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Epithelial mesenchymal transition of non-small-cell lung cancer cells A549 induced by SPHK1

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ABSTRACT

Objective: To explore the effect and molecular mechanism of SPHK1 in the invasion and metastasis process of non-small-cell lung cancer cells (A549). **Methods:** Recombinant retrovirus was used to mediate the production of A549/vector, A549/SPHK1, A549/scramble, and A549/SPHK1/RNAi that stably expressed or silenced SPHK1. The invasion and migration capacities of A549 cells overexpressing or silencing SPHK1 were determined using Transwell invasion assay and scratch wound repair experiment. The protein and mRNA expression levels of E-cadherin, fibronectin, vimentin in A549/vector, A549/SPHK1, A549/scramble, A549/SPHK1/RNAi were detected with Western blot (WB) and quantitative PCR (QPCR) methods, respectively. **Results:** Transwell invasion assay and scratch wound repair experiments showed that over-expression of SPHK1 obviously enhanced the invasion and migration capacities of A549 cells. WB and QPCR detection results showed that, the expression of E-cadherin (a molecular marker of epithelial cells) and fibronectin, vimentin (molecular markers of mesenchymal cells) in A549 cells was upregulated after overexpression of SPHK1; while SPHK1 silencing significantly reduced the invasion and metastasis capacities of A549 cells, upregulated the expression of molecular marker of epithelial cells, and downregulated the expression of molecular marker of mesenchymal cells. **Conclusions:** SPHK1 promotes epithelial mesenchymal transition of non-small-cell lung cancer cells and affects the invasion and metastasis capacities of these cells.

1. Introduction

Tumor metastasis is a multi-stage, multi-step process involving a variety of genes, it can be summarized as follows: *in situ* tumor invasion into the basement membrane and stroma, crossing through the vascular wall and entering peripheral circulation, then forming thrombus in blood vessels and adhering to vascular endothelial cells, breaking through vascular walls and affecting the stroma in secondary tissue or organ, ultimately forming secondary tumors[1,2].

Lung cancer is the first malignant cause of death, it's estimated that the annual incidence rate is nearly 1.23 million. The existing therapeutic strategies for non-small cell lung cancer include surgery, radiotherapy, chemotherapy and physical therapy. The survival rate of non-small-cell lung cancer patients is less than 1%. Radiotherapy and chemotherapy were not completely effective for treating non-small-cell lung cancer[3-9]. Therefore it is crucial to find molecular markers for predicting the metastasis and prognosis of lung cancer.

As an oncogene, SPHK1 exerts important biological functions and is highly expressed in a variety of malignant tumors, it also assists in the regulation of tumor growth, invasion, metastasis and apoptosis[10-12]. Preliminary studies of our research group found that SPHK1 is highly expressed in lung cancer cells and tissues. Although SPHK1 contributes to regulate cell proliferation and growth, the role of SPHK1 in the invasion, metastasis, and epithelial mesenchymal transition (EMT) of lung cancer cells is scarcely reported, and the regulatory mechanism has not

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been fully elucidated. Therefore, this study aims to explore the underlying mechanism of SPHK1 on the invasion and metastasis of virus-mediated lung cancer cell line A549. The effect of SPHK1 on the SPHK1-overexpressing and SPHK1-silencing lung cancer cells was investigated, which provides evidence for studying the pathogenesis of lung cancer.

2. Materials and methods

2.1. Cells and plasmids

HBE bronchial epithelial cells were purchased from the Cell Bank of Cancer Institute of Chinese Academy of Medical Sciences. A549, 95C and 95D cells were provided from ATCC Cell Bank. Retroviral packaging cell 293FT and pMSCV-puro-SPHK1, pSUPER-puro-SPHK1 and the corresponding viral packaging system were presented as a gift by Guangzhou Boke Cancer Institute.

2.2. Reagents

Fetal bovine serum, RPMI-1640 medium, Transwell culture plate (GIBCO Company); Matrigel (BD Biosciences); Puromycin (Invitrogen); E-cadherin, fibronectin, vimentin antibody, goat anti-rabbit secondary antibody (Abcam); real-time quantitative PCR (QPCR) kit (Guangzhou Fulen Gene) were all used in this study. Other reagents and supplies were purchased from Guangzhou Laura Biotech Ltd.

2.3. Establishment of stable cell lines A549

The virus was packaged with calcium phosphate transfection method, then the retroviral packaging plasmid PIK and retroviral expression plasmids (pMSCV-puro-SPHK1, pMSCV-Vector, pSUPER-puro-SPHK1, pSUPER-puro-vector) were transfected into 293FT cells for 5 hours. The culture medium was replenished 5 hours later. The virus solution was collected 24 hours later, filtered using 0.45 μm filter, and stored at -80 ℃.

The non-transfected A549 cells were conventionally cultured. When the cells reached a 70% confluence, recombinant virus solution and polybrene (8 μg/mL) were added to transfect the cells for 48 hours. The transfected cells were then digested and cultured in the medium containing 0.5 μg/mL puromycin (Sigma). Cells at passage 5 were harvested and total RNA was extracted.

2.4. Cell function assays

2.4.1. Scratch wound repair experiment

Cells in good growth state were collected and digested, then seeded in a 6-well culture plate at the density of 5×10^5 cells, in 37 ℃ incubator with 5% CO_2. After the cultured cells began to adhere, the original culture medium was removed, cells were rinsed with PBS and starved in serum-free 1640 medium for 24 hours. A scratch wound was made on the monolayer cells using 100 μL sterile pipette, and the cells were cultured in serum-free medium after the exutive cells were removed with PBS washing. The repairing process of scratch wound was observed under an inverted microscope. The cells were photographed at 0 hour and 24 hours to record and compare repair speed and manner in different cells.

2.4.2. Transwell invasion assay

Cells at logarithmic phase were digested. Cell suspension was seeded in the Matrigel-coated Transwell chamber, in which the underlayer was supplemented with RPMI1640 culture containing 10% fetal bovine serum as chemoattractant solution. After the cells were cultured for 24 hours, the chamber was removed and Matrigel was discarded with a cotton swab. Cultured cells were fixed in formalin and stained with hematoxylin. The number of cells penetrating the membrane was calculated under the microscope.

2.5. Expression of E-cadherin, fibronectin, vimentin in transfected cells detected by QPCR

Cells at logarithmic phase were collected and digested. Total RNA was isolated from whole cells using Trizol. RNA concentration was measured with UV spectrophotometer and preserved at -20 ℃ for further use. The housekeeping gene β-Actin was served as the reference. The expression levels

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Primer sequences were shown as follows:

Gene	Forward primer	Reverse primer
E-cadherin	5′CTGCTGCAGGTCTCCTCTTG 3′	5′TGTCGACCGGTGCAATCTTC 3′
Fibronectin	5′GACCACATCGAGCGGATCTG 3′	5′GTCTCTTGGCAGCTGACTCC 3′
Vimentin	5′AAGGCGAGGAGAGCAGGATT 3′	5′GGTCATCGTGATGCTGAGAAG 3′
β-Actin	5′TGGCACCCAGCACAATGAA-3′	5′CTAAGTCATAGTCCGCCTAGAAGCA-3′

of E-cadherin, fibronectin, vimentin in different cell lines were determined.

2.6. Expression of E-cadherin, fibronectin, vimentin in stable cells detected by Western blot (WB)

Cells at logarithmic growth phase were lysated and protein concentration was detected using the BCA method. A total of 20 μg total proteins were electrophoresized in 9% SDS-PAGE gel at 70 V for 4 hours, then transferred to another film and incubated with E-cadherin, fibronectin, vimentin antibodies. Subsequently cells were developed with ECL, to analyze the expression of target proteins.

2.7. Statistical analysis

Data were statistically analyzed using SPSS 18.0 software for windows (IBM). Measurement data were expressed as mean±SD. The difference among samples was compared with one-way analysis of variance. A two-tailed $P < 0.05$ was considered statistically significantly different.

3. Results

3.1. SPHK1 protein and mRNA expression in lung cancer cell lines and para-carcinomatous tissue

The results showed that, SPHK1 mRNA and protein expression in lung cancer tissue was significantly higher than that in para-carcinomatous tissue (Figure 1B, Figure 1D). In addition, SPHK1 protein and mRNA expression in lung cancer cell lines and normal lung epithelial cells were detected with QPCR and western blot analysis, respectively. The results showed that, SPHK1 protein and mRNA expression in lung cancer cell lines was increased when compared with normal lung cells (Figure 1A, Figure 1C).

3.2. SPHK1-overexpression and SPHK1-silencing cell lines were successfully established

The viruses carrying SPHK1-overexpression and SPHK1-silencing genes were transfected into A549 cells. The transfected cells were then screened with 0.5 μg/mL puromycin and total RNA was extracted from the 7-generation cells. QPCR and western blot assay showed that, SPHK1 was expressed less in control strain A549/vector than in A549/SPHK1, while SPHK1 expression in A549/SPHK1/RNAi was significantly decreased when compared with control strain A549/scramble. Experimental results indicated that, SPHK1-overexpression and SPHK1-silencing cell lines were successfully established (Figure 2).

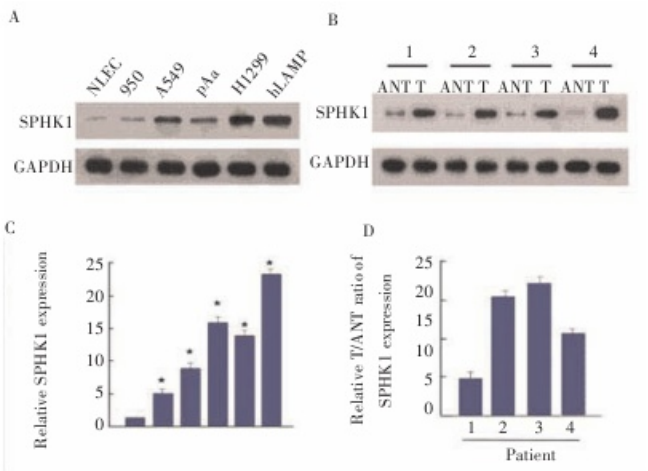

Figure 1. SPHK1 protein and mRNA expression levels.
A, B: SPHK1 protein and mRNA expression levels in para-carcinomatous tissue by western blot analysis; C, D: SPHK1 protein and mRNA expression levels in lung cancer cell lines by QPCR detection.

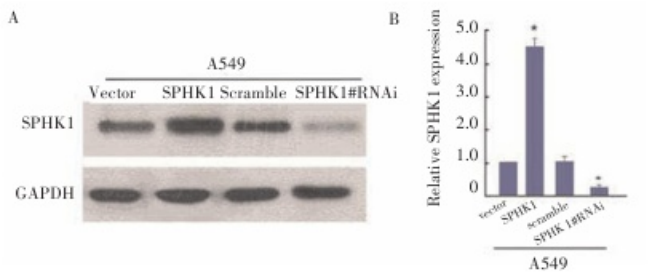

Figure 2. Identification of stable cell lines.
A: Western blot detection of SPHK1protein expression in A549 cells; B: QPCR detection of SPHK1 mRNA expression in A549 cells.

3.3. Invasion and migration of SPHK1-overexpression and SPHK1-silencing cell lines

Transwell assay showed that, more GPC-transduced A549/SPHK1 cells, rather than A549/vector, penetrated through the basement membrane; the number of A549/SPHK1/RNAi penetrating the basement membrane was significantly lower than A549/scramble (Figure 3A). In scratch wound repair experiments, the scratch wound in control strain A549/vector was repaired slowly than that in A549/SPHK1; but A549/SPHK1/RNAi cells migrated slowly than the A549/scramble (Figure 3B). Our findings suggested that overexpression of SPHK1 could promote cell migration and invasion, and the

effect was enhanced along the increasing expression of SPHK1.

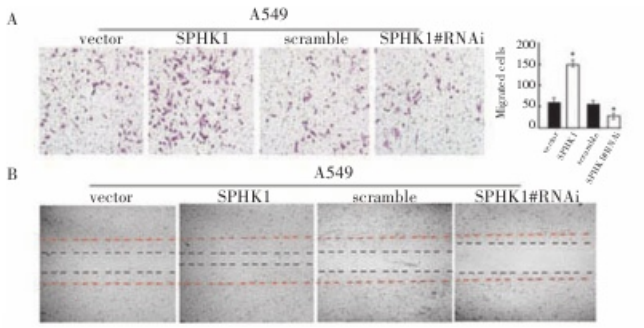

Figure 3. SPHK1 regulates the migration and metastasis of A549 cells.
A: Transwell invasion assay; B: scratch wound repair experiments.

3.4. Effect of SPHK1 overexpression on the EMT

WB and QPCR methods were used to detect the expression of EMT-related markers in A549 cells after SPHK1 overexpression and silencing. The results showed that, overexpression of SPHK1 significantly decreased the expression of E-cadherin (marker of epithelial cells) in A549 cells, while significantly increased the expression of vimentin and fibronectin (markers of mesenchymal cells) (Figure 4). The results suggested that overexpression of SPHK1 obviously promoted the EMT process.

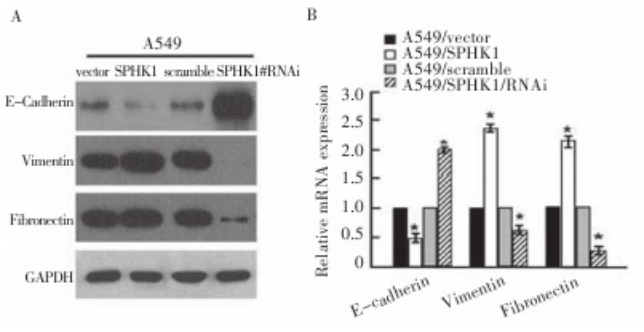

Figure 4. Western blot analysis (A) and QPCR (B) detection of the expression of EMT-related molecular markers following SPHK1 over-expression.

4. Discussion

Non-small-cell lung cancer occupies 80% of all lung cancers according to the pathology classification[2], therefore it is critically important to emphasize non-small-cell lung cancer models. Growing evidence demonstrated that, the majority of lung cancer patients die of tumor invasion and metastasis. Human body is an integrated regulatory body, the occurrence and metastasis of tumor cells are extremely complex and cumbersome, involving both external and internal factors[13,14]. The most crucial event triggering tumor invasion and metastasis is the alterations of phenotype molecule and adhesion molecule in epithelial cells, which is also called EMT. When the tumor metastasis function is changed, EMT-associated molecules are changed accordingly, such as increasing the expression of mesenchymal cells specific proteins (Vimentin, fibronectin), and decreasing the expression of epithelial cell adhesion protein (E-cadherin)[15,16]. The down-regulation of adhesion protein's expression may lead to the loss of intercellular adhesion, and actually tumor cells are shed and migrate to distant parts, resulting in tumor metastasis and exerting a super-strong invasive ability[17-19]. The migration and invasion capacities of tumor cells educed with functional changes are regarded as EMT[20].

Preliminary studies of our research group have found that, SPHK1 was highly expressed in lung cancer cell lines and para-carcinomatous tissue, and it can regulate cell metastasis. This evidence indicates that SPHK1 expression may be associated with metastasis. In this study, we established SPHK1-overexpressing and SPHK1-silencing lung cancer cell models A549/SPHK1 and A549/SPHK1/RNAi with retrovirus mediated method[21-24]. QPCR and western blot assay results showed that, lung cancer cells still highly expressed SPHK1 even after 7 passage of culture. Using these highly expressed cells, we ensured the reproducibility of experimental results of cell function assays and related markers. The cell morphology maintained unchanged in the course of routine culture. In Transwell invasion assay and scratch wound repair experiments, the migration and invasion capacities of A549 cells were obviously inhibited after silencing SPHK1, while obviously enhanced after overexpressing SPHK1. QPCR and western blot analysis were used to detect the expression of EMT-related molecules after over-expressing and silencing SPHK1. The results found that over-expression of SPHK1 obviously down-regulated the expression of epithelial cell marker E-cadherin in A549 cells, while significantly upregulated the expression of mesenchymal cell markers, vimentin and fibronectin. Silencing SPHK1 achieved the reverse effects, increasing E-cadherin expression, decreasing vimentin and fibronectin expression in A549 cells. Our findings suggest that SPHK1 can promote the invasion and metastasis of lung cancer cells A549, and trigger EMT.

In summary, we observed the changes of EMT phenotype and surface receptor signaling molecules, and found that SPHK1 expression contributed to regulate EMT in A549 cells,

and affected the invasion and metastasis of tumor cells, leading to cell functional changes. This study will provide a theoretical basis for future investigations on non-small-cell lung cancer mechanism, diagnosis, treatment and prognosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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