SIRT5 promotes non-small cell lung cancer progression by reducing FABP4 acetylation level

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This study evaluates the role of SIRT5 in non-small cell lung cancer (NSCLC) progression and explores the underlying mechanism. The expression and correlation of SIRT5 and FABP4 in lung cancer were analyzed by the GEPIA database. The expression levels of SIRT5 and FABP4 in NSCLC cells were measured by qRT-PCR and western blot. The effect of SIRT5 and FABP4 on NSCLC cell development was determined. The interaction between SIRT5 and FABP4 was analyzed by co-immunoprecipitation (Co-IP). Tumor mass and volume were measured in nude mice to study the effect on the growth of NSCLC transplanted tumors. GEPIA database analysis showed that SIRT5 was highly expressed, while FABP4 was lowly expressed in lung cancer, which was consistent with the detection results of SIRT5 and FABP4 expressions in NSCLC cell lines. The expression of SIRT5 was negatively correlated with FABP4. Transfection of sh-SIRT5 in NSCLC cells led to a decrease in NSCLC cell malignancy, which was counteracted by sh-FABP4 transfection. Western blot and Co-IP showed that SIRT5 reduced FABP4 expression by inducting the deacetylation of FABP4. Nude mice in the sh-SIRT5 + sh-FABP4 group had significantly reduced tumor mass and volume compared with those in the sh-FABP4 group, while the tumor mass and volume in the sh-SIRT5 + sh-FABP4 group were increased in comparison to those in the sh-SIRT5 group. To conclude, collected evidence showed that SIRT5 promoted NSCLC cell development by reducing FABP4 acetylation level.

Key words: SIRT5, FABP4, acetylation, non-small cell lung cancer, invasion

Lung cancer is pathologically divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which is one of the most common malignant tumors [1]. Among them, NSCLC accounts for 80–90% of lung cancers and mainly manifests as lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), and lung large cell carcinoma (LULC) [2]. Despite improvements in treatment modalities (such as surgical resection, platinum-based double chemotherapy, and targeted therapy), long-term survival of NSCLC remains unsatisfactory, with a 5-year survival rate of less than 15% owing to a cancer relapse and metastasis [3]. Therefore, it is imperative to understand the molecular mechanisms underlying NSCLC progression to identify novel therapeutic targets.

Sirtuins belong to a class of nicotinamide adenine dinucleotide (NAD+)-dependent enzymes, which have the activities of deacetylase, desuccinylase, deglutarylase, and demalonylase [4], and play a crucial part in regulating cancer cell metabolism and tumor microenvironment [5]. Among them, SIRT5 was reported to be elevated in some cancers: NSCLC [6], breast tumor [7], Waldenstrom's macroglobulinemia [8], and colorectal cancer (CRC) [9], while SIRT5 was lowly expressed in others: endometrial carcinoma [10], head and neck squamous cell carcinoma (HNSCC) [11], and hepatocellular carcinoma (HCC) [12]. These findings reflected that, like other sirtuins, SIRT5 may play a specific role in human cancers [13]. Acetylation is one of many acyl modifications and recent proteomic surveys of mitochondrial protein acylation have identified the regulation of protein acetylation by SIRT3 and SIRT5 [14]. A study showed that the specific deacetylation of lactate dehydrogenase B (LDHB) by SIRT5 was essential for CRC [15]. But the function of SIRT5-mediated acetylation has not been studied in NSCLC. Based on a previous study, we speculated that SIRT5 would also affect NSCLC progression through acetylation.
Fatty acid-binding protein 4 (FABP4) and FABP3 belong to the FABP family, which controls the metabolism and transportation of long-chain fatty acids [16]. Reportedly, FABP4 played an important role in tumor development, for example, FABP4 could suppress the proliferation and migration of endometrial cancer (EC) cells by the PI3K/Akt pathway [17]. Also, FABP4 was lowly expressed in HCC tissues and overexpression of FABP4 inhibited tumor growth in vivo [18]. Of note, Ni et al. have demonstrated decreased expression of FABP4 in NSCLC tissues [19]. However, the regulatory mechanisms of FABP4 in NSCLC remain unclear. Data analysis on the GEPIA database illustrated that the expression of SIRT5 was negatively correlated with FABP4. Given that SIRT5 could regulate protein acetylation to affect cancer development, we posited that SIRT5 may regulate the acetylation level of FABP4 in NSCLC. However, the effect of the SIRT5/FABP4 axis on NSCLC progression has not been investigated. This study aimed to explore the possible effect of the SIRT5/FABP4 axis on NSCLC progression.

Materials and methods

Analysis of data from the GEPIA database. The expressions of SIRT5 and FABP4 and their correlation in NSCLC were analyzed using the GEPIA database (http://gepia.cancer-pku.cn/index.html).

Cell culture. The verified human NSCLC cells, SK-MES-1, H520, H226, and human normal lung bronchial epithelial cells BEAS-2B were all purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). Cells were cultured under 37 °C and 5% CO\(_2\) in DMEM (Gibco, Grand Island, NY, USA) in which 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Wilmington, DE, USA) and antibiotics (Gibco, Grand Island, NY, USA) were supplemented.

Cell transfection. SIRT5 overexpression vector (SIRT5), negative control vector of SIRT5 (vector), SIRT5 knockdown vector (sh-SIRT5), FABP4 knockdown vector (sh-FABP4), and the negative control of sh-SIRT5 and sh-FABP4 (sh-NC) were all purchased from GenePharma (Shanghai, China). Cell transfection was performed based on the instruction on Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA).

qRT-PCR. Total RNA of tissues and cells was extracted by TRIZOL reagent and reverse transcribed into cDNA on the basis of the specifications of the reverse transcription kit (TaKaRa, Tokyo, Japan). The samples were subjected to real-time fluorescence quantitative PCR (ABI 7300, ABI, Foster City, CA, USA) by using SYBR GreenMix (Takara). Each experiment was run in triplicate. GAPDH was used as the internal reference and gene expression was analyzed using the 2\(^{-\Delta\Delta Ct}\) method. \(\Delta\Delta Ct\) = experimental group (Ct target gene – Ct internal control) – control group (Ct target gene – internal control). Primer information is listed in Table 1.

Co-immunoprecipitation (Co-IP). Cells were lysed with pre-cooled RIPA lysis buffer and a 15 min centrifugation (at 14,000×g, 4 °C) was performed, and then the supernatant was removed to a new tube. Anti-SIRT5 (8782S, 1:1000, Cell Signaling Technology, CST, Boston, USA) and anti-FABP4 (2120S, 1:1000, CST) were added to 1 ml cell lysate, and IgG antibody (ab172730, 1:100, Abcam, USA) was added to the negative control group. The antigen-antibody mixture was slowly shaken overnight at 4 °C or 2 h at room temperature on a shaker. After that, the mixture was added with 100 μl Protein A/G agarose beads (prepared in PBS solution with a concentration of 50%) and mixed at 4 °C overnight or incubated for 1 h. The agarose beads-antigen-antibody complex was harvested after a 5 s centrifugation and supernatant removal. Next, the complex was washed three times with pre-cooled RIPA buffer (800 μl) and suspended with 2× loading buffer (60 μl). The sample was boiled for 5 min and centrifuged to collect the remaining agarose beads, and the supernatant was boiled again for 5 min and then electrohoresed. Protein expression was tested by western blot.

Western blot. After cells were lysed on ice for 15 min with RIPA lysis (Beyotime) and centrifugated at 13,000×g for 5 min, a BCA detection kit (Beyotime) was used to detect the protein concentration. Afterward, the loading buffer was added and the protein was denatured in a boiling water bath for 10 min. The volume of each sample was calculated according to protein loading quantity. After sample loading, electrophoresis was performed at 80 V for 30 min and continued at 120 V for 90 min after bromophenol blue entered the separation gel. The protein was transferred onto a PVDF membrane in an ice bath with a current of 250 mA for 100 min, after which the membrane was rinsed three times, each for ~1–2 min. After 2 h incubation with blocking buffer, the membranes were cultured with the primary antibodies of SIRT5 (8782S, 1:1000, CST), FABP4 (2120S, 1:1000), and acetylated-Lysine Antibody (9441S, 1:2000, CST) at 4 °C overnight. Next, the membranes were washed three times with TBST for 10 min each. Then horseradish peroxidase-labeled goat anti-rabbit IgG (A0208, 1:1000, Beyotime, Shanghai) was added and incubated for 2 h. The membranes were washed 3 times again for 10 min each and then analyzed by Bio-Rad after enhanced chemiluminescence developing solution (ECL, P0018FS, Beyotime, Shanghai) was added.

Transwell assay. Cells (5×10\(^4\)) were suspended in a serum-free DMEM and then inoculated in Transwell chambers coated with Matrigel (BD Biosciences, Bedford, MA, USA),
and 600 µl medium containing 10% FBS was added into the lower chamber. After 48 h incubation, the cells passing through the membrane were fixed in 100% methanol and stained with 0.1% crystal violet. The non-invaded cells above the membrane were removed with cotton swabs. The fixed cells were photographed using a microscope (Olympus, Tokyo, Japan) in five randomly selected fields at a 200× magnification.

**Scratch test.** The transfected cells were inoculated onto 6-well plates. The cells with 90% confluency were scratched with a 200 µl pipette tip. The exfoliated cells were washed once in serum-free medium, and then observed under a low-power phase contrast microscope (Olympus MK, Tokyo, Japan) and photographed. Afterward, the cells were cultured in a serum-free medium at 37°C and 5% CO₂ for 24 h and photographed again. Image-Pro Plus software was used to measure the distance the cell migrated. The migration ability of cells in the experimental groups was accordingly calculated.

**Tumor formation of nude mice.** Healthy male BALB/c nude mice (4–6 weeks old, n=30), weighing 16±2 g, were obtained from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. All mice were fed in a specific pathogen-free (SPF) laminar flow chamber under standard conditions (constant temperature of 25–28°C, humidity of 50%, and 12:12 h dark/light cycle) with free access to food and water. The paddings were replaced every 3 days and all operations were carried out under sterile conditions. All animal experiments obeyed the rules and regulations of experimental animal management and operating standards as well as the ethical requirements of experimental animals of the First Hospital of Changsha. Nude mice were randomly divided into 5 groups (SK-MES-1, sh-NC, sh-SIRT5, sh-FABP4, and sh-SIRT5 + sh-FABP4 groups) and anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). After anesthesia, the skin of the armpit and the back of the right forelimb was routinely disinfected with iodophor. Mice in the SK-MES-1 group were inoculated with 0.2 ml SK-MES-1 cell suspension (1×10⁶ cells). An equal number of SK-MES-1 cells transfected with sh-SIRT5, sh-FABP4, sh-SIRT5 + sh-FABP4, or sh-NC was injected into the mice in sh-SIRT5, sh-FABP4, sh-SIRT5 + sh-FABP4, and sh-NC groups respectively. After injection, the needle was slowly removed to prevent leakage and the injection site was marked. Five weeks later, the nude mice were sacrificed by cervical dislocation. Subcutaneous tumors were separated and weighed, and the tumor volume was calculated. Tumor volume = 1/2 × long diameter × short diameter².

**Statistical analysis.** Statistical analysis was conducted with GraphPad Prism7 software, and all data were exhibited as mean ± standard deviation (χ ± s). t-test and one-way analysis of variance were used for comparisons between two

Figure 1. The expressions of SIRT5 and FABP4 are evaluated in NSCLC cells. A) The expression of SIRT5 in lung cancer cells was analyzed in the GEPIA database; B) the FABP4 profile in lung cancer cells was analyzed in the GEPIA database; C) the correlation analysis of SIRT5 and FABP4 in the GEPIA database. D, E) SIRT5 expression in different cell lines was detected by qRT-PCR and western blot. F–G) FABP4 expression in different cell lines was detected by qRT-PCR and western blot. *p<0.05, **p<0.01, compared with BEAS-2B. Abbreviation: NSCLC—non-small cell lung cancer.
groups and among groups, respectively. Tukey's multiple comparisons test was used for post hoc analysis. A p-value <0.05 was considered to have statistically significance.

Results

High expression of SIRT5 and low expression of FABP4 in NSCLC. First, the GEPIA database was used to analyze the expression and correlation of SIRT5 and FABP4. The results showed that SIRT5 was highly expressed, while FABP4 was lowly expressed in NSCLC tissues compared with normal tissues (Figures 1A, 1B). The expression of SIRT5 was negatively correlated with FABP4 (Figure 1C). Subsequently, the expressions of SIRT5 and FABP4 were measured in NSCLC cell lines by qRT-PCR and western blot. Results showed that the expressions of SIRT5 mRNA and protein in H226, H520, and SK-MES-1 cells were significantly increased compared with BEAS-2B cells (Figures 1D, 1E, *p<0.05, **p<0.01), but the expressions of FABP4 mRNA and protein were significantly decreased (Figures 1F, 1G, *p<0.05, **p<0.01). SIRT5 and FABP4 expressions altered more significantly in H520 and SK-MES-1 cells than those in H226 cells, and thus H520 and SK-MES-1 cells were selected for the following experiments. Taken together, SIRT5 and FABP4 played a vital role in the development of NSCLC, and there may be a certain relationship between the two genes.

SIRT5 knockdown reduces the malignancy of NSCLC cells. In order to study the effect of SIRT5 in NSCLC cells, sh-SIRT5 was transfected into SK-MES-1 and H520 cells. qRT-PCR and western blot results showed that sh-SIRT5 significantly reduced the expression levels of SIRT5 mRNA and protein in SK-MES-1 and H520 cells compared with the sh-NC group (Figures 2A–2D, *p<0.05, **p<0.01). Scratch test and Transwell assay showed that transfection with sh-SIRT5 in SK-MES-1 and H520 cells led to inhibited cell malignancy compared with the sh-NC group (Figures 2E, 2G, *p<0.05). The above results showed that sh-SIRT5 could inhibit the progression of NSCLC cells.

SIRT5 decreases the acetylation level of FABP4. In view of the above results, the mechanism of SIRT5 promoting the development of NSCLC cells was further explored. First, we transfected SIRT5 or sh-SIRT5 into SK-MES-1 and H520 cells to observe their effect on FABP4 protein expression. qRT-PCR and western blot results showed that transfection of SIRT5 overexpression vectors in SK-MES-1 and H520 cells signally increased SIRT5 mRNA and protein expression levels (Figures 3A–3D, **p<0.01, ***p<0.001). In addition, compared with the vector group or the sh-NC group, the

Figure 2. SIRT5 knockdown suppresses the progression of NSCLC cells. A–D) The transfection efficiency of sh-SIRT5 in SK-MES-1 and H520 cells was assessed by qRT-PCR and western blot. E–G) The migration and invasion abilities of SK-MES-1 and H520 cells after sh-SIRT5 transfection were assessed by the scratch test and Transwell assay. *p<0.05, **p<0.01, compared with sh-NC. Abbreviation: NSCLC-non-small cell lung cancer.
SIRT5 group had decreased expression of FABP4 protein, and the sh-SIRT5 group showed significantly increased expression of FABP4 protein (Figures 3E, 3F, *p<0.05, #p<0.05). Co-IP results showed the interaction between SIRT5 and FABP4 in SK-MES-1 and H520 cells. The interaction between SIRT5 and FABP4 was detected by Co-IP (Figures 3G, 3H). Then, we studied the effect of SIRT5 or sh-SIRT5 on FABP4 acetylation in SK-MES-1 and H520 cells to investigate whether SIRT5 can deacetylate FABP4. The results showed that SIRT5 overexpression signally reduced FABP4 acetylation in SK-MES-1 and H520 cells, while SIRT5 knockdown signally promoted FABP4 acetylation (Figures 3I, 3J). These results suggested that SIRT5 could decrease the acetylation level of FABP4 in NSCLC cells.

SIRT5 facilitates NSCLC cell progression by reducing FABP4. It has been found that SIRT5 can reduce FABP4 acetylation levels in NSCLC cells. To further explore the related mechanism of SIRT5 promoting NSCLC cell malignancy, sh-SIRT5 and sh-FABP4 were simultaneously transfected into SK-MES-1 and H520 cells. qRT-PCR and western blot results showed that sh-FABP4 markedly reduced the expression levels of FABP4 mRNA and protein in SK-MES-1 and H520 cells (Figures 4A–4D, *p<0.05). The results of the scratch test and Transwell assay showed that the aggressiveness of SK-MES-1 and H520 cells was markedly repressed in the sh-SIRT5 group compared with the sh-NC group (Figures 4E–4G, *p<0.05, **p<0.01). The malignancy of SK-MES-1 and H520 cells was markedly enhanced in the sh-FABP4 group compared with the sh-NC group (Figures 4E–4G, **p<0.01). The aggressiveness of SK-MES-1 and H520 cells was restored in the sh-SIRT5+sh-FABP4 group compared with the sh-SIRT5 group (Figures 4E–4G). Those results suggested that SIRT5 enhanced the development of NSCLC cells by reducing FABP4.

SIRT5 promotes xenograft tumor growth by reducing FABP4 expression. To further evaluate whether SIRT5 regulates NSCLC progression by reducing FABP4 expres-
Stably infected with sh-SIRT5 and sh-FABP4 were injected into nude mice. qRT-PCR and western blot respectively detected the expression of FABP4 mRNA and protein in vivo and the results showed that SIRT5 knockdown increased the expression levels of FABP4 mRNA and protein (Figures 5A, 5B, \( *p<0.05 \)), while sh-FABP4 injection decreased the expressions of FABP4 mRNA and protein (Figures 5A, 5B, \( **p<0.01 \)). Interestingly, when sh-SIRT5 and sh-FABP4 were simultaneously injected into nude mice, sh-FABP4 reversed the enhancive effect of sh-SIRT5 on FABP4 mRNA and protein expressions compared with the injection of sh-SIRT5 alone (Figures 5A, 5B, \( \#p<0.05 \)). In addition, compared with the sh-NC group, the volume and weight of the transplanted tumor in the sh-SIRT5 group were significant lower (Figures 5C, 5D, \( **p<0.01 \) ), but that in the sh-FABP4 group were significantly increased (Figures 5C, 5D, \( \#\#p<0.01 \) ). However, the sh-SIRT5 + sh-FABP4 group showed a larger transplanted tumor volume and a heavier tumor weight than the sh-SIRT5 group (Figures 5C, 5D, \( \#\#p<0.01 \) ). On parallel, decreased tumor volume and weight were found in the sh-SIRT5 + sh-FABP4 group when compared with the sh-FABP4 group (Figures 5C, 5D, \( \#\#p<0.05 \), \( \#\#\#p<0.001 \)). The above results further suggested that SIRT5 regulated NSCLC progression by decreasing FABP4 expression.

**Discussion**

In China, lung cancer accounts for the highest morbidity and mortality among all types of cancers [20]. NSCLC, a subtype of lung cancer, is prone to drug resistance, metastasis, and recurrence, leading to poor survival rates [21]. Due to the lack of effective long-term treatment strategies and poor survival rates, finding new treatment options is crucial to facilitate the prognosis of patients with NSCLC. In the present study, it was concluded that SIRT5 was increased but FABP4 was decreased in NSCLC cells, and knockdown of SIRT5 may reduce the malignancy of NSCLC cells. In addition, it was further confirmed that SIRT5 promoted the growth of xenograft tumor and participated in NSCLC cell development via reducing FABP4 acetylation level.
Cell invasion and migration are known to be key factors in malignant progression and metastasis. This study focused on exploring the effect of SIRT5 and FABP4 on NSCLC cell migration and invasion. SIRT5, a member of the sirtuin family, is abnormally expressed in most cancers. A prior study has shown that SIRT5 was increasingly expressed in CRC tissues [22]. It was consistent with our measurement result that SIRT5 was highly expressed in NSCLC cells, indicating the implication of SIRT5 in NSCLC. Additionally, FABP4 was found to be lowly expressed in NSCLC cells and negatively correlated with SIRT5 expression. Taken the above results together, it was interesting to probe the possible interaction between SIRT5 and FABP4.

Post-translational modifications in the nucleosome of chromatin and modifications in N-terminal tails are emerging as vital epigenetic modulatory mechanisms, including acetylation, phosphorylation, ubiquitination, ADP-ribosylation, deamidation, and isomerization [23]. Acetylation is one of the most frequent modifications, taking place in approximately 85% of eukaryotic proteins [24]. The sirtuins regulate gene expression by deacetylation of histones and a variety of non-histones [25]. SIRT5 could support melanoma cell behavior by influencing chromatin dynamics [26]. Downregulated SIRT5 mediated vimentin acetylation, which may affect the progression of HCC [27]. SIRT5-mediated cell proliferation of CRC cells was dependent on the deacetylation of LDHB at the K329 [15]. SIRT5 may regulate the progression of prostate cancer cells through acetyl-CoA acetyl-transferase 1 [28]. In our study, the interaction of SIRT5 and FABP4 has been measured by Co-IP and the effect of overexpression of SIRT5 and sh-SIRT5 on FABP4 acetylation has been studied. Our study confirmed that SIRT5 reduced FABP4 acetylation level in NSCLC cells. All results indicated that SIRT5 facilitated the development of NSCLC cells by reducing the FABP4 acetylation level.

The classical xenograft tumor model is a cell line-derived tumor xenograft model which is an animal model formed by injecting a tumor cell line cultured in vitro into an immunodeficient mouse [29]. In order to further confirm our in vitro results, the xenograft tumor model of nude mice has been established and the growth of transplanted tumor has been evaluated. It was found that SIRT5 promoted tumor growth by reducing FABP4.

In conclusion, these results suggested that SIRT5, as a deacetylase, reduced FABP4 acetylation level to decrease FABP4 expression, thereby promoting NSCLC progression.
Our findings provided an important preclinical basis for NSCLC treatment.

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