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Original Article

Identification of adipophilin as a potential diagnostic tumor marker for lung adenocarcinoma

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Abstract: In our previous study, the upregulation of adipophilin in lung adenocarcinoma were identified compared with normal lung tissues by quantitative proteomics. In this study, our aim was to verify the result from quantitative proteomics, further investigate the relationship between adipophilin expression and clinicopathologic factors of lung cancer patients. The expression levels of adipophilin were examined in 10 pairs of lung adenocarcinoma and normal lung tissues using western blotting and the expression and cellular distribution of adipophilin were determined by IHC in 62 formalin-fixed and paraffin embedded primary lung cancer specimens. Adipophilin expression was significantly higher in lung adenocarcinoma specimens than in normal tissues and lung squamous cell carcinomas ($P<0.05$). There were no significant difference of adipophilin expression between lung squamous cell carcinomas and normal lung tissues. The expression of adipophilin in lung cancer did not correlate with any clinicopathologic factors such as lymph node metastasis, patients' age, gender, tumor size, grade, and TNM stage. In Conclusion, Adipophilin was upregulated in lung adenocarcinoma, suggesting that adipophilin play an important role in tumorigenesis of lung adenocarcinoma and may serve as a potential marker for lung adenocarcinoma.

Keywords: Adipophilin, lung cancer, western blotting, immunohistochemistry

Introduction

Lung cancer is currently one of the most common types of cancer and remains the leading cause of cancer-related mortality worldwide. Lung cancer accounts for 13% of the total cancer cases and 18% of the total cancer deaths [1]. Adenocarcinoma is the most common histologic type of lung cancer, accounting for almost half of all lung cancers [2]. Most lung cancer patients are initially diagnosed at an advanced stage. Although there has been considerable progress in treatment for advanced lung cancer, its prognosis remains poor, with the 5-year survival of about 16% [3]. Lung adenocarcinoma tend to grow and spread faster than lung squamous cell carcinoma and the pathogenesis remains unclear. Therefore, it is urgent to investigate the molecular mechanism of carcinogenesis and development in lung adenocarcinoma.

Proteomics approaches are important and very useful in the large-scale study of proteins, including differential protein expression, post-translational modifications. Recently, there has been a tendency to focus on organellar proteomics to provide information about the protein contents of organelles, substructures, or compartments isolated from cells [4]. The iTRAQ-labeling combined with two-dimensional liquid chromatography tandem mass spectrometry (2D-LC-MS/MS) technology has been widely used in many proteomics study including subcellular proteomics such as membrane proteomics and mitochondrial proteomics [5-8].

In our previous study, we performed iTRAQ labeling combined with 2D LC-MS/MS to identify differential protein expression profiles of cell membrane from lung adenocarcinoma and matched normal lung tissue samples. The result showed Adipophilin (alternative name(s): perilipin-2; adipose differentiation-related protein) was significantly upregulated in lung adenocarcinoma (2.46-fold) compared with normal lung tissues [9]. The MS/MS spectra on adipophilin is listed in **Figure 1**. Adipophilin was first charac-

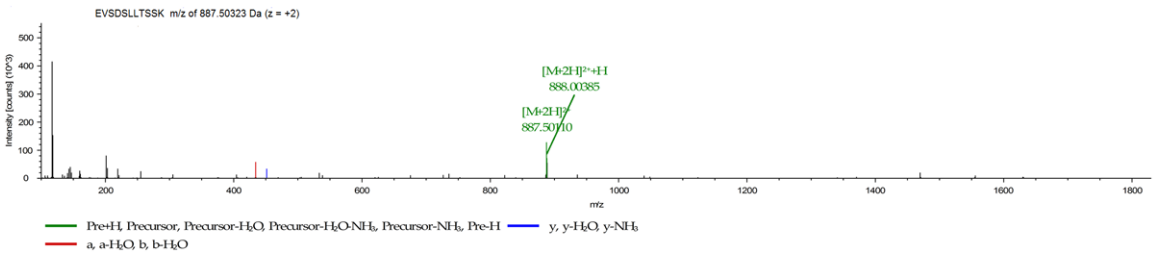

Figure 1. MS/MS spectra of identified peptides for adipophilin. The MS spectra of Sequence: EVSDSLLTSSK with m/z of 887.50323 Da (z = +2).

terized as a novel protein induced early during the process of differentiation [10, 11]. Several studies shown that adipophilin was expressed at high levels in adipose tissue and also in a variety of cells including murine MA-10 Leydig cells, Chinese hamster ovary (CHO) fibroblasts, human HepG2 hepatoma cells and restrictedly expressed in lactating mammary epithelial cells, adrenal cortex cells, Sertoli and Leydig cells of the male reproductive system, and steatosis or fatty change hepatocytes in tissues [12, 13]. In recent years, it has been shown that adipophilin is involved in carcinogenesis [14, 15].

However, up to now, the clinical impact of adipophilin expression in lung cancer remains yet unknown. In the present study, we verified the upregulation of adipophilin in lung cancer samples and further investigated the relationships between adipophilin expression and clinicopathologic factors of lung cancer patients. The study may provide important information on the role of adipophilin in carcinogenesis and development of lung cancer.

Materials and methods

Tissue specimens

For western blotting, a total of 10 fresh primary lung adenocarcinoma and matched adjacent normal lung tissues undergoing surgical resection were collected from the second affiliated hospital, Xi'an Jiaotong University, China and stored at -80°C until use. None of the patients received chemotherapy, radiotherapy prior to surgery. The formalin-fixed and paraffin embedded 62 primary lung cancer specimens included 41 lung adenocarcinoma and 21 lung squamous cell carcinoma and 24 cases of normal lung tissues were used for immunohistochemical analysis. These paraffin embedded samples were obtain from the second affiliated hospital, Xi'an Jiaotong University and shaanxi cancer hospital, China. All lung cancer samples were confirmed by histopathology. The normal lung tissues were collected at least 5 cm far away from the cancer. Informed consent was obtained from each patient and the study was approved by the local ethnics committee. Patients were staged according to the TNM staging system of the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC). The tumors were histologically subtyped and graded according to the World Health Organization guidelines.

Western blotting

We detected the expression of adipophilin in 10 pairs of fresh lung adenocarcinoma and normal lung tissues by western blotting. In brief, 60 μg of protein were separated by SDS-PAGE, and then electroblotted onto membranes. The membranes were blocked with 5% nonfat dry milk in TBST buffer for 2 h at room temperature, and then incubated with the anti-adipophilin antibody (1:400) overnight at 4°C, followed by incubation with the horseradish peroxidase-conjugated secondary antibody (1:4000) after three washes with TBST. The signals were visualized using the enhanced chemiluminescence method. The expression of β-actin was used as loading control.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using standard SP methods. In Brief, 4 um thick paraffin embedded sections were dewaxed, rehydrated with graded alcohol, and antigen was retrieved with a microwave. The intrinsic peroxidase activity was blocked using 3% hydrogen peroxide solution at room temperature for 10 min. After incubation with normal

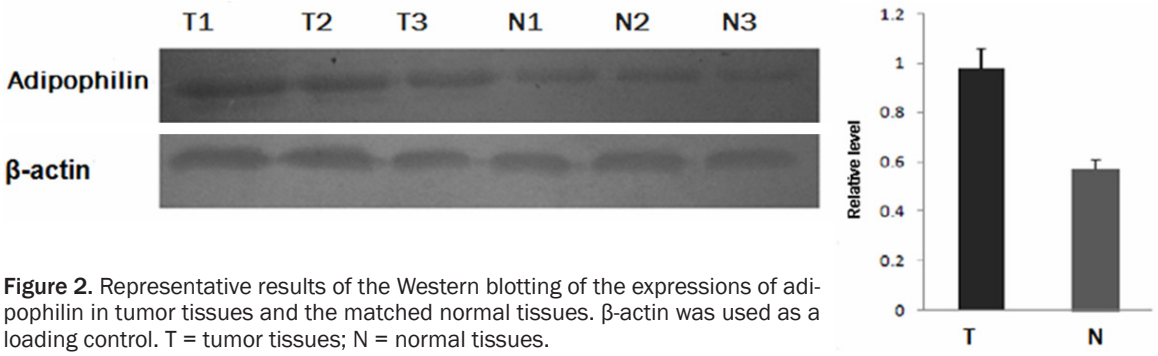

Figure 2. Representative results of the Western blotting of the expressions of adipophilin in tumor tissues and the matched normal tissues. β-actin was used as a loading control. T = tumor tissues; N = normal tissues.

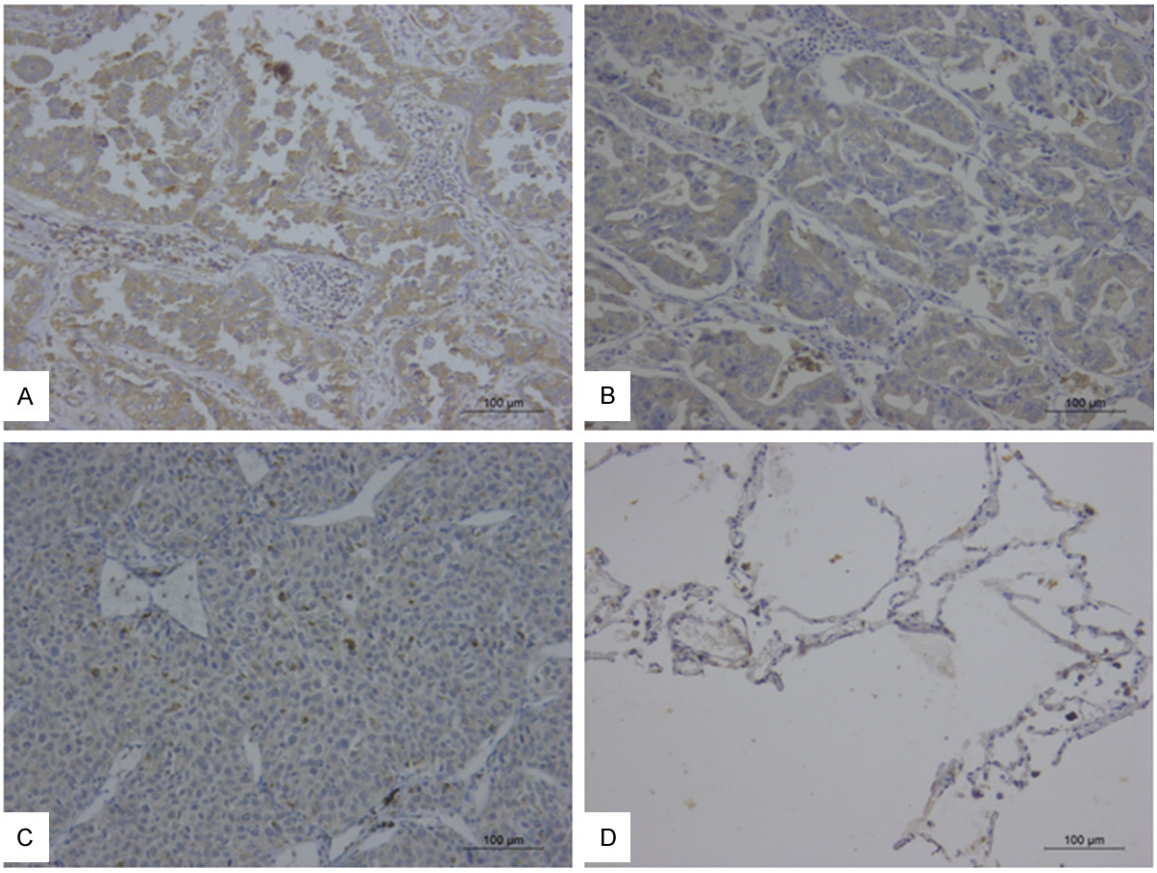

Figure 3. Immunohistochemical staining of adipophilin in lung adenocarcinoma (A, B), lung squamous carcinomas (C), and normal lung tissue (D) (IHC ×200).

goat serum, the sections were incubated with anti-adipophilin antibody (1:200) overnight at 4°C. IHC was carried out using the SP9001 Rabbit kit (Zhongshan Jinqiao biotech company, Beijing, China). The immunoreaction was visualized using 3, 3'-diaminobenzidine (DAB) staining. Finally, then sections were counterstained with hematoxylin, dehydrated. For negative controls, the primary antibodies were replaced with PBS. All sections were examined microscopically and immunostaining were evaluated as described previously [16]. Intensity was graded as follows: neg, negative stain; 1+, focally weakly positive stain; 2+, focally intensely or diffusely weakly positive stain; 3+, diffusely intensely positive stain.

Statistical analysis

Statistical analysis was performed using SPSS (Version 16.0; Chicago, IL, USA). The associa-

Table 1. Adipophilin expression in lung cancer and normal lung tissues

Groups	N	Expressional levels				P*
		neg	1+	2+	3+	
LAC	41	6	23	7	5	0.001[a]
LSCC	21	7	12	2	0	0.335[b]
Normal lung tissues	24	11	12	1	0	0.007[c]

[a]LAC versus normal lung tissue; [b]LSCC versus normal lung tissue; [c]Lung cancer versus normal lung tissue; *P<0.05 by Mann–Whitney U test; LAC, lung adenocarcinoma; LSCC, lung squamous cell carcinoma; Lung cancer, LAC+LSCC.

Table 2. Correlation between clinicopathological characteristics and adipophilin expression in lung cancer

Parameters	N	Expressional levels				P*
		neg	1+	2+	3+	
Age						
<60	32	7	18	5	2	0.802
≥60	30	6	17	4	3	
Gender						
Male	41	8	23	8	2	0.722
Female	21	5	12	1	3	
Grade						
G1+G2	34	9	19	4	2	0.171
G3	28	4	16	5	3	
Histology						
LAC	41	6	23	7	5	0.026
LSCC	21	7	12	2	0	
Lymphatic invasion						
N0	33	7	17	7	2	0.666
N+	29	6	18	2	3	
Tumor size						
≤3	22	3	13	5	1	0.654
3<T≤7	31	8	17	2	4	
>7	9	2	5	2	0	
TNM stage						
I+II	35	7	19	7	2	0.675
III+IV	27	6	16	2	3	

*P<0.05 by Mann–Whitney U test.

tions between the adipophilin expression status and clinicopathological factors were analyzed using Mann–Whitney U test or Kruskal-Wallis test. The significant difference of adipophilin protein expression levels between lung cancer and normal tissue were analyzed using Mann–Whitney U test. In all tests, two-sided *P*-values <0.05 were considered statistically significant.

Results

Validation of adipophilin expression by western blotting

To confirm the differential expression of adipophilin, we performed western blotting analysis to detect the differential expression in 10 pairs of lung adenocarcinoma and normal lung tissues. Consistent with the previous findings, adipophilin is significantly increased in tumor tissues compared with adjacent normal tissues (P<0.01, **Figure 2**).

Detection of the expression of adipophilin in lung cancer and normal lung tissues by IHC

The expression and cellular distribution of adipophilin were determined by IHC in 62 lung cancer samples including 41 lung adenocarcinoma specimens and 21 lung squamous cell carcinomas and 24 normal lung tissues. In 12 lung adenocarcinoma tissues, adipophilin were diffusely intensely or weakly stain in the cytoplasm or the vicinity of the cell membrane in most tumor cells (**Figure 3A** and **3B**). However, adipophilin were only focally or weakly positive stain in most of lung squamous cell carcinomas and normal lung specimens (**Figure 3C** and **3D**). Compared with normal specimens, the expression level of adipophilin in adenocarcinoma was significantly increased (P=0.001). The upregulation of adipophilin were also observed in the lung cancer (lung adenocarcinoma + squamous cell carcinoma) compared with normal lung specimens (P=0.007) (**Table 1**). While there were no significant difference of adipophilin expression between lung squamous cell carcinoma and normal lung specimens. Furthermore, we analyzed the association between clinicopathological characteristics and adipophilin expression in lung cancer. The correlation between the clinicopathological characteristics and adipophilin expression is listed in **Table 2**. The results indicated the upregulation of adipophilin in lung adenocarcinoma compared with squamous cell carcinoma (P=0.026). The expression of adipophilin did not correlated with other clinicopathologic factors such as lymph node metastasis, patients' age, gender, tumor size, grade, and TNM stage.

Discussion

We performed iTRAQ-based quantitative proteomic analysis to identify the differentially

expressed membrane proteins between lung adenocarcinoma and matched lung normal tissues. Adipophilin was significantly upregulated for 2.46 fold in lung adenocarcinoma tissues compared with normal tissues. In this study, we confirmed adipophilin expression level in the two types tissues, then further evaluated the association between adipophilin expression and clinicopathological factors in lung cancer patients.

Adipophilin is a member of PAT (PERILIPIN, ADRP, and TIP47) family. It was first identified in 1246 Cells during adipocyte differentiation and found as a membrane-associated protein by investigating localization [10, 11]. Adipophilin was expressed not only at high levels in adipose tissue but also at lower level in many different types of cells and various tissues such as lung, liver, testes, etc [12, 13].

Several studies suggested that adipophilin might be involved in uptake of long chain fatty acids, the formation or stabilization of lipid droplets in adipocytes and serve as a saturable transport component for long chain fatty acids [17, 18]. Adipophilin also play an important role in this novel mechanism for the transfer of lipids from lipid droplet to the type 2 lung epithelial cells for the production of surfactant phospholipids, but such a role has not been substantiated [19, 20]. The study by Torday et al [21] shows that adipophilin derived from lipofibroblast coordinates alveolar type II epithelial' synthesis of surfactant phospholipid and surfactant protein-B. Recently, few studies showed adipophilin significantly expressed in clear-cell renal carcinoma and colorectal cancer cells [14-16].

In this study, we performed western blotting analysis to verify the result from quantitative proteomics. Consistent with our previous findings, the result showed that adipophilin in lung adenocarcinoma was significantly higher than in normal lung tissues. Furthermore, we evaluated the association between adipophilin and clinicopathological characteristics using immunohistochemistry. The results showed that the expression of adipophilin was increased in lung adenocarcinomas compared with normal lung tissues and lung squamous cell carcinomas. While we did not found significant difference of adipophilin expression between lung squamous cell carcinomas and normal lung tissues. In addition, our results indicated the expression of adipophilin in lung cancer did not correlate with other clinicopathologic factors such as lymph node metastasis, age, gender, tumor size, grade, and TNM stage. Few studies reported adipophilin was upregulated in clear-cell renal carcinoma and colorectal cancer cells and associated with the differentiated grade [14, 16], but we did not found the association with grade. Our data indicated that adipophilin might play an important role in carcinogenesis of lung adenocarcinoma. To our knowledge, this is the first report to investigate adipophilin expression in lung cancer and indicate the correlation with pathological type of lung cancer. It has been demonstrated that adipophilin are inducible by hypoxia-inducible factor 1 (HIF-1), a heterodimer of HIF-1α and ARNT (Ah receptor nuclear translocator; HIF-1β) [22]. In addition, HIF-1α plays an important role in lung cancer progression and metastasis through activation of many target genes which are involved in important aspects of cancer biology [23]. Although the exact mechanism of the interactions between adipophilin and HIF in cancer has not been investigated, we speculated adipophilin may play a potential role in tumorigenesis through adaptation to hypoxia which is essential for tumor progression. Several studies showed dietary fat including dairy products, saturated fats, and lipids increased the risk for lung cancer [24-26] and adipophilin might be involved in uptake of long chain fatty acids. Taken together, we hypothesize adipophilin may play a possible role in carcinogenesis by involving in fatty acid uptake and transport in lung.

However, there are some limitations in the present study. Firstly, only few IIIB and IV stages specimens were used because surgery was mostly involved in patients with early stage lung cancer, which may results in selection bias, secondly our results may be still preliminary results because of relatively small sample sizes and experimental investigations involving a larger group of patients is needed to further evaluate.

In conclusions, we confirmed the result from quantitative proteomics that adipophilin were upregulated in lung adenocarcinoma by western blotting and immunohistochemistry. Our results indicate that the upregulation of adipophilin was potentially involved in the carcino-

genesis of lung adenocarcinoma and adipophilin may serve as a potential marker for distinguishing between lung adenocarcinoma and lung squamous cell carcinoma. However, a study of larger populations is required to further confirm our results.

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Disclosure of conflict of interest

None.

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