Expression of Polymerase I and Transcript Release Factor (PTRF) in Lung Adenocarcinoma

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Abstract

Aim: In our study we found the Lung cancer is a most common malignant tumor and important cause of cancer-related death worldwide [1]. The prognosis for lung cancer is still low despite the important amelioration that has been made in the diagnosis and treatment of this disease [2]. Lung Adenocarcinoma is the most ordinary type of lung cancer. Adenocarcinoma is also a histologically, and genetically heterogeneous disease, packed by gradual accumulation of various genetic and epigenetic alterations leading to the activation of several molecular pathways and resulting in markedly different responses to the same treatment [3]. PTRF was cloned in 1998 and was first described to be involved in RNA transcription machinery [4]. Polymerase I and Transcript Release Factor (PTRF) is a cytoplasmic protein required for Cav1-dependent formation of caveolae. The effects of PTRF related to caveolae suggest that this protein may play important roles in health and disease. PTRF was initially reported to be involved in the termination of the transcription process [5]. PTRF is a resident protein in caveolae [6] and is widely expressed in a range of tissues, with highest expression in adipocytes, cardiac and skeletal muscles and osteoblasts [7]. The functional role of PTRF in caveolae formation has only recently been described. Loss of PTRF is accompanied by reduced numbers of caveolae [8]. Re-expression of PTRF in cell lines that have reduced or lack PTRF results in caveolae formation [4]. PTRF is an essential protein for caveolae formation and the Caveolae are specialized membrane subdomains involved in cellular functions such as migration, signaling and traffic. PTRF was highly expressed in various cells, including adipocytes, osteoblasts and smooth muscle cells [5]. Low expression in carcinoma of prostate, breast and colon has been demonstrated to be related with tumor progression [6]. Lack of PTRF expression is reported in prostate cancer, and ectopic PTRF expression in prostate cancer cells inhibits tumor growth and metastasis. Expression of PTRF reduces the motility of PC3 cells, a metastatic prostate cancer cell line [9]. On the other side, the PTRF expression is up regulated in the glioblastoma tissue and pancreatic carcinoma [10]. All these results indicate the PTRF may play important roles in carcinogenesis.

Materials and Methods

Tissue: Thirty cases of fresh lung AdC and corresponding normal lung tissues from lung AdC patients received neither chemotherapy nor radiotherapy were obtained from Department of Cardiothoracic Surgery, Xiang ya Hospitals of Central South University, China, and stored until use. Five pairs of lung AdC and corresponding normal lung tissues were used for Western blotting. An additional set of formalin-fixed and paraffin embedded archival tissue specimens including 60 cases of lung AdC and 60 corresponding normal lung tissues from the lung AdC patients undergoing curative surgery, and used for immunohistochemistry.

Cell lines: The H1975, LTEPA2, A549, HBE cell lines were obtained from ATCC.

Keywords: PTRF, IHC, Adenocarcinoma

Introduction

Lung cancer is a most important cause of cancer-related death worldwide [1]. The prognosis for lung cancer is still low despite the important amelioration that has been made in the diagnosis and treatment of this disease [2]. Lung Adenocarcinoma is the most ordinary type of lung cancer. Adenocarcinoma is also a histologically, and genetically heterogeneous disease, packed by gradual accumulation of various genetic and epigenetic alterations leading to the activation of several molecular pathways and resulting in markedly different responses to the same treatment [3]. PTRF was cloned in 1998 and was first described to be involved in RNA transcription machinery [4]. Polymerase I and Transcript Release Factor (PTRF) is a cytoplasmic protein required for Cav1-dependent formation of caveolae. The effects of PTRF related to caveolae suggest that this protein may play important roles in health and disease. PTRF was initially reported to be involved in the termination of the transcription process [5]. PTRF is a resident protein in caveolae [6] and is widely expressed in a range of tissues, with highest expression in adipocytes, cardiac and skeletal muscles and osteoblasts [7]. The functional role of PTRF in caveolae formation has only recently been described. Loss of PTRF is accompanied by reduced numbers of caveolae [8]. Re-expression of PTRF in cell lines that have reduced or lack PTRF results in caveolae formation [4]. PTRF is an essential protein for caveolae formation and the Caveolae are specialized membrane subdomains involved in cellular functions such as migration, signaling and traffic. PTRF was highly expressed in various cells, including adipocytes, osteoblasts and smooth muscle cells [5]. Low expression in carcinoma of prostate, breast and colon has been demonstrated to be related with tumor progression [6]. Lack of PTRF expression is reported in prostate cancer, and ectopic PTRF expression in prostate cancer cells inhibits tumor growth and metastasis. Expression of PTRF reduces the motility of PC3 cells, a metastatic prostate cancer cell line [9]. On the other side, the PTRF expression is up regulated in the glioblastoma tissue and pancreatic carcinoma [10]. All these results indicate the PTRF may play important roles in carcinogenesis.

Material and Methods

Immunohistochemistry and western blot were used to verify the PTRF expression in lung adenocarcinoma (LADC) and normal lung (NL). And PTRF expression status was also detected in the lung adenocarcinoma cells by western blot.

Results: Totally 41 differentially expression proteins were identified. Eighteen proteins are up regulated in the lung adenocarcinoma and 23 proteins are down regulated in the lung adenocarcinoma. Western blot and IHC shows the PTRF expression is down regulated in the lung adenocarcinoma. PTRF expression has close relationship with the gender and T stage.

Conclusion: PTRF expression is down-regulated in lung adenocarcinoma tissue and lung carcinoma cells and the loss of PTRF expression occur with progression of lung adenocarcinoma. PTRF expression has a close relationship with gender and T stage of lung adenocarcinoma. PTRF may be apotential target in the future.

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Western blot analysis

Tissue samples preparation: lung adenocarcinoma and adjacent

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normal lung tissue, 10 cases; 2) protein extract: tissue lysate was added (1 mmol/L EDTA, 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 0.5% Triton X-100, 0.5% NP40, 1 mM PMSE, 25 μg/mL Aprotinin and 25 μg/mL Leupeptin) to lung adenocarcinoma and adjacent normal lung tissue, cracking ice for an hour, 12,000 rpm, centrifuged at 4 °C for 30 minutes to extract the supernatant of tissue total cell protein, protein concentration measured using the Bradford method, -70 °C refrigerator spare. 3) SDS-PAGE electrophoresis: 50 μg of total protein with an equal volume of loading buffer (135 mM Tris-HCl pH6.8, 20% glycerol, 0.02% bromophenol blue, 6% SDS, 10% β- mercaptoethanol) after thermal denaturation then separated by electrophoresis on 10% SDS-PAGE, the protein electrophoresis after power was transferred to PVDF blots. 4) PVDF membrane with 5% skim milk at room temperature for 2 hours closed. 5) Anti PTRF (1:1000; Abeam) was incubated overnight at 4 °C, then with TBST wash buffer film 3× 10 min; 6) The film 1:4000 diluted HRP labelled secondary antibody for 1 hour at room temperature, TBST buffer the membrane was washed 3 × 10 min; 7) Uniformly covered with the developing PVDF membrane brightening agent, exposure to the darkroom 3-5 minutes, then developed until significant bands, the final fixing, CAV1 and ITGB1 to β-actin (1:5000) as an internal reference, FLOT1 to GAPDH (1:4000) is as an internal reference. The experiment was repeated three times. 8) Image J software analysis of protein bands of grey value, to calculate the relative strength of each protein. The experiment was repeated three times. 8) Image J software analysis of protein bands of grey value, to calculate the relative strength of each protein.

Western blot to identify the cell membrane of purity, the following steps

1) Sample: next to the previously-purified lung adenocarcinoma and normal lung tissue cell membrane 2) Protein extraction: Sample added insoluble membrane lysate see solution preparation, cracking ice for 2 hours, 12 000 g, and 4 °C for 30 min centrifugation supernatant is plasma membrane proteins, RC-DCTM assay kit containing SDS the membrane protein concentration.

One-dimensional gel electrophoresis: sample per well membrane protein 50 μg, separating gel concentration of 10%, reduced the use of plastic at a concentration of 4.8%. SDS-PAGE of plasma membrane proteins were separated on the gel. 4) Transfer membrane: membrane protein electrically transferred to nitrocellulose membranes (PVDF) on. 5) Closed: PVDF membrane was taken after the transfer film, placed in blocking solution overnight at 4 °C or more, or at least stand for 2 hours. 6) Plus antibody: Remove the film after the closing, 75KDa PVDF membrane was cut in half at the high molecular weight half into a small plastic membrane under two antigens (Na + / K + -ATPase (112KDa) and prohibiting (30KDa) molecular size, bag and then Anti-Na + / K + -ATPase antibody ([μl] L stock solution was diluted with the antibody dilution 1 mL). half of the low molecular weight membrane into the other sachet, was added 4 ng Anti-prohibiting antibody (the antibody was treated with 4 L diluent into 2 mL), the reaction shaking one hour, the solution was washed three times with TBST, 5 minutes each. remove the diaphragm, on the back surface of the pouch into a new, 0.3 ng with horseradish peroxidase was added (HRP) labelled secondary antibody (diluted with 4 L antibody dilution solution to 2 mL), shaking for 45 minutes, and finally washed 3 times with TBST, 5 minutes. 7) Development: ECL reagents light, developing and fixing.

Immunohistochemistry analysis

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded tissue sections using a standard immunohistochemical technique. Briefly, 4 μm of tissue sections were deparaffinised, rehydrated and treated with an antigen retrieval solution (10 mmol/l sodium citrate buffer, pH 6.0). The sections were incubated with anti-PTRF antibody (1:40) overnight at 4°C and were then incubated with 1:1,000 dilution of biotinylated secondary antibody followed by avidin-biotin peroxidase complex (DAKO) according to the manufacturer’s instructions. Finally, tissue sections were incubated with 3’, 3’-diaminobenzidine (Sigma-Aldrich) until a brown color developed and counterstained with Harris modified haematoxylin. In negative controls, primary antibodies were omitted. Immunostaining was blindly evaluated by two investigators in an effort to provide a consensus on staining patterns by light microscopy. A quantitative score was performed by adding the score of staining area and the score of staining intensity for each case to assess the expression levels of the proteins as previously described by us.

Results

Screening of lung adenocarcinoma and adjacent normal lung tissue plasma membrane proteins

Differential screening of plasma membrane protein expression to simultaneously satisfy the following conditions: 1. In two technical replicates were identified as the plasma membrane protein; protein identification; 2. Membrane proteins based on two or more specific peptide; 3. Membrane proteins differentially expressed proteins in the average change in the ratio of two experiments 1.5 or ≤ 0.667 (t -test, p<0.05). According to the differences in protein screening requirements, the present studies were screened to 41 differentially expressed proteins, the results in (Tables 1-3). In these differentially expressed proteins, 30 proteins were up-regulated in normal lung tissues, 27 proteins were down-regulated in lung adenocarcinoma. Wherein Polymeerase I and transcript release factor (PTRF) normal lung tissue down-regulated in lung cancer tissue than the next.

Western blotting confirmed the PTRF expression in the cell lines of lung

The PTRF expression is lower in the adenocarcinoma cells (A549 cells, H1975 cells and LTEPA2 cells) compared with the normal bronchial epithelial cells (HBE) (Figure 1). The difference has statistical significance (P<0.05)

Western blotting confirmed the PTRF expression in the tissue of adenocarcinoma: The expression of PERF was lower compared with in the corresponding normal lung tissues. The difference has statistical significance (P<0.05).

Immunohistochemical analysis

The result of immunohistochemistry shows the expressive levels of PTRF in normal lung tissue is strong positive, while the expression of PTRF in lung adenocarcinoma presented differential appearance, from moderate positive to negative (Figure 2).

A: Normal B: non-metastasis metastasis C: lung AdC with metastasis (LM AdC).

Figure 3A-1 and 3A-2 shows strong staining of PTRF in normal lung tissue; Figure 3B-1 shows moderate staining of PTRF in primary AdC tissue; Figure 3B-2 shows weak staining of PTRF in primary AdC tissue. Figure 3C-1 shows moderate staining of PTRF in lymph node metastasis AdC tissue. Figure 3C-2 shows weak staining of PTRF in lymph node metastasis AdC tissue. Figure 1-6A representative result of immunohistochemistry shows the expressive levels of PTRF in normal lung tissue (NLT), lung AdC non metastasis (non-LNM AdC), and lung AdC with metastasis (LM AdC). Representative
results of immunohistochemistry of PTRF in tissue specimens. PTRF was expressed in membrane and cytoplasm. Presence of the specific protein is indicated by the amount of brown staining. Nuclei were counterstained with haematoxylin (blue) for visualization purposes.

### Results

#### Expression of PTRF and the relationship between the level of expression and the clinicopathological factors of lung adenocarcinoma

The proteins that met the lung adenocarcinoma with lymph node metastasis were considered as differential proteins between the

| Acession Number | Protein name | Mean | SD  | P     | Subcellar source |
|-----------------|--------------|------|-----|-------|------------------|
| P05023          | Sodium/potassium-transporting ATPase subunit alpha-1 | 2.456 | 0.131 | 0.003 | PM               |
| P05362          | Intercellular adhesion molecule 1 | 9.032 | 0.271 | 0     | PM               |
| Q00610          | Clathrin heavy chain 1 | 2.607 | 0.026 | 0     | PM               |
| P06731          | Carcinoembryonic antigen-related cell adhesion molecule 5 | 13.094 | 0.081 | 0     | PM               |
| P15941          | Mucin-1 | 9.713 | 0.163 | 0     | PM               |
| P05556          | Integrin beta-1 | 1.583 | 0.111 | 0     | PM               |
| P08575          | Leukocyte common antigen | 3.375 | 0.057 | 0     | PM               |
| P06733          | Alpha-elastase | 1.72 | 0.016 | 0     | M                |
| Q99828          | Calcium and integrin-binding protein 1 | 5.191 | 0.056 | 0     | PM               |
| P10871          | Ig mu chain C region | 2.24 | 0.106 | 0.002 | PM               |
| P01833          | Polymeric immunoglobulin receptor | 6.916 | 0.078 | 0     | PM               |
| P08311          | Cathepsin G | 2.503 | 0.041 | 0     | M                |
| O00299          | Chloride intracellular channel protein 1 | 2.619 | 0.11 | 0.002 | PM               |
| Q75955          | Flotillin-1 | 2.281 | 0.018 | 0     | PM               |
| P04083          | Annexin A1 | 2.419 | 0.273 | 0.012 | M related        |
| Q16853          | Membrane primary amine oxidase | 0.147 | 0.007 | 0     | PM               |
| O00159          | Myosin-IVc | 0.341 | 0.011 | 0     | PM               |
| Q99758          | ATP-binding cassette sub-family A member 3 | 0.393 | 0.006 | 0     | PM               |
| Q03135          | Caveolin-1 | 0.115 | 0.004 | 0     | PM               |
| P62158          | Calmodulin | 0.285 | 0.003 | 0     | PM               |
| P21964          | Catechol O-methyl-transferase | 0.236 | 0.01 | 0     | PM               |
| P10301          | Ras-related protein R-Ras | 0.086 | 0.002 | 0     | PM               |
| P62834          | Ras-related protein Rap-1A | 0.436 | 0.006 | 0     | PM               |
| P22748          | Carbonic anhydrase 4 | 0.204 | 0.011 | 0     | PM               |
| P00387          | NADH-cytochrome b5 reductase 3 | 0.22 | 0.013 | 0     | PM               |
| P07099          | Epoxide hydrolase 1 | 0.387 | 0.001 | 0     | PM               |
| Q9BVC6          | Transmembrane protein 109 | 0.442 | 0.009 | 0     | PM               |
| P08758          | Annexin A5 | 0.223 | 0.02 | 0.007 | M related        |
| P50148          | Guanine nucleotide-binding protein G(q) subunit alpha | 0.123 | 0.009 | 0     | PM               |
| P27105          | Erythrocyte band 7 integral membrane protein | 0.496 | 0.046 | 0.003 | Secreted        |
| O04373          | Periplakin | 0.285 | 0.01 | 0     | M                |
| O96009          | Napsin-A | 8.956 | 0.13 | 0.004 | Secreted        |
| A7Y9J9          | Mucin-5AC | 3.39 | 0.1587 | 0.006 | Secreted        |
| P04406          | Glyceraldehyde-3-phosphate dehydrogenase | 3.575 | 0.038 | 0.005 | Cytoplasm       |
| Q99536          | Synaptic vesicle membrane protein VAT-1 homolog | 0.49 | 0.007 | 0.012 | Cytoplasm       |
| Q09666          | Neuroblast differentiation-associated protein ANK | 0.056 | 0.002 | 0.002 | Nucleus         |

| Table 1: The identified differentially expressed plasma membrane proteins between the LAdc and adjacent normal lung tissue. | Table 2: The difference of PTRF expression in normal lung tissues and lung adenocarcinoma. |
three types of tissues: normal lung tissues, and lung adenocarcinoma. We detected that the expression of PTRF was significantly up-regulated in normal lung tissues and down-regulated in lung adenocarcinoma. But there is no difference between the AdC and with lymph node metastasis.

From the data available PTRF expression has the relationship with the gender, and primary tumor size, but not with age, smoking, tumor differentiation, regional lymph node metastasis and clinical stage.

### Discussion

The aim of this study was to investigate the expression of PTRF occur with the development and progression of lung adenocarcinoma. There are 41 differentially expression proteins were identified with 18 proteins up regulated and 23 proteins down regulated in the lung adenocarcinoma. PTRF was one of above identified differentially expressed plasma membrane proteins. Western blot and immunohistochemistry analysis demonstrate that PTRF expression is lower in lung adenocarcinoma than the coupled normal lung tissues. And PTRF expression is down regulated in the cell lines of lung adenocarcinoma compared with the normal epithelial cell line. PTRF (Polymerase I and Transcript Release
Factor), is a protein essential for RNA transcription [4] is necessary for the formation of caveolae [5] and, reduction the expression of the tissues and cells of lung adenocarcinoma. This introversion of the cell surface are associated with vesicular transport process, cholesterol homeostasis, signal transduction [6] and the control of lipolysis [7]. Bai and his colleagues not long ago reported that the promoter methylation was associated with down-regulation of PTRF protein in cell lines of breast cancer and breast tumor tissue [9]. Prostate cancer was associated with progression in case of loss of expression of PTRF [10]. It had recently been demonstrated that the loss of expression of PTRF in HBE tumorigenic cells compared to normal human bronchial epithelial cells [11]. PTRF has been implicated in a variety of pathophysiological processes. In contrast, PTRF expression was recently demonstrated to have relationship with metastatic potential of pancreatic cancer cells and the higher expression of PTRF was demonstrated in glioblastoma tissues [12]. PTRF is necessary for the formation of caveolae and, as expected, a reduction of the expression of PTRF in adenocarcinoma tissues and cells lines have no immune reactivity was detected in tissues and cells. We suggest that, the loss of PTRF associates with the progression of adenocarcinoma. Zeyad D and his colleagues have previously demonstrated that the expression PTRF in cancerous prostate cells that can down-regulation the proteins that control the growth, significantly decreased cell migration and reduces tumor growth, and metastasis in vivo [12]. And it has been shown that the PTRF deficit expression Decreases the migration of prostate cancer cells. And it should be noted that the PTRF not only plays role information of caveolae, and may regulate signal transduction. Caveolae as platforms for locating or sequester other molecules signaling or to orchestrate signaling, such as the metabolism of lipids. In the non-attendance of caveolae, caveolin and cell surface receptors transiently associate with several mobile lipid rafts on the surface of the cell membrane and the loss of PTRF will result in a decrease in associations alteration of signaling pathways within the membrane which can cause a change in the performance of the cell and caveolae, so that the shedding of caveolae can contribute to or facilitate tumor growth, Thus PTRF may play an indirect role in the biology of the cell by regulating the number of caveolae present. The evidence that PTRF and other protein do not exhibit exactly the same pathophysiology, while PTRF is missing in cancer progression but caveolin expression is unaltered or increased and that PTRF appears to be associated with certain proteins, suggest that PTRF may also have specific effects within caveolae. Moreover, these effects may differ between cells, for example the relationship between PTRF and hormone sensitive lipase in adipocytes. One idea to explain this is that PTRF and caveolin are necessary for the formation of caveolae but within each caveolae PTRF associates and regulates specific cell signaling pathway. Besides, the targeting PTRF can provide new opportunities to develop agents’ specific treatment for diseases such as cancer. PTRF expression is down-regulated in lung adenocarcinoma tissue and lung carcinoma cells. PTRF expression has a close relationship with gender and T stage of lung adenocarcinoma.

### Conclusion

In conclusion the expression of PTRF is down-regulated in lung adenocarcinoma tissue and lung carcinoma cells and the loss of PTRF expression occur with progression of lung adenocarcinoma, So there is correlation between PTRF expression, gender and the T stage of lung adenocarcinoma.

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