Expression of Autophagy-Related Proteins Beclin-1 and LC3-II in Metastatic Pleural Effusion of Lung Cancer (Postprint)

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Abstract

Background Malignant pleural effusion is one of the important markers of advanced-stage tumors and metastasis. Existing methods for diagnosing the nature of pleural effusion have defects and limitations, preventing timely and effective judgment of pleural effusion properties. Most malignant pleural effusions are caused by metastatic lung cancer. The autophagy nucleation key protein Beclin-1 and LC3- , which is used to monitor cellular autophagy levels, play important roles in the progression of lung cancer, but their application in metastatic pleural effusion has not been reported in relevant literature and requires further investigation.

Objective To analyze the expression of autophagy-related protein Beclin-1 and microtubule-associated protein 1 light chain 3- (MAP1LC3-) in lung cancer metastatic pleural effusion and explore their value in early clinical diagnosis of the disease.

Methods From May 2022 to July 2023, bioinformatics data for Beclin-1 and LC3- were obtained through analysis using the GEO database, GEPIA2, and GeneMANIA database. The collected pleural effusion samples were grouped using liquid-based thin-layer cytology smears combined with clinical data into a malignant assay group (95 cases) and a benign control group (190 cases). Expression of Beclin-1 and LC3- at the gene and protein levels was detected by RT-PCR and Western Blot methods, respectively, and the expression quantity of LC3 protein was confirmed using immunofluorescence assays.

Results Bioinformatics analysis results showed that both Beclin-1 and LC3 exist in lung cancer differentially expressed gene datasets, with differential expression between lung cancer tissues and normal tissues. RT-PCR results demonstrated that gene expression of Beclin-1 and LC3 was higher in the benign control group than in the malignant assay group (P<0.05). Western Blot results showed that protein expression of Beclin-1 and LC3- was higher in the benign control group compared to the malignant assay group, with statistically significant differences (P<0.05). Furthermore, the number of fluorescein isothiocyanate-labeled LC3- fluorescent spots representing autophagosome quantity was higher in the benign control group than in the malignant assay group.

Conclusion By analyzing the differential expression of autophagy-related proteins Beclin-1 and LC3- in benign pleural effusion and lung cancer metastatic malignant pleural effusion, they can be used for early diagnosis of malignant pleural effusion, providing a new perspective for the differential diagnosis and targeted treatment of benign and malignant pleural effusions.

Full Text

Expression of Autophagy-Related Proteins Beclin-1 and LC3-II in Lung Cancer Metastatic Pleural Effusion

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Abstract

Background: Malignant pleural effusion (MPE) represents a critical clinical indicator of advanced-stage malignancy and metastatic progression. Current diagnostic methods for pleural effusion characterization suffer from significant limitations, preventing timely and accurate diagnosis. Metastatic lung cancer is the primary cause of MPE. While the roles of autophagy-related proteins Beclin-1 and LC3-II in lung cancer pathogenesis have been established, their expression patterns and diagnostic potential in MPE remain largely unexplored.

Objective: To analyze the expression of autophagy-related proteins Beclin-1 and microtubule-associated protein 1 light chain 3-II (MAP1LC3-II) in lung cancer-associated malignant pleural effusion and evaluate their potential value in early clinical diagnosis.

Methods: From May 2022 to July 2023, we performed bioinformatics analysis of Beclin-1 and LC3 using GEO, GEPIA2, and GeneMANIA databases. Pleural effusion samples were classified into malignant (n=95) and benign (n=190) groups based on liquid-based cytology and clinical data. mRNA and protein expression levels of Beclin-1 and LC3-II were quantified by RT-PCR and Western blot, respectively. LC3 puncta formation was further validated by immunofluorescence assay.

Results: Bioinformatics analysis confirmed that both Beclin-1 and LC3 were present in lung cancer differentially expressed gene datasets, showing differential expression between lung cancer tissues and normal tissues. RT-PCR revealed significantly higher mRNA expression levels of both Beclin-1 and LC3 in benign controls compared to malignant cases ($P<0.05$). Western blot analysis demonstrated elevated Beclin-1 and LC3-II protein abundance in benign specimens relative to the malignant group ($P<0.05$). Additionally, immunofluorescence showed higher FITC-labeled LC3-II puncta, representing autophagosome numbers, in benign controls versus malignant cases.

Conclusion: Analysis of expression differences in autophagy-related proteins Beclin-1 and LC3-II between benign pleural effusion and lung cancer metastatic malignant pleural effusion may facilitate early diagnosis of MPE and provide novel perspectives for differential diagnosis and targeted therapeutic strategies.

Keywords: Lung neoplasms; Pleural effusion; Autophagy; Beclin-1; LC3-II

Introduction

Lung cancer is one of the most common malignant tumors and represents a leading cause of cancer-related mortality worldwide [?], with a five-year survival rate of only 3% [?]. The majority of patients are diagnosed with advanced-stage disease or distant metastasis at initial presentation [?]. Although novel therapeutic approaches have improved outcomes for advanced lung cancer patients [?], prognosis remains poor [?]. Notably, malignant pleural effusion (MPE) caused by lung cancer accounts for 50-65% of all MPE cases [?] and serves as an important marker of advanced disease and metastasis [?].

Current diagnostic methods for pleural effusion, including routine examinations and biochemical analysis, lack specificity, while diagnostic biomarkers exhibit insufficient sensitivity [?]. Cytomorphological assessment is further limited by the nature of the effusion itself and the technical expertise required, making accurate characterization challenging and significantly impacting clinical decision-making [?]. Therefore, investigating the mechanisms underlying MPE formation and identifying highly specific diagnostic markers are of significant clinical importance for precise diagnosis and treatment planning.

Autophagy is a degradation and recycling process in which lysosomes degrade cytoplasmic proteins and damaged organelles under the regulation of autophagy-related genes (ATG) [?]. Numerous studies have demonstrated that autophagy plays crucial roles in tumor initiation, progression, and metastasis [?], suggesting that investigating autophagy-related protein expression in lung cancer metastatic pleural effusion could elucidate autophagy mechanisms in lung cancer and serve as an effective diagnostic strategy. Beclin-1 is a key protein in autophagy initiation and regulation, functioning as a marker of autophagy activation. Microtubule-associated protein 1 light chain 3 (LC3) represents a downstream effector regulated by Beclin-1. The cytosolic form LC3-I is conjugated to phosphatidylethanolamine, modifying it into the membrane-bound LC3-II protein [?]. LC3-II activity positively correlates with cellular autophagy levels and serves as an excellent indicator of autophagic activity [?]. However, the expression patterns and functional roles of autophagy-related proteins Beclin-1 and LC3-II in lung cancer metastatic pleural effusion remain unclear. Therefore, this study utilized minimally invasive pleural effusion specimens to examine Beclin-1 and LC3-II expression levels in different types of effusions, aiming to provide novel molecular evidence for distinguishing benign from malignant pleural effusion.

Methods

1.1 Bioinformatics Analysis

1.1.1 GeneMANIA Database Analysis of Beclin-1 and LC3 Interacting Genes From May 2022 to July 2023, we utilized the GeneMANIA database to analyze interacting proteins using parameters including physical interactions, co-expression, predicted associations, co-localization, pathway interactions, genetic interactions, and shared protein domains. Network diagrams were constructed, revealing that Beclin-1 primarily functions in autophagy, phosphatidylinositol 3-kinase complex formation, response to nutrient levels and starvation, cellular response to extracellular stimuli, autophagosome formation, and regulation of cell division. LC3 primarily participates in macroautophagy, autophagosome organization and formation, and cellular responses to starvation and stimuli [Figure 1: see original paper].

1.1.2 GEO Database Bioinformatics Analysis Microarray data were obtained from the National Center for Biotechnology Information (NCBI) GEO database using "Lung cancer" and "adjacent normal lung tissue" as search keywords. The GSE19188 dataset, based on the GPL570 platform, was selected for analysis. This dataset contains expression profiles from 91 tumor samples and 65 adjacent normal lung tissue samples. Differentially expressed genes were identified using adjusted $P<0.05$ as the cutoff, and volcano plots were generated using the ggplot package in R [Figure 2: see original paper]. Both Beclin-1 and LC3 were present in this differentially expressed gene dataset .

1.1.3 TCGA Database Analysis Using the GEPIA2 online tool (gepia2.cancer-pku.cn), we analyzed TCGA data including 483 lung adenocarcinoma cases and 347 normal controls, as well as 486 lung squamous cell carcinoma cases and 338 normal controls. The Box plot function under Expression DIY was used for analysis. Beclin-1 and LC3 were entered in the "Gene" field, while lung adenocarcinoma and lung squamous cell carcinoma were selected in the "Datasets Selection" field. The resulting PDF box plots were downloaded for further analysis.

1.2 Experimental Subjects A total of 400 pleural effusion samples were collected from untreated patients at the Clinical Laboratory of the First Affiliated Hospital of Baotou Medical College between 2018 and 2023. After applying inclusion and exclusion criteria, 285 cases were selected for the study, comprising 190 benign controls and 95 malignant cases. This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Baotou Medical College (Approval No.: 2022-5).

1.2.1 Inclusion Criteria

(1) Patients with confirmed pleural effusion by chest X-ray, CT scan, or ultrasound, with effusion samples obtained via thoracentesis. Benign control effusions were defined as those showing no malignant cells or abnormal tumor markers in cytological or biochemical analysis, with clinical diagnosis confirming benign disease. (2) Malignant cases were defined as effusions in which cancer cells were identified by cytology or pleural biopsy, with pathological and clinical data confirming metastatic lung cancer as the cause.

1.2.2 Exclusion Criteria

(1) Cases without definitive clinical diagnosis; (2) Cases with pleural effusion caused by other diseases; (3) Cases who had undergone surgery, chemotherapy, or molecular targeted therapy.

1.3 Experimental Procedures

1.3.1 Sample Collection and Processing Qualified pleural effusion specimens (100 mL) were aliquoted into 5 tubes, mixed with 2 mL PBS phosphate buffer, and centrifuged at 2000 r/min for 10 minutes. The supernatant was discarded, and the precipitate was divided into 5 tubes. Two tubes were stored at -80°C for future use, while the remaining 3 tubes were immediately processed for liquid-based thin-layer slide preparation and microscopic examination.

1.3.2 Liquid-Based Cytology for Pleural Effusion Characterization Pleural effusion smears were prepared using the EasyFix liquid-based cytology system, fixed in 95% ethanol for 15 minutes, and stained with

Wright-Giemsa stain. Microscopic examination revealed that benign pleural effusions predominantly contained mesothelial cells, lymphocytes, neutrophils, and macrophages with normal morphology. Malignant pleural effusions showed clustered cell aggregates with irregular morphology, significantly enlarged and variable cell size, abundant basophilic cytoplasm with vacuoles, increased nuclear-cytoplasmic ratio, unevenly distributed chromatin, prominent nucleoli, and irregularly thickened nuclear membranes [Figure 3: see original paper]. Based on cytological results and clinical-pathological data, 190 cases were classified as benign controls (119 male, 71 female; mean age 74.4$\pm$10.4 years) and 95 cases as malignant (54 male, 41 female; mean age 67.8$\pm$12 years).

1.3.3 Real-Time PCR for Gene Expression Primer sequences are listed in . Total RNA was extracted using RNAiso Plus, and concentration was measured. Reverse transcription to cDNA was performed on ice with the following parameters: (1) 95°C for 30 s; (2) 40 cycles of 95°C for 5 s and 60°C for 34 s; (3) 95°C for 15 s; (4) 60°C for 60 s; (5) 95°C for 15 s. Gene expression was normalized to β-actin as a housekeeping gene, and relative expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method.

1.3.4 Western Blot for Protein Expression Total protein was extracted using radio-immunoprecipitation assay (RIPA) buffer, and protein concentration was determined by bicinchoninic acid (BCA) assay. Forty micrograms of protein were loaded for gel electrophoresis. Polyvinylidene fluoride (PVDF) membrane transfer was performed as follows: LC3-II at 300 mA for 25 minutes, Beclin-1 at 300 mA for 75 minutes, and GAPDH at 300 mA for 45 minutes. After blocking with fish gelatin, membranes were incubated with primary antibodies at 4°C overnight, followed by secondary antibodies at room temperature for 2 hours. After washing with Tris-buffered saline with Tween-20 (TBST), bands were visualized using an automated chemiluminescence imaging system.

1.3.5 Immunofluorescence Detection of LC3-II Liquid-based cytology slides were fixed, air-dried, and permeabilized with autofluorescence quencher solution A in a dark box. After washing and drying, slides were blocked with 5% BSA, incubated with LC3-II antibody (1:200) at 4°C overnight, and then with FITC-conjugated goat anti-rabbit IgG secondary antibody at room temperature for 2 hours. After applying autofluorescence quencher solution B, washing, and drying, slides were mounted and examined under a Zeiss Imager M2 microscope. Images were captured and analyzed using lsis software (Version V5.8.11).

1.4 Statistical Analysis Data were analyzed using SPSS 26.0 and GraphPad Prism 9 software. Normally distributed data are expressed as mean ± standard deviation ($\bar{x} \pm s$) and compared between groups using paired t-tests. Non-normally distributed data are expressed as median (P25, P75) and compared using paired rank-sum tests. $P<0.05$ was considered statistically significant.

PCR data were normalized to β-actin and analyzed using 2^(-ΔΔCt) values. Western blot bands were quantified for gray values using ImageJ software.

Results

2.1 GEPIA2 Analysis of TCGA Database Analysis of TCGA data using GEPIA2, including 483 lung adenocarcinoma cases and 347 normal controls, as well as 486 lung squamous cell carcinoma cases and 338 normal controls, revealed that Beclin-1 mRNA expression was significantly higher in both lung adenocarcinoma and lung squamous cell carcinoma tissues compared to normal lung tissue ($P<0.05$). In contrast, LC3 mRNA expression was significantly lower in both cancer types compared to normal tissue ($P<0.05$). These results for Beclin-1 contradicted findings from most experimental studies [Figure 4: see original paper], prompting further experimental validation of protein expression differences.

2.2 Real-Time PCR Results Real-time PCR data, normalized to β-actin, showed that Beclin-1 mRNA expression was higher in benign pleural effusion [0.79 (0.59, 2.86)] compared to malignant effusion [0.61 (0.21, 1.34)]. Similarly, LC3-II mRNA expression was higher in benign [1.94 (0.35, 2.48)] versus malignant [0.88 (0.21, 1.49)] effusions. These differences were statistically significant ($P<0.05$) [Figure 5: see original paper].

2.3 Western Blot Results Western blot analysis further investigated protein-level expression differences. Normalized to GAPDH, Beclin-1 protein expression was higher in the benign group (0.936$\pm$0.335) compared to the malignant group (0.683$\pm$0.442). LC3-II protein expression was also higher in the benign group (0.796$\pm$0.410) versus the malignant group (0.4923$\pm$0.358). These differences were statistically significant ($P<0.05$) [Figure 6: see original paper].

2.4 Immunofluorescence Following immunofluorescence processing, LC3-II protein appeared diffusely distributed in the cytoplasm under basal conditions. During autophagy induction, LC3-II formed green punctate or patchy fluorescent signals representing autophagosomes, with the number of green fluorescent spots indicating autophagic activity. Cell nuclei stained with DAPI appeared blue. Benign pleural effusions showed increased FITC-labeled LC3-II fluorescent puncta compared to malignant effusions [Figure 7: see original paper].

Discussion

The median survival time for lung cancer patients with MPE is only 5 months [?]. Cytological examination of pleural effusion is the most common clinical practice for diagnosing benign disease and malignant MPE, but its sensitivity is only 63% [?]. Due to similar morphological features, abnormal mesothelial cells are easily confused with tumor cells [?], and cytological diagnosis is relatively subjective. Therefore, investigating more accurate and less invasive methods to differentiate pleural effusion characteristics is essential. Such methods could also corroborate tumor diagnosis, reducing the invasiveness and high risk associated with lung biopsy, which remains the diagnostic gold standard. Identifying novel minimally invasive biomarkers or therapeutic targets for lung cancer diagnosis would significantly benefit patients.

Autophagy is triggered primarily through adenosine monophosphate (AMP)-activated protein kinase and PI3K/AKT/mTOR signaling pathways. When autophagy is activated, expression of LC3-II, a component of the autophagosome double membrane, increases to form autophagosomes that fuse with lysosomes to clear damaged organelles [?]. Autophagy plays a dual role in tumor metastasis: it can suppress cancer by removing oncogenic substrates or degrading unfolded proteins and damaged organelles to maintain genetic stability [?], or promote tumor metastasis by recycling metabolic nutrients, modulating mitochondrial energy metabolism, and inducing cellular dormancy [?]. Under stress conditions, cells initially attempt to repair damage and survive through autophagy; however, when this fails, autophagy-mediated programmed cell death, apoptosis, and necrosis may drive damaged cells toward carcinogenesis [?].

Beclin-1, the first mammalian autophagy-related protein discovered, plays an indirect but important role in cellular metabolism and directly participates in autophagy initiation. LC3-II is involved throughout the entire autophagy process and serves as a specific marker for detecting autophagy. This study examined pleural effusion specimens to investigate Beclin-1 and LC3-II expression levels in different effusion types to aid in distinguishing benign from malignant pleural effusion. Compared to traditional cytology, molecular testing offers greater accuracy and efficiency.

Current research on MPE pathogenesis primarily focuses on the immune microenvironment, with detection targets mainly consisting of immunological markers and tumor markers. However, few studies have approached MPE pathogenesis from the perspective of autophagy, using autophagy-related protein expression as an entry point for molecular-level investigation. Our detection of Beclin-1 in pleural effusion revealed significantly higher expression in benign effusions compared to lung cancer metastatic malignant effusions, consistent with previous findings of low Beclin-1 expression in tumor cells. Studies have shown that downregulating Beclin-1 can reduce autophagic activity and promote tumor cell proliferation, leading to faster tumor growth rates in adenocarcinoma mouse models with defective self-sustained quiescence [?]. Loss of Ambra1, a Beclin-1 regulator that maintains Beclin-1 stability, accelerates tumor growth and reduces survival in melanoma mouse models by increasing cell invasion and epithelial-mesenchymal transition (EMT)-like processes [?]. Recent research has identified Beclin-1 as a valuable independent biomarker for lung cancer patients [?], highlighting its important role in tumor development and metastasis and its potential as a tumor suppressor gene and therapeutic target in the autophagy pathway.

Our study also found that LC3-II expression was significantly lower in lung cancer-associated malignant pleural effusion compared to benign effusions, consistent with previous studies on LC3-II expression in tumors and GEPIA2 database analysis [?]. Research has shown that increasing LC3-II expression in two colorectal cancer cell lines can induce cell cycle arrest and autophagy, thereby inhibiting colorectal cancer metastasis [?]. LC3-II interacts with the selective autophagy substrate p62, promoting its specific degradation; accumulation of p62 promotes tumor development and interacts with the pro-EMT protein Twist-1 to facilitate cell migration [?]. Studies have demonstrated that sertraline and the cytostatic drug erlotinib synergistically increase LC3-II accumulation and autolysosome formation, effectively promoting autophagy, reducing tumor growth, and prolonging survival, providing a therapeutic strategy for non-small cell lung cancer [?].

In this study, both Beclin-1 and LC3-II showed higher relative expression at the gene level in benign pleural effusions compared to malignant effusions. This trend may be related to the increased tumor risk associated with Beclin-1 haploinsufficiency [?]. Research has shown that exogenous Beclin-1 gene transfection can inhibit tumor cell growth [?], and LC3-II expression negatively correlates with lung cancer progression [?], possibly because decreased Beclin-1 expression in tumor cells reduces LC3-I to LC3-II conversion [?]. At the protein level, expression differences mirrored those at the gene level, likely due to reduced mRNA levels encoding Beclin-1 and LC3-II proteins, as well as potential gene mutations affecting Beclin-1 and LC3-II in disease states [?]. Immunofluorescence results consistently showed higher LC3-II expression in benign versus malignant pleural effusion groups. Beclin-1 and LC3-II act synergistically in the autophagy pathway to regulate cellular autophagy.

Analysis of Beclin-1 and LC3-II expression differences between benign pleural effusion and lung cancer metastatic malignant pleural effusion may facilitate differential diagnosis and serve as an effective basis for auxiliary diagnostic biomarkers in malignant tumors. This study also provides new perspectives for targeted treatment of benign versus malignant pleural effusion and offers novel insights for tumor diagnosis and therapy, which is of significant importance for improving patient quality of life.

Author Contributions

YAO Wenjing and GAO Jinliang performed the experiments and implemented the research. WANG Cuifeng and REN Meiying conceived the research proposition and designed the study; WANG Cuifeng took overall responsibility for the article. JING Xuefen revised the final version. FU Yuhua performed statistical analysis and prepared figures and tables.

Conflicts of Interest: None declared.

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Figure Legends:

Figure 1 Diagram of the interacting protein network of Beclin-1 with LC3. Note: Panel A shows the interacting protein network of Beclin-1; Panel B shows the interacting protein network of LC3. CASP10=caspase 10, BCL2L1=B-cell lymphoma 2-like protein 1, AMBRA1=Beclin-1 regulator 1, UVRAG=UV resistance-associated gene, PIK3R4=phosphoinositide 3-kinase regulatory subunit 4, TSC1=tuberous sclerosis complex 1, NRBF2=nuclear receptor binding factor 2, SQSTM1=sequestosome 1, RETREG1=reticulophagy regulator 1.

Figure 2 Volcano map of differential genes. Note: Red dots represent upregulated genes, blue dots represent downregulated genes, and black dots represent genes with no significant change. tumor=tumor, health=healthy, P value=P value, fold change=fold change.

Figure 3 Microscopic cell morphology of benign and malignant pleural effusion. Note: Panel A shows benign pleural effusion at 100X magnification; Panel B shows malignant pleural effusion at 100X magnification.

Figure 4 GEPIA2 analysis of Beclin-1 and LC3 mRNA expression in the TCGA database. Note: Panel A shows Beclin-1 mRNA expression analysis; Panel B shows LC3 mRNA expression analysis. LUAD=lung adenocarcinoma, LUSC=lung squamous cell carcinoma.

Figure 5 The expression levels of Beclin-1/LC3-II mRNA in benign/malignant pleural effusion. Note: A: Relative expression of Beclin-1 mRNA; B: Relative expression of LC3-II mRNA. a indicates $P<0.05$, b indicates $P<0.01$.

Figure 6 The expression levels of Beclin-1/LC3-II protein in benign/malignant pleural effusion. Note: A: Western blot bands for Beclin-1 protein expression; B: Western blot bands for LC3-II protein expression; C: Relative expression of Beclin-1 protein; D: Relative expression of LC3-II protein. a indicates $P<0.05$, b indicates $P<0.01$.

Figure 7 Expression of LC3-II in benign/malignant pleural effusion under fluorescence microscopy. Note: Panel A shows benign pleural effusion at 10X magnification; Panel B shows benign pleural effusion at 100X magnification; Panel C shows malignant pleural effusion at 10X magnification; Panel D shows malignant pleural effusion at 100X magnification.

Table 1 Beclin-1 and LC3 related parameters in the GSE19188 database. Note: LC3=microtubule-associated protein 1 light chain 3.

Table 2 PCR primer sequence table.

Note: Figure translations are in progress. See original paper for figures.

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