tubes and was allowed to clot at room temperature for 30 minutes and then centrifuged for 15 min at 1000 g. The separated sera were stored in aliquots using 1 ml cryotubes at -80 °C for later biochemical assays of survivin and fibulin-3.

B) ELISA assays of survivin and fibulin-3

Quantitative determinations of serum survivin and fibulin-3 were achieved using commercially available ELISA assay kits supplied by Chongqing Biospes Co., Ltd, China with catalog numbers BYE3519 and BYEK2017, respectively. The assays were performed using a microplate ELISA reader (EMR 500, USA), according to the manufacturer protocol.

C) Western blotting assessments of local survivin and fibulin-3 expression levels

Lung and pleural biopsies were homogenized in ice-cold RIPA lysis buffer(Sigma-Aldrich, Milan, Italy), containing 1% protease inhibitor cocktail (Cell Signaling Technology, Inc., MA, USA) using a Potter-Elvehjem rotor-stator homogenizer (glass/Teflon homogenizer) and were preserved at -70 °C for subsequent analysis of tissue nuclear survivin and fibulin-3 expression by western blotting technique.

Proteins in each corresponding lung or pleural tissue homogenates were denatured at 95 °C for 5 min in 2 × Laemmli buffer followed by the addition of 5% 2-mercaptoethanol. SDS–PAGE electrophoresis was achieved by loading 50 µg protein per lane at 75 volts through resolving gel (18% for survivin and 15% for fibulin-3) followed by 125 volts for approximately 2 h and transferred to a PVDF membrane using a T-77 ECL semidry transfer unit (Amersham BioSciences UK Ltd) for 2 h. immunoblotting was performed by incubating the PVDF membrane in TBS buffer containing 0.1% Tween and 5% non fat milk for one hour at 4 °C, followed by overnight incubation at 4 °C with rabbit anti-survivin polyclonal antibody (Bioss Inc., Massachusetts, USA) and rabbit anti-fibulin-3 polyclonal antibody (Novus Biologicals, LLC, Littleton, CO, USA) at a dilution of 1:1500. After washing with the TBST buffer three times, each membrane was incubated at room temperature for 1 h with an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Novus Biologicals, LLC, Littleton, CO, USA) at 1:5000 dilution, the membrane-bound antibody was detected after being washed four times in TBST with a commercially available BCIP / NBT substrate detection Kit (Genemed Biotechnologies, Inc., CA, USA). Equivalent protein loading has been confirmed for each lane by stripping and re-blotting each membrane at 4 °C against the mouse monoclonal anti-β-actin antibody (Santa Cruz Biotechnology, Inc., CA, USA) at 1:5000 dilution. The analysis has been replicated 3 times to ensure findings are reproducible. Quantification was performed using ImageJ software and expressed as the band density relative to that of β-actin (17,18).

Statistical analysis

Data entry and data analysis are done using SPSS version 19 (Statistical Package for Social Science).

Data were presented as number, percentage, mean and standard deviation for parametric data. Chi-

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square test and Fisher exact test were used to compare qualitative variables. Independent t-test was used to compare quantitative variables between the two groups. Pearson correlation was done to measure the correlation between quantitative variables. The Medcalc Program was used to calculate sensitivity, specificity and positive and negative predictive values with calculation of the AUC (95% CI). P-values were considered statistically significant when < 0.05 .

Results

Demographic data of the study groups

The current study was conducted on 219 age- and sex- matched patients (141 males and 78 females) with various benign and malignant lung and pleural diseases. They were categorized into four groups; group A included 63 patients with lung cancer (42 males and 21 females, with their mean age was 57.67 ± 7.51 years); group B included 63 patients with various benign lung diseases (39 males and 24 females, with their mean age was 51.62 ± 11.14 years); Group D included 45 patients with malignant pleural mesothelioma (MPM), (27 males and 18 females, with their mean age was 53.73 ± 4.95 years) and the last group formed of 48 patients having various benign pleural diseases serve as group E (33 males and 15 females, with their mean age was 52.13 ± 4.11 years). For 60 healthy controls (30 males and 30 females) matched with the study patients for age and sex, with mean age was a 55.5 ± 7.88 year and categorized as group C.

Clinical data of the patients' groups

Analysis of the clinical data of the patients' groups with benign respiratory diseases (groups B and E) revealed that, chronic lung abscess, bronchiectasis and post-T.B cavitory lesion were diagnosed in 11 cases (17.4%) each, emphysematous bulla in 25 cases (39.7%) and arteriovenous malformation in 5 cases (8%) in group B. While, among group E, empyema was present in 15 cases (31.3%), chronic pleurisy was diagnosed in 24 cases (50%) and pleural fibroma was diagnosed in 9 cases (18.8%).

Analysis of the tumor stage of the patients' groups with malignant respiratory diseases (groups A and D) revealed that, 27 cases (42.9%) of the included patients with lung cancer had stage-II, 6 cases (9.5%) had stage-III and 30 cases (47.6%) had stage-IV. While those with MPM, 12 cases (26.7%) had stage-III and 33 cases (73.3%) had stage-IV. In terms of the histopathological types, adenocarcinoma was present in 36 cases (57.1%) and squamous cell carcinoma was present in 27 cases (42.9%) of the included patients with non-small cell lung cancer (NSCLC). While among the included patients with MPM, epithelial mesothelioma was present in 30 cases (66.7%) and sarcomatoid mesothelioma in 15 cases (33.3%).

Survivin and fibulin-3 expression levels in the study groups

As regards patients with lung diseases, there were statistically significant higher mean \pm SD serum levels of survivin among patients with lung cancer (681.93 ± 274.79 pg/ml) when compared with both patients with benign lung diseases and the controls (423.34 ± 239.73 and 269.22 ± 8.01 pg/ml, respectively), ($p <$

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when compared with the controls ($p = 0.006$). On the contrary, there were significantly lower serum fibulin-3 levels among patients with lung cancer (3.56 ± 2.18 pg/ml) when compared with both patients with benign lung diseases and the controls (10.16 ± 7.83 and 8.99 ± 2.2 pg/ml, respectively), ($p < 0.001$ for both), with non-significant difference in the serum levels of fibulin-3 between patients with benign lung diseases and the controls ($p = 0.364$), (**Table.1**).

Regarding to patients with pleural diseases, there were statistically significant higher mean \pm SD serum levels of survivin among patients with MPM (582.75 ± 71.91 pg/ml) when compared with both patients with benign pleural diseases and the controls (259.7 ± 17.78 and 269.22 ± 8.01 pg/ml, respectively), ($p < 0.001$ for both), with non-significant difference in the serum levels of survivin between patients with benign pleural diseases and the controls ($p = 0.873$). In addition, there were significantly higher serum fibulin-3 levels among patients with MPM (14.5 ± 3.07 pg/ml) when compared with both patients with benign pleural diseases and the controls (8.9 ± 2.3 and 8.99 ± 2.2 pg/ml, respectively), ($p < 0.001$ for both), with non-significant difference in the serum levels of fibulin-3 between patients with benign pleural diseases and the controls ($p = 0.949$), (**Table.2**).

Using western blot analysis technique, the local survivin and fibulin-3 expression levels in patients with benign and malignant respiratory diseases were consistent with the circulating levels. Local survivin expression levels were significantly higher among patients with lung cancer and MPM. Interestingly, there were significantly lower local fibulin-3 expression levels among patients with lung cancer, and significantly higher among MPM's patients (**Figure.1**).

There were no significant differences in the serum survivin and fibulin-3 levels in the included patients with lung cancer or MPM in terms of their histological types, $p \geq 0.05$ for all.

Correlation analysis of survivin and fibulin-3 expression levels in the study groups

There were no significant correlations between survivin and fibulin-3 serum levels in each included study group, $p \geq 0.05$ for all. Additionally, no significant correlations between the measured biochemical markers with the tumor stage could be detected among the included patients with lung cancer and those with MPM, $p \geq 0.05$ for all.

Diagnostic utilities of survivin and fibulin-3 expression levels in patients with respiratory diseases

The sensitivity, specificity, PPV, NPV and AUC for survivin at cut-off point > 279.7 pg/ml in diagnosing lung cancer were 71.43%, 100.0%, 100.0%, 62.5% and 0.743, respectively, while for fibulin-3 at cut-off point ≤ 6.68 pg/ml were 64.29%, 90.0%, 93.1%, 54.5% and 0.736, respectively, $p \geq 0.05$ (Fig. 2A **and B**). While, the sensitivity, specificity, PPV, NPV and AUC for survivin at cut-off point > 263.6 pg/ml in predicting MPM were 82.86%, 75.0%, 87.9%, 66.7% and 0.804, respectively, and for fibulin-3 at cut-off point > 11.7 pg/ml were 37.14%, 100.0%, 100.0%, 42.1% and 0.680, respectively, $p \geq 0.05$ (Fig. 2C **and D**).

Receiver Operating Characteristic (ROC) curves for serum survivin and fibulin-3 in discriminating lung cancer from malignant pleural mesothelioma revealed that the sensitivity, specificity, PPV, NPV and AUC for survivin at cut-off point > 632 pg/ml were 52.38%, 86.67%, 84.6%, 56.5% and 0.603, respectively, while for fibulin-3 at cut-off point ≤ 9.16 pg/ml were 100.0%, 93.3%, 95.5%, 100.0% and 0.994, respectively, $p \leq 0.001$ (Fig. 3A and B).

Discussion

Lung cancer is the second leading illness that contributes to years of life lost due to premature mortality (19). The prognosis of MPM is even much worse. Therefore, search of additional biomarkers for the development of novel strategies for its early detection is mandatory.

Although the incidence of adenocarcinoma (50.60%) during the period 1998–2007 was about twice that of squamous cell carcinoma (25.24%), the former did not attract as much attention as the latter, which is associated with cigarette smoking, according to data from the National Cancer Registry (20). In the current study, adenocarcinoma was present in 57.1% and squamous cell carcinoma was present in 42.9% of the included patients with non-small cell lung carcinoma (NSCLC). Recently, there is an increased incidence of MPM, associated with a poor prognosis. It is classified pathologically into epithelioid, sarcomatoid, or biphasic types, 55, 15, and 30% of cases of MPM, respectively (21,22). In the current study, 66.7% of the included MPM patients had epithelial mesothelioma, while the remaining 33.3% had sarcomatoid type.

Survivin over expression in different malignant tumors pretends to be a causal association between survivin up-regulation, higher malignant grades and decreased survival rates (23). In this study, the expression levels of survivin and fibulin-3 were evaluated in oncologic and non-oncologic diseases of the lung and the pleura. Our findings revealed a significantly higher circulating and local expression levels of survivin in bronchogenic carcinoma patients compared to those with benign lung diseases; with a non-significant difference in its serum levels as regards the histological types and a non-significant correlation with the tumor stage. Many investigators were in line with our findings (24–26).

Although a meta-analysis performed by Duan et al.(27) indicates survivin expression was correlated with tumor stage, but not pathological type and tumor size. They also reported higher survivin expression in patients with NSCLC compared with normal controls. In addition, Naumnik et al.(28) reported no correlation between serum survivin concentrations and the histological type or staging of lung cancer, in contrary, they reported that survivin concentrations were the same in patients with NSCLC as in healthy people. The discrepancy may be attributable to differences in the tumor entities, different experimental settings such as patients' selection criteria and study design. In these sense, putative survivin antagonists under study are showing promising antitumoral potential (29).

Notably, our results showed significantly higher serum survivin levels in patients with benign lung diseases when compared with the healthy controls. This confirms similar findings reported by Terasaki et

al,(30) who described its high expression as a key mediator of cytoprotection in acute lung injury that is partly dependent on apoptosis inhibition.

The findings of the present study revealed significantly lower circulating and local fibulin-3 expression levels in patients with lung cancer when compared with those having benign lung lesions with non-significant differences in its serum levels in term of histological types and no significant correlation with the stage of lung cancer. Additionally, there was a non-significant difference in fibulin-3 serum levels between patients with benign lung lesions compared to the healthy controls. Our results were consistent with other studies documenting downregulation of fibulin-3 in lung cancer due promoter hypermethylation (10,31,32). Participation of fibulin-3 in cancer may depend on the pathways, protein-protein interactions and tumor microenvironment involved (33). In lung cancer, downregulation of fibulin-3 is needed to enhance invasion and metastasis through Wnt/ β -catenin activation and matrix metalloproteinase-7 (MMP-7) expression (11).

Regarding the included patients with pleural diseases, there were significant higher circulating and local expression levels of both survivin and fibulin-3 in patients with MPM when compared with both those had benign pleural lesions and the healthy controls with lack of association of survivin and fibulin-3 with the MPM stage or histological types. Additionally, there were no significant differences in the circulating fibulin-3 between benign pleural diseases group and the healthy controls. Many researchers were in consistent with our findings (13,34,35,36).

To the best of our knowledge, this is the first study to report absence of significant correlations between survivin and fibulin-3 in various benign or malignant respiratory diseases.

Area under curve (AUC) of summary receiver operating characteristics (sROC) is an index that specifies the overall diagnostic value of a test [37]. The literature researches lack the validity of survivin and fibulin-3 in diagnosing lung cancer and MPM. We reported non-significant difference in the diagnostic validities of survivin and fibulin-3 in diagnosing lung cancer or predicting MPM. Although no published data regarding the utility of survivin in diagnosing lung cancer, humoral fibulin-3 test data published revealed high variability among studies and still remains a contentious issue (38). A meta-analysis study reported that blood fibulin-3 is useful for MPM diagnosis and its validity was higher than soluble mesothelin-related peptides (SMRP) and osteopontin (38). Few studies could be traced in literature and reported similar findings (37, 39).

To the best of our knowledge, this is the first report comparing the validity of serum survivin vs fibulin-3 in discriminating lung cancer from MPM and revealed that sensitivity, specificity and AUC for survivin at cut-off point > 632 pg/ml were 52.38%, 86.67%, and 0.603, respectively, while for fibulin-3 at cut-off point ≤ 9.16 pg/ml were 100.0%, 93.3%, and 0.994, revealed that fibulin-3 a more valid biomarker in differentiating MPM from lung cancer.

In conclusion, the current research showed that serum survivin and fibulin-3 could be used for lung cancer differences in the diagnostic validity of serum

survivin and fibulin-3 were found in predicting lung cancer or MPM from corresponding benign lesions, while serum fibulin-3 was more helpful in differentiating lung cancer from MPM. Furthermore, the present study confirms the lack of associations between survivin and fibulin-3 expression levels with both the histological types and tumor stage.

Study Limitation

Relatively small sample size and single centre study were the main study limitations.

Abbreviations

Inhibitors of the apoptosis protein (IAP); malignant pleural mesothelioma (MPM); epithelial-mesenchymal transition (EMT); transforming growth factor beta (TGF- β); glycogen synthase kinase 3β (GSK 3β); erythrocyte sedimentation rate (ESR); complete blood count (CBC); radioimmunoprecipitation assay buffer (RIPA buffer); Polyvinylidene difluoride (PVDF) membrane; Tris-buffered saline (TBS); Tris-buffered saline with tween (TBST); 5-Bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium (BCIP / NBT); non-small cell lung carcinoma (NSCLC); matrix metalloproteinase-7 (MMP-7).

Declarations

Ethics approval and consent to participate

The study was approved by the Faculty of Medicine's Ethics Committee, South Valley University, Qena, Egypt, and it was carried out in accordance with the Declaration of Helsinki. Every subject included has obtained informed written consent. Ethical approval code: SVU-MED-MBC004-4-20-7-57.

Consent for publication

Not applicable

Availability of data and materials

The data sets that were used and/or analyzed during the current study are available are included in this published article.

Competing interests

The author(s) have declared no possible conflicts of interest regarding this article's work, authorship and/or publication.

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Author contributions

Study concept and design: MMH, BEME and MA-B; Patients' selection and clinical assessments: MA-B, MW, MB and KM; Blood and tissue samples collection and processing: MMH, BEME, THA-E, MA-B, MW, MB and KM; Biochemical and molecular assays: MMH and BEME; Statistical analysis: MMH, SA and MA-B; literature search: MMH; BEME and SA: First manuscript draft: MMH; All authors had revised and approved the final manuscript version.

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Tables

Table.1 Mean±SD of serum survivin and fibulin-3 among patients with benign and malignant lung diseases vs. the control group

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Measured biochemical markers	Group A (N=63)	Group B (N=63)	Group C (N=60)	*P1	*P2	*P3
Serum survivin (Pg/ml), (mean±SD)	681.93±274.79	423.34±239.73	269.22±8.01	<0.001**	0.006**	<0.001**
Serum Fibulin-3 (Pg/ml), (mean±SD)	3.56±2.18	10.16±7.83	8.99±2.2	<0.001**	0.364	<0.001**

*P1= A vs. C

*P2= B vs C

*P3= A vs B

**p<0.05 : significant; p>0.05: Non-significant.

N.B: Group A: patients with lung cancer; group B: patients with benign lung diseases; group C: control group.

Table.2 Mean±SD of plasma survivin and fibulin-3 among patients with benign and malignant pleural diseases vs. the control group

Measured biochemical markers	Group D (N=45)	Group E (N=48)	Group C (N=60)	*P1	*P2	*P3
Serum survivin (pg/ml), (mean±SD)	582.75±71.91	259.7±17.78	269.22±8.01	<0.001**	0.873	<0.001**
Serum fibulin-3 (pg/ml), (mean±SD)	14.5±3.07	8.9±2.3	8.99±2.2	<0.001**	0.949	<0.001**

*P1= D vs. C

*P2= E vs C

*P3= D vs E

**p<0.05 : significant; p>0.05: Non-significant.

N.B: Group D: patients with malignant pleural mesothelioma (MPM); group E: patients with benign pleural disease; group C: control group.

Figures

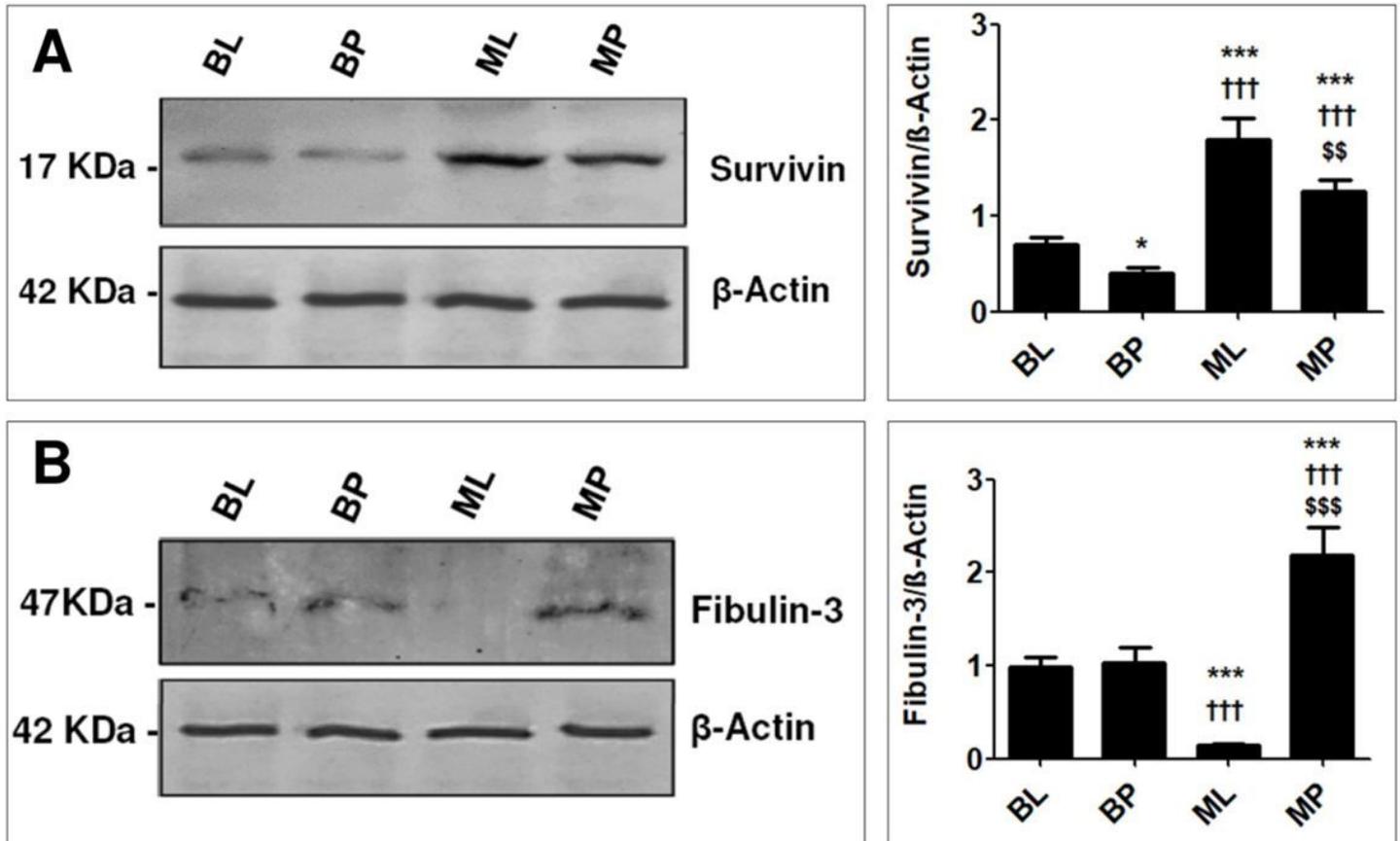


Figure 1

Representative western blot analysis of survivin (A) and fibulin(B) expression in different groups. β -Actin was used in parallel as internal control. The right panels represent the corresponding quantification of each analysis measured by the Image J software and expressed as the relative band density to β -actin. The levels of significance were accepted with $p < 0.05$ and all relevant results were graphically displayed as mean \pm SD ($n=3$). *, † and \$ indicate significant changes from the BL, BP, and ML groups respectively. *, † and \$ indicate significant change at $p < 0.05$; **, †† and

*indicates significant changes at $p < 0.01$; * * *, † † † and*

\$ indicate significant changes at $p < 0.001$. BL, benign lung diseases; BP, benign pleural diseases; ML, malignant lung diseases; MP, malignant pleural diseases.

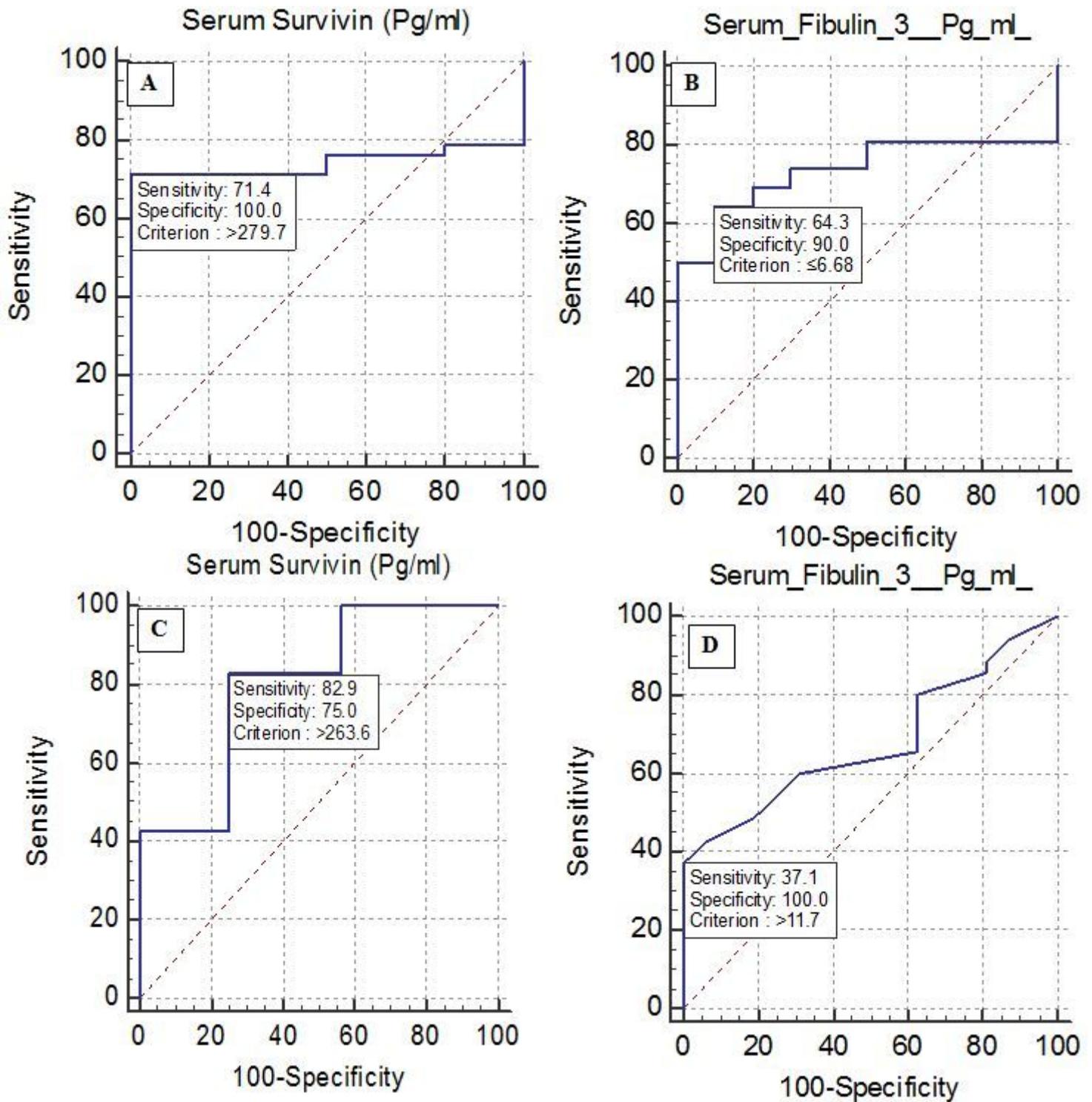


Figure 2

Receiver Operating Characteristic (ROC) curves for serum survivin (A) and fibulin-3 (B) in predicting lung cancer, $p \leq 0.05$ and in diagnosing malignant pleural mesothelioma (C) & (D), $p \leq 0.05$.

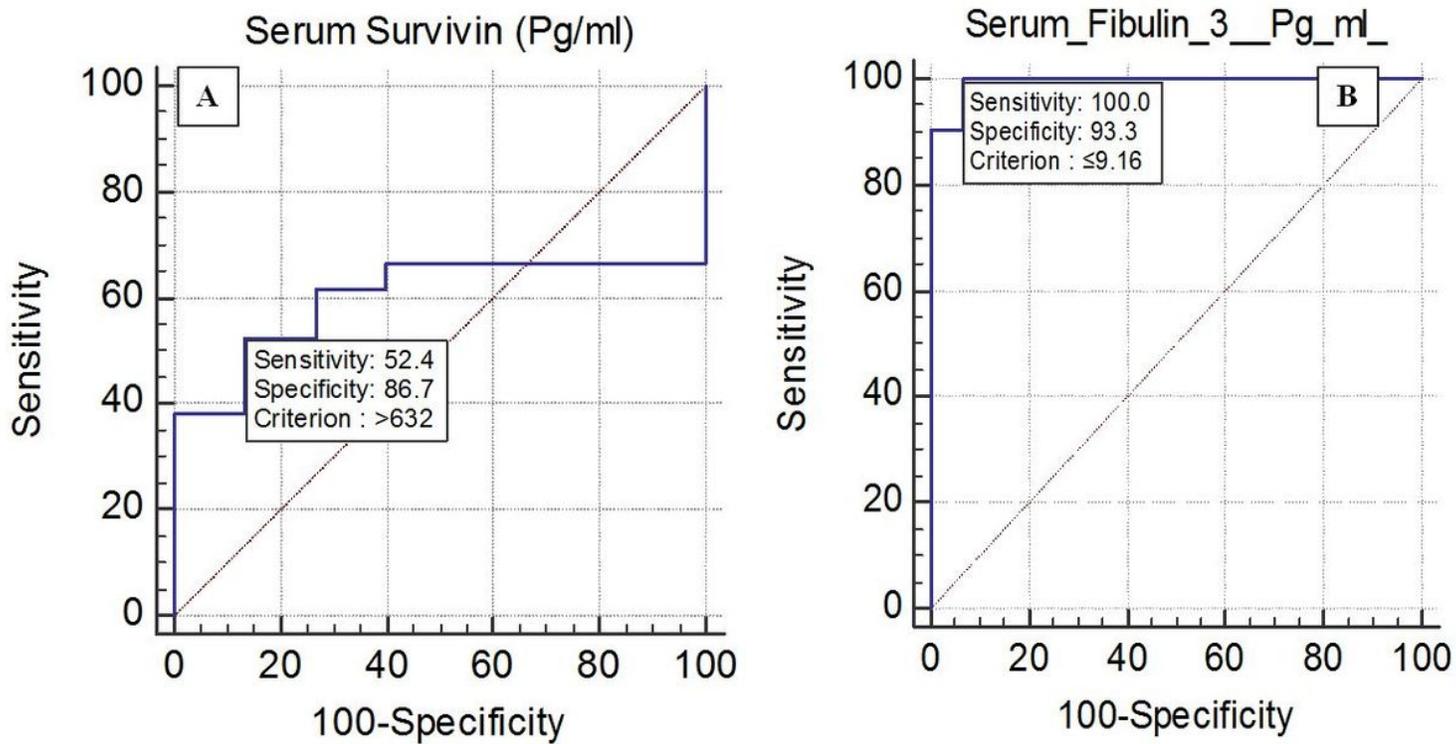


Figure 3

Receiver Operating Characteristic (ROC) curves for serum survivin (A) and fibulin-3 (B) in discriminating lung cancer from malignant pleural mesothelioma, Fibulin-3 was superior to survivin, $p < 0.001$.