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Increased expression of phosphorylated adducin in tumor cells

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Abstract

Objective: This preclinical research was designed to study the phosphorylation level of adducin in cancer tissues, healthy tissues, and malignant tumor cells to determine the relationship between adducin and cancer.

Methods: Western blotting was used to detect the expression level of phospho-adducin in tissues and cell lines.

Results: Phospho-adducin at Ser662 was detected in all tumor cells and cancer tissues. The main type of phospho-adducin at Ser662 was γ-adducin in healthy lung tissue, and α-adducin in both lung cancer tissue and para-lung cancer tissue. Phosphorylation of adducin at Thr445 was observed in healthy lung tissue, adjacent healthy tissue, and cancer tissue, but was not detected in any other malignant cells. Additionally, more phosphorylation of adducin at Thr445 was seen in cancer tissue than in adjacent healthy tissue.

Conclusion: The abnormal expression of phospho-adducin at Ser662 and Thr445 may be associated with tumorigenesis, suggesting a novel approach for the diagnosis and treatment of tumors.

Keywords

Lung cancer, carcinogenesis, adducin, phosphorylation, Ser662, Thr445

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Introduction

Adducin is a membrane skeletal protein localized at spectrin–actin junctions that is found in many tissue types. It is composed of three subunits, α-adducin (ADD1), β-adducin (ADD2), and γ-adducin (ADD3), which have molecular weights of 120 kDa, 110 kDa, and 80 kDa, respectively. Adducin self-associates into α/β or α/γ heterodimers or heterotetramers. It functions downstream of protein kinase (PK) A, PKC, Rho-kinase, and Ca^{2+}/Calmodulin in cytoskeletal meshwork formation, cellular signal transduction, ionic transportation, cell motility, cellular proliferation, and cell–cell junctions.[1–4]

Recently, adducin has been suggested to play a crucial role in carcinogenesis. However, few studies have identified changes in levels of adducin and/or phospho-adducin in cancers. ADD1 was found to be up-regulated in ovarian cancer and to hinder cell proliferation, the formation of soft agar colonies, and tumor invasion, suggesting that it may act as a tumor suppressor.[5] In another study, total adducin levels were reported to decrease while Ser660-phosphorylated adducin levels increased in renal carcinoma.[6] These studies indicated that adducin is involved in tumorigenesis and tumor development.

The present study aimed to measure levels of adducin and phospho-adducin in different tumor cells to determine the role of adducin in tumorigenesis.

Materials and methods

Cells and tissues

Red blood cells, the A549 human lung carcinoma cell line, the human embryonic kidney cell line 293, the human breast cancer cell line MCF, the human intestinal adenocarcinoma cell line CaCo2, and the human prostatic adenocarcinoma cell line LNCaP were purchased from the biological sciences division of the Chinese Academy of Sciences (Shanghai, China). All cells were maintained in Dulbecco's modified Eagle's medium (Corning, Corning, NY, USA) containing 10% calf serum, streptomycin, and 100 IU/mL penicillin (Hyclone Laboratories Inc., Logan, UT, USA). MCF cells were transfected with pEF-BOS-HA-adducin with the pSVIISRα vector (Invitrogen Corp., Carlsbad, CA, USA) containing the neomycin resistance gene using Lipofectamine (GIBCO® Cell Culture, Carlsbad, CA, USA) according to the manufacturer's instructions. Neomycin-resistant clones were selected using 100 µg/mL for 7 days and named MCF+adducin. Clinical specimens, including lung cancer tissues, adjacent tissues, and healthy lung tissues, were obtained from Zhejiang Cancer Hospital. Lung protein was extracted by lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 1% w/v NP-40, 2 mM vanadate, and 2× complete protease inhibitor cocktail [Roche, Basel, Switzerland]) for 24 hours with a polytron (Ultra-Turrax T25; IKA-Werke, Staufen, Germany).

Western blot

Lysates of cells were prepared in phosphate-buffered saline (pH 7.2) with 1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, and 1× Complete Protease Inhibition tablets (Roche Diagnostics GmbH, Mannheim, Germany), resolved by SDS polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. Protein loading was adjusted according to the internal reference protein. The membrane was blocked with 5% fetal bovine serum at room temperature for 1 hour, then probed with an appropriate primary antibody as follows overnight at 4°C: rabbit anti-α-adducin antibody at a

1:1,000 dilution (Millipore, Billerica, MA, USA), rabbit anti-β-adducin antibody or rabbit anti-γ-adducin antibody (at a 1:1,000 dilution; abcam, Cambridge, MA, USA), anti-phospho-adducin (Ser662) at a 1:1,000 dilution (Millipore), anti-phospho-adducin (Thr445) at a 1:1,000 dilution (Millipore), purified anti-α-adducin (BioLegend, San Diego, CA, USA), or mouse anti-β-actin antibody at a 1:2,500 dilution. Immunoreactive proteins were detected with either goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hour, and visualized with the ECL detection system (Millipore). ImageJ software was used to measure the optical density of individual bands.

Bioinformatics analysis

Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/index.html) was used to screen the expression levels of adducin in other types of tissues based on The Cancer Genome Atlas and Genotype–Tissue Expression data.

Ethical statements

All techniques were performed in accordance with relevant institutional guidelines. Informed consent was obtained from all participants prior to their inclusion in this study. Clinical specimens were approved for use by the Ethics Committee of Zhejiang Cancer Hospital on 30 May 2018.

Results

Adducin expression in different cell lines and tissues

Adducin expression in various cell lines, cancer tissues, adjacent healthy tissues, and healthy tissues is shown in Figure 1. Abundant ADD1 expression was detected in the A549 cell line, while slightly reduced ADD1 expression was detected in 293 cells. Healthy lung tissue was found to only express ADD3, while ADD1 was detected in cancer tissues and adjacent healthy tissues.

Phospho-adducin (Ser662) expression in different cell lines and tissues

Phospho-adducin (Ser662) expression in seven cell lines is shown in Figure 2a. Red blood cells and MCF+adducin were found to only express ADD1, while all three subunits of adducin were detected in 293, MCF, CaCo2, LNCaP, and A549 cell lines. The concentration of phospho-adducin was found to vary in different tissues, as shown in Figure 2b and 2c. The main subunit expressed in healthy lung tissue is ADD3, whereas cancer tissues and adjacent healthy tissues mainly express ADD1.

Phospho-adducin (Thr445) expression in different cell lines and tissues

Phospho-adducin (Thr445) expression in seven cell lines and tissues is shown in Figure 3. No phospho-adducin (Thr445)

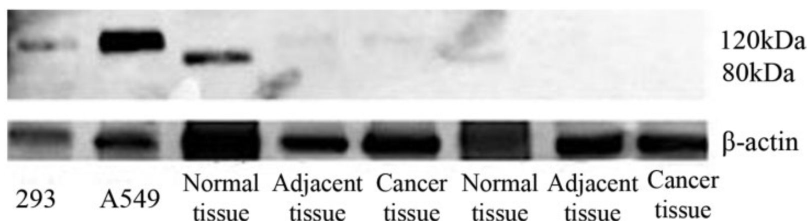

Figure 1. The expression of adducin in different cell lines and tissues. Western blot was used to detect α-adducin at 120 kDa and γ-adducin at 80 kDa.

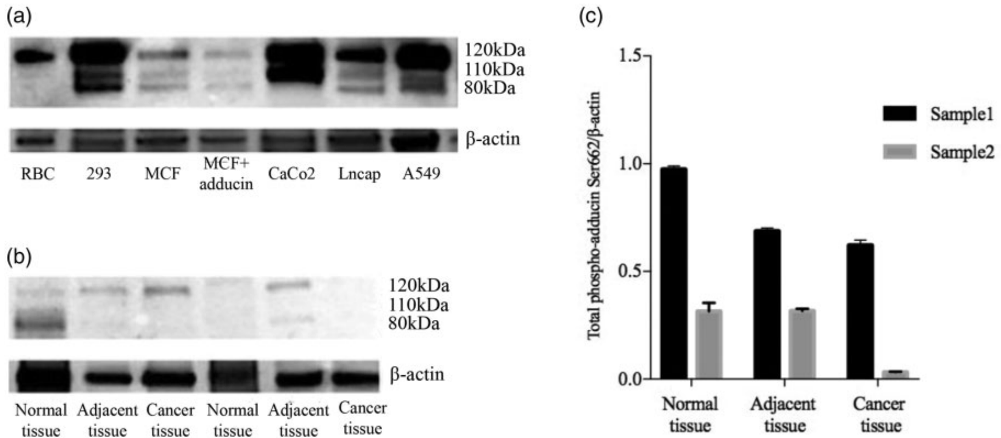

Figure 2. Different expression of phospho-adducin at Ser662 in cell lines and tissues.
a: Phospho-adducin at Ser 662 in seven cell lines. b: Phospho-adducin at Ser 662 in lung tissues.
c: Densitometric quantification of total phospho-adducin at Ser 662 in lung tissues.

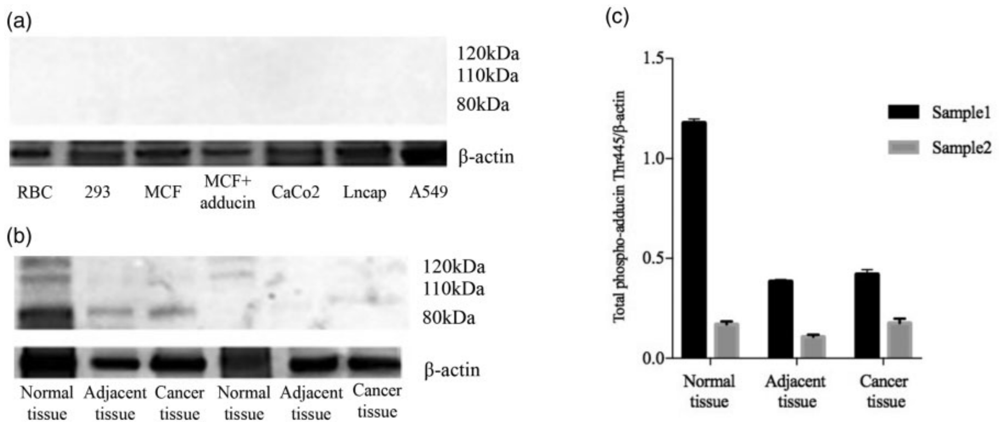

Figure 3. Different expression of phospho-adducin at Thr445 in cell lines and tissues.
a: Phospho-adducin at Thr445 in seven cell lines. b: Phospho-adducin at Thr445 in lung tissues.
c: Densitometric quantification of total phospho-adducin at Thr445 in lung tissues.

expression was detected in any cell lines, but it was observed in healthy tissue, cancer tissue, and adjacent healthy tissue.

Phospho-adducin (Thr445) expression in cancer tissue and adjacent healthy tissue from lung cancer patients

To further explore the expression of phospho-adducin (Thr445), we examined cancer tissue and adjacent healthy tissue from four lung cancer patients. As shown in Figure 4, its expression was higher in cancer tissues of all four patients compared with adjacent healthy tissue.

Identification of ADD3 in other types of cancer tissues

Because of a lack of clinical specimens, we analyzed the expression of ADD3 in other

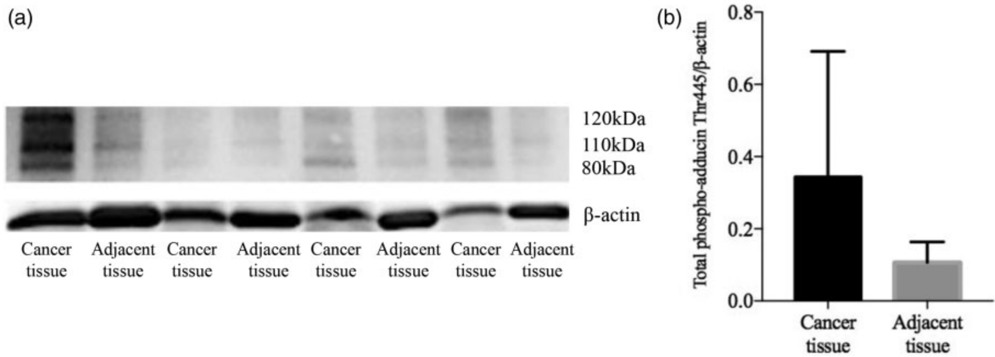

Figure 4. The expression of phospho-adducin at Thr445 in cancer tissue and paired adjacent tissue from four lung cancer patients.

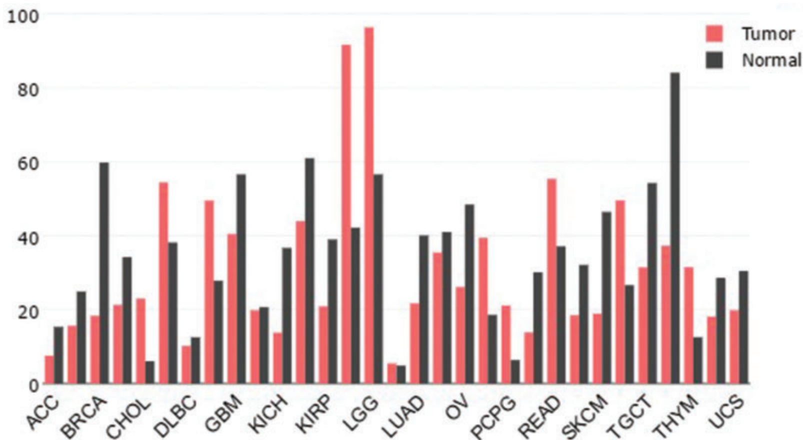

Figure 5. Gene expression profile of ADD3 across different tumor samples and paired healthy tissues.

kinds of cancers using GEPIA. We found that ADD3 was down-regulated in lung cancer, as well as most other cancers including invasive breast carcinoma, kidney renal papillary cell carcinoma, and ovarian cancer (Figure 5).

Discussion

Changes in protein phosphorylation are one of the most common predisposing factors in tumorigenesis. The abnormal regulation of phosphorylation can result in instability and unusual protein activity, leading to irregular cell proliferation, dysdifferentiation, and apoptosis suppression.

Adducin functions through changing its states between phosphorylation and dephosphorylation. It plays an important role in the regulation of cell–cell adhesion,[6] and ADD1 and ADD3 in particular are enriched at intercellular junctions in epithelial cells and mucosal epithelia. Previous studies have indicated that adducin also plays a role in tumorigenesis, invasion, and metastasis. Therefore, the present study investigated the differential expression of adducin and phospho-adducin in

various cells and tissues to examine its function in tumorigenesis.

Bowen et al.[7] showed that *ADD1* is synchronously upregulated with the overexpression of Ki-67, keratin 6, and keratin 16 in basal cell carcinoma and squamous carcinoma, suggesting that *ADD1* is involved in cell proliferation. Additionally, high levels of ADD1 were found to be related to the malignant biological behavior of tumors.[8–10] The phenomenon of ADD1 Ser660 phosphorylation in renal cancer indicates that abnormal phosphorylation of adducin may have a role in tumorigenesis.[8] Differing from this study focus, our current study focused on the phosphorylation site of Ser662. We showed that ADD1 was expressed at higher levels in lung cancer tissue than in healthy lung tissue and para-lung cancer tissue, which is in agreement with previous findings.[11] Our results also revealed the expression of ADD1 in multiple malignant cells, such as A549, MCF7, CaCo2 and LNCaP cell lines, suggesting that Ser662 activation is relevant in tumorigenesis. However, whether ADD1 Ser662 can be used as a biological marker for tumor prophylaxis or as a therapeutic target requires further investigation.

Rani et al. detected the downregulation of ADD3 in metastatic glioblastoma cells. Their study demonstrated that miR-145 can be tumor suppressive in glioblastoma as it reduces the proliferation, adhesion, and invasion of glioblastoma cells, apparently by suppressing the activity of ADD3 and oncogenic protein Sox9.[12] *ADD3*, depleted of exon 14, was reported to be downregulated in non-small cell lung cancer (NSCLC) compared with healthy lung tissue.[13] Additionally, a study by Tao showed that expression levels of ADD3 and ADD3-Ib were decreased in colorectal cancer (CRC) tissues compared with healthy mucosa, while the ADD3-Ia/ADD3-Ib ratio was increased in CRC tissue.[14] In a recent study by Lechuga et al.,[15] ADD3 was found to be markedly downregulated in NSCLC cells with the invasive mesenchymal phenotype, while ADD3-depleted NSCLC cells were extracellular matrix adhesion-independent, revealing a negative regulator function of adducin in NSCLC cell migration and invasion. Our study observed down-regulated expression of ADD3 in lung cancer tissue compared with healthy tissue, which was further confirmed by GEPIA analysis. These findings indicate that low levels of ADD3 expression could accelerate tumor growth and development. It can be speculated that ADD3 functions in cell proliferation, motility, adhesion, and signal transduction through its changes in Ser662 phosphorylation, but it remains unclear whether tumors can be treated by regulating the activity of ADD3 as a tumor suppressor.

Our study also showed that adducin phosphorylation at Thr445 occurs in healthy lung tissue, para-lung cancer tissue, and cancer tissue, but that the extent of phosphorylation is much greater in cancer tissue. Previous quantitative research demonstrated that Thr445 is mainly phosphorylated by Rho-kinase in a transduction pathway believed to participate in tumorigenesis, invasion, and metastasis.[16] Taken together, these findings suggest that high levels of Thr445 phosphorylation suppress the malignant biological behavior of cells. Because our study found no adducin phosphorylation at Thr445 in any cell lines, this indicates that the activation of adducin may require extracellular stimuli in the interstitium but not within cells. However, our results are limited by the small number of cells and tissues analyzed, so further study is needed to determine why phospho-adducin (Thr445) is only expressed in tissues and not in cell lines.

In summary, the abnormal phosphorylation of adducin appears to be associated with tumorigenesis. These findings provide

useful knowledge for research into tumorigenesis and the prophylaxis and treatment of tumors.

Availability of data and materials

The data and materials in the current study are available from the corresponding author on reasonable request.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Ethics and consent

All techniques were performed in accordance with relevant institutional guidelines. Informed consent was obtained from all participants prior to their inclusion in this study. Clinical specimens were approved for use by the Ethics Committee of Zhejiang Cancer Hospital on 30 May 2018.

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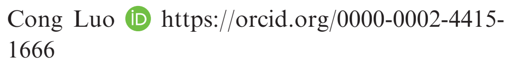

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