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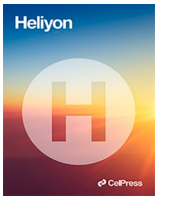

Research article

SIRT5 participates in the suppressive tumor immune microenvironment of EGFR-mutant LUAD by regulating the succinylation of ACAT1

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ABSTRACT

Epidermal growth factor receptor (EGFR)-mutant lung adenocarcinoma (LUAD) exhibits a poor response to immune checkpoint inhibitors (ICIs) by shaping a suppressive tumor immune microenvironment (TIME), which characters as lacking immune cell infiltration; however, the underlying mechanism remains to be elucidated. Here, we demonstrated that Sirtuin 5 (SIRT5), a member of the deacetylase SIRT family, functions as a desuccinylase of acetyl-CoA acetyltransferase 1 (ACAT1) and enhances the enzymatic activity of ACAT1 to activate the NRF2 pathway, inhibiting the secretion of the chemokines CCL5 and CXCL10, which are important for recruiting $CD8^+$ T cells, thereby participating in the formation of an inhibitory TIME in *EGFR*-mutant LUAD. In conclusion, we propose that the combination of a SIRT5 inhibitor with ICIs therapy may be a promising therapeutic approach for patients with *EGFR*-mutant LUAD.

1. Introduction

Epidermal growth factor receptor (EGFR) gene mutations are the most common driver mutations in non-small cell lung cancer (NSCLC) and are commonly found in lung adenocarcinoma (LUAD) patients [1]. Although EGFR tyrosine kinase inhibitors (TKIs) can significantly improve the survival of these patients, acquired resistance is still inevitable. Combination strategies may be considered for some known resistant mechanisms. For example, the vascular endothelial growth factor (VEGF) pathway has been recognized as a key mediator of angiogenesis that contributes to the emergence of resistance to EGFR TKIs. However, although some clinical trial data supported that dual EGFR/VEGF pathway inhibition has demonstrated consistent efficacy in prolonging progression-free survival in patients with *EGFR*-mutant NSCLC, the overall survival benefit has not been demonstrated [2,3]. Besides, several clinical studies have demonstrated the benefits of dual blockade of EGFR using anti-EGFR monoclonal antibodies (mAbs) coupled with EGFR-TKIs in overcoming treatment resistance. However, a single treatment option may not result in the same clinical benefits in all patients with acquired resistance. Further investigation of potential biomarkers may allow patient selection for those who could benefit from this combination treatment [4]. In addition, activation of other by-pass pathways, such as fibroblast growth factor receptor (FGFR) and

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PI3K/AKT pathways, are interconnected and can be potential targets for TKIs resistance. However, the therapeutic benefit of combining FGFR and Akt inhibitors is only observed in the preclinical experiments and further clinical trials of this strategy are still needed [5]. Immune checkpoint inhibitors (ICIs) have gained attention as a novel treatment strategy for patients with *EGFR* mutations for whom targeted therapy has failed. However, unfortunately, multiple clinical trials have suggested limited efficacy of ICIs in patients with *EGFR* mutations. For instance, the Checkmate-057 study, which aimed to evaluate the differential effects of second-line treatment using nivolumab and docetaxel monotherapy in advanced nonsquamous NSCLC patients, showed that there was no benefit from ICIs (HR = 1.18) among 82 patients who had previously received *EGFR*-targeted therapy or platinum-based therapy and who progressed. Similar results were also found in the Keynote-010 study; among 86 patients with advanced NSCLC harboring *EGFR* mutations, no significant improvement in overall survival was observed with ICIs. Moreover, meta-analyses of several large clinical trials have indicated that the efficacy of ICIs monotherapy in patients with *EGFR* mutations is limited [6,7]. In conclusion, the immunotherapeutic efficacy is suboptimal in patients with *EGFR*-mutant LUAD, emphasizing the urgent need to elucidate its underlying mechanisms and explore potential therapeutic targets in clinical practice.

The tumor immune microenvironment (TIME), which is a complex network that consists of various cell types and factors that play important roles in tumor survival, is closely related to the efficacy of immunotherapy. *EGFR*-mutated tumors reportedly lack tumor-infiltrating lymphocytes, especially effector T cells ($CD8^+$ T cells) [8,9], leading to a suppressive TIME, which may be a potential reason for poor sensitivity to ICIs therapy. The infiltration of $CD8^+$ T cells into tumors requires the recruitment of chemokines, especially CCL5 and CXCL10, which are important ligands that can effectively guide $CD8^+$ T-cell mobilization from regional lymph nodes to tumor tissues [10]. Therefore, the chemokine-mediated TIME influences the efficacy of ICIs, and the regulatory mechanism needs to be clarified [11].

Nuclear factor erythroid 2-like 2 (NRF2), a key transcription factor in cellular antioxidant, metabolic, cytoprotective, and anti-inflammatory pathways, is well known to be associated with enhanced tumor growth, aggressiveness, and refractoriness to cancer-directed therapy, such as radiation, chemotherapy, and targeted therapy [12,13]. Moreover, high NRF2 expression can inhibit the infiltration of various immune cells [14], while NRF2 deficiency significantly promotes $CD8^+$ T-cell infiltration [15]. It was also reported that NRF2 and its downstream target genes are closely related to the synthesis and secretion of CCL5 and CXCL10 [16–19], suggesting its potential role in regulating chemokines.

Sirtuin 5 (SIRT5) is a unique member of the SIRT family of deacetylases that possesses weak deacetylase activity but strong desuccinylase activity. Studies have confirmed its high expression in the malignant behaviors of various tumors, such as lung cancer, ovarian cancer, and prostate cancer [20–23]. Recent studies have shown that SIRT5 can also regulate the activity and differentiation of innate immune and T cells [24,25], indicating its involvement in the TIME. We have previously shown that SIRT5 could activate the NRF2 pathway to function as an oncogene in ovarian cancer [21]. Besides, succinylation is regarded as a novel posttranslational modification that participates in the TIME by modulating gene expression and protease activity [26]. Overall, these evidences suggested the potential role of SIRT5 in the suppressive TIME of *EGFR*-mutated LUAD. However, whether it can function as a desuccinylase and its target substrate enzyme needs to be explored. Hence, this study aimed to clarify the role and potential mechanism of SIRT5 in the formation of the inhibitory TIME in *EGFR*-mutated LUAD, providing molecular targets and a theoretical basis for improving the efficacy of ICIs in LUAD patients harboring *EGFR* mutations.

2. Materials and methods

2.1. Cell culture and treatment

The human NSCLC cell lines A549, CALU-1, PC-9, and H1975 were purchased from Wuhan Pricella Biotechnology (Wuhan, China). PC-9 and H1975 cells were cultured in RPMI-1640 medium (Gibco, MA, USA), A549 cells were cultured in Ham's F-12K medium (Gibco), and CALU-1 cells were cultured in McCoy's 5A medium (Gibco). All media were supplemented with 10 % fetal bovine serum (Gibco). The culture environment was maintained at 37 °C and 5 % CO_2.

Lipofectamine 3000 reagent (Invitrogen, MA, USA) was used to perform transient transfection according to the manufacturer's instructions. A human SIRT5 expression vector and an empty vector (GeneChem, Shanghai, China) were used for SIRT5 overexpression and negative control, respectively. For knockdown experiments, cells were transfected with SIRT5-specific siRNA or scrambled control siRNA (Genecreate, Wuhan, China) for 72 h.

To inhibit NRF2 signaling, cells were treated with the NRF2 inhibitor ML385 (MCE, China) in DMSO (Sigma–Aldrich, Germany). Cells were treated with a final concentration of 5 μM ML385 for 12, 24, or 48 h after transfection.

To inhibit ACAT1 activity, cells were treated with 7.5 μM avasimibe (MCE) for 12, 24, or 48 h.

Table 1
PCR primers.

	Forward 5′-3′	Reverse 5′-3′
GAPDH	CCACCCATGGCAAATTCC	GATGGGATTTCCATTGATGACA
SIRT5	TCGTGGTCATCACCCAGAAC	GCCACAACTCCACAAGAGGTAC
CCL5	TTGCCTGTTTCTGCTTGCTC	TGTAACTGCTGCTGTGTGGT
CXCL10	GAAATTATTCCTGCAAGCCAATTT	TCACCCTTCTTTTTCATTGTAGCA

2.2. Quantitative real-time polymerase chain reaction (qRT–PCR)

TRIzol (Invitrogen) was used to extract total RNA from NSCLC cells. Reverse transcription was performed using PrimeScript RT Master Mix (Takara, Otsu, Japan). qRT–PCR was performed using SYBR qPCR Mix (TOYOBO, Shanghai, China) on a real-time PCR system (MX3000P, Agilent Technologies, CA, USA). The thermocycling conditions were as follows: 30 s at 95 °C, followed by 40 cycles at 95 °C for 5 s and 65 °C for 30 s. The mRNA ratio of the target genes to GAPDH was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in Table 1.

2.3. Immunofluorescence staining

A549 and H1975 cells were seeded in 20 mm culture plates, washed with PBS, fixed with 4 % paraformaldehyde for 15 min, and permeabilized in 0.1 % Triton X-100 for 5 min. After blocking with 5 % bovine serum albumin for 1 h at room temperature, the cells were incubated with primary antibodies against SIRT5 or ACAT1 (diluted 1:100) overnight at 4 °C. Then, the cells were incubated with FITC- or TRITC-conjugated secondary antibodies (diluted 1:200) for 1 h in the dark at room temperature, and the cells were stained with 4′,6-diamidino-2-phenylindole for 5 min to visualize the nuclei. Images were captured using a fluorescence microscope.

2.4. Coimmunoprecipitation (Co-IP) assay

Lysates of cultured A549 and H1975 cells were harvested and subjected to immunoprecipitation using anti-SIRT5 or anti-ACAT1 antibodies (4 μg). Antibody-protein complexes were obtained using 20 μL protein A + G sepharose beads (P2012, Beyotime, Shanghai, China). Immunoprecipitates were then analyzed by Western blotting.

2.5. Western blotting

Total protein from NSCLC cells was extracted in RIPA (Beyotime, Shanghai, China) lysis buffer supplemented with 1 % phenylmethanesulfonyl fluoride. 40 μg of proteins were separated via 10 % SDS–PAGE, transferred onto 0.45 μm polyvinylidene fluoride membranes (Millipore, MA, USA), and incubated overnight at 4 °C with primary antibodies, followed by incubation with a horseradish peroxidase-conjugated anti-rabbit/mouse IgG secondary antibody. ImageJ software was used to evaluate the gray value of each band.

2.6. Chemotaxis assay

Peripheral blood mononuclear cells were isolated from the peripheral blood of healthy volunteers using Ficoll-Paque (GE Healthcare, CA, USA) according to the instructions. Transwell chambers (5 μm pore size, Corning, NY, USA) were used to perform the chemotaxis assay. 2×10^5 cells were loaded onto the top chamber and cell-free supernatant after different treatment was added to the lower chamber. The cells were allowed to migrate for 8 h at 37 °C. Then the migrated cells were harvested and resuspended in PBS containing 0.5 % BSA and tested with FACS Canto II cytometer (BD, NJ, USA) using antihuman-CD8-FITC (BD) and antihuman-CD3-APC (BD), to determine the percentage of CD3+/CD8+ T cells in the migrated cells and subsequent analysis was performed using FlowJo software.

2.7. Enzyme-linked immunosorbent assay (ELISA)

To evaluate the concentrations of CCL5 and CXCL10 in the cell culture supernatant after different treatments, ELISA kits (human CCL5/RANTES ELISA Kit and human CXCL10/IP-10 ELISA Kit, Multi Sciences, Hangzhou, China) were used according to the instructions supplied by the manufacturers [27].

2.8. Measurement of ACAT1 activity

ACAT1 activity was measured in a reaction buffer containing 50 mM Tris-HCl (pH 8.1), 20 mM MgCl2, 40 mM KCl, 10 μM acetoacetyl-CoA (pH 7.0), and 60 μM CoA. The reaction was maintained at 25 °C in 96 wells and was monitored by measuring the absorption at 303 nm [28] using a microplate reader (CLARIOstar, BMG LABTECH, UK).

2.9. Immunohistochemistry

EGFR mutant and wild-type tumor specimens were collected and cut into 4-mm sections. The sections were incubated with antibodies against NRF2 (Proteintech,16396-1-AP) or ACAT1 (Proteintech,16215-1-AP) at 1/100 dilution overnight at 4 °C. Then, the reaction was visualized using the Elivision super HRP IHC Kit (Maixin-Bio) and 3,3-diaminobenzidine (DAB); nuclei were counterstained with hematoxylin. The sections were dehydrated in ethanol before mounting.

2.10. Online database

A series of online databases were used in this study, as shown in Table 2.

2.11. Statistical analysis

All experiments were repeated at least three times. The data were analyzed using GraphPad Prism 8.0 software and are presented as the mean ± standard deviation. Student's *t*-test was used to compare the differences between the two groups. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. SIRT5 was highly expressed in EGFR-mutant LUAD

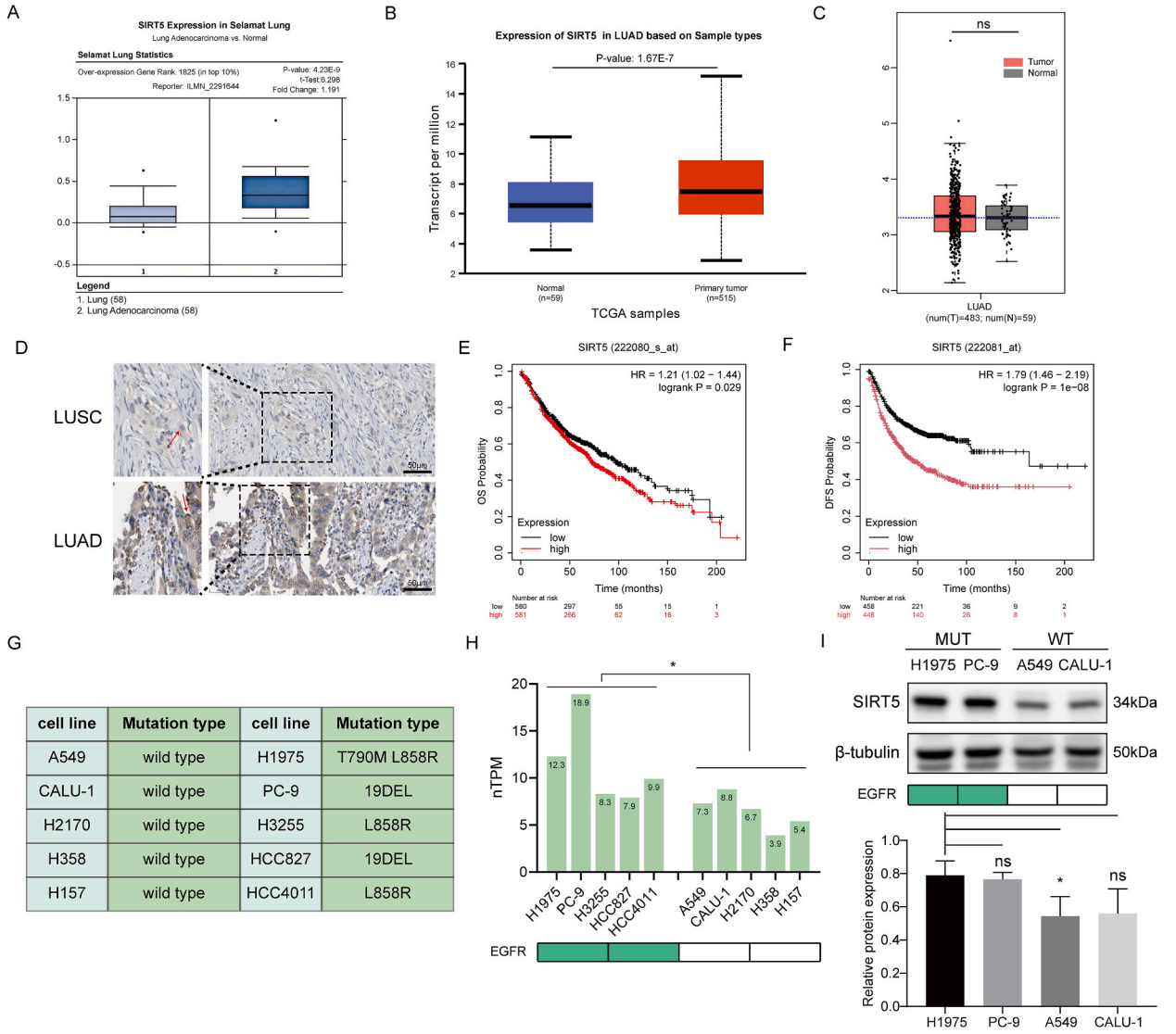
First, the mRNA expressions of SIRT5 were analyzed through three public databases. Both the Oncomine and UALCAN databases revealed that the mRNA expressions of SIRT5 were significantly higher in LUAD patients than in their normal counterparts whereas the expressions of SIRT5 in LUAD tumors were just subtle higher than that in normal ones, and the difference was not statistics significant (Fig. 1A–C). Moreover, as depicted in Fig. 1D, the immunohistochemistry findings from the HPA database affirmed that the protein expression of SIRT5 in LUAD was positive, while it was nearly negative in LUSC, indicating a higher protein expression of SIRT5 in LUAD compared to LUSC. Furthermore, the results from the Kaplan–Meier plotter database showed that high SIRT5 expression was associated with both worse overall survival and disease-free survival in patients with LUAD, suggesting the poor prognostic value of SIRT5 (Fig. 1E and F). As it was recently reported that SIRT5 is involved in tumor immunity [24,25], we wondered whether SIRT5 is related to the TIME of *EGFR*-mutant LUAD. Ten typical and representative cell lines were chosen from the CCLE database (Fig. 1G). Both the mRNA and protein expression of SIRT5 in these cell lines were verified by the HPA database and western blotting, respectively. The results indicate that SIRT5 was highly expressed in *EGFR*-mutant LUAD (Fig. 1H and I). Collectively, these results revealed that SIRT5 was highly expressed in LUAD and *EGFR*-mutant cell lines, suggesting a potential role of SIRT5 in the development of *EGFR*-mutant LUAD.

3.2. SIRT5 was related to the suppressive TIME and negatively regulated chemokines in $CD8^+$ T cells in EGFR-mutant LUAD

First, since *EGFR*-mutant LUAD was reported to have a poor response to immune therapy through the establishment of a suppressive TIME, which is characterized by a lack of CD8+T cell infiltration, we found that SIRT5 expression was significantly negatively correlated with $CD8^+$ T cell abundance in 517 patients with LUAD based on the results from the TISIDB database (Fig. 2A). In addition, we also found that most of the chemokines that are important for shaping the TIME, especially CCL5 and CXCL10, which are two potent chemokines for recruiting $CD8^+$ T cells into the TIME [29], had a negative relationship with SIRT5 expression in LUAD (Fig. 2B and C). These findings were confirmed via the GEPIA database (Fig. 2D). Then, we verified these observations by up- and downregulating SIRT5 in *EGFR*-wild-type cells and *EGFR*-mutant cells via transfection of a SIRT5 expression vector or a specific siRNA, respectively (Fig. 2E–G). The Chemotaxis assay showed that overexpression of SIRT5 attenuated CD3+/CD8+ T cells recruitment, whereas the opposite effects were observed in SIRT5 knockdown groups (Fig. 2H). These findings provided direct evidence that SIRT5 could regulate the $CD8^+$ T cells recruitment negatively. Besides, the upregulation of SIRT5 resulted in decreasing mRNA levels of CCL5 and CXCL10 (Fig. 2I). These effects were reversed by SIRT5 knockdown (Fig. 2J). Then, the secretion levels of these chemokines in the cell culture medium were tested by ELISA. Consistent with the above findings, overexpression of SIRT5 inhibited the secretion of CCL5 and CXCL10, whereas SIRT5 downregulation had the opposite effect (Fig. 2K). Moreover, the in vivo experiment demonstrated that the combination of PD-1 inhibitor and SIRT5 inhibitor exerted a superior effect compared with PD-1 inhibitor monotherapy (Fig. S1). These results indicated that SIRT5 was related to the suppressive TIME and negatively regulated chemokines of $CD8^+$ T cells in *EGFR*-mutant cell lines.

Table 2
The list of online databases used in the study.

Name	Website	Application
CCLE	https://sites.broadinstitute.org/ccle	To identify the mutation type of NSCLC cell lines used in the study.
Oncomine	https://www.oncomine.org/	To observe the expression of SIRT5 in lung cancer and normal tissues.
UALCAN	https://ualcan.path.uab.edu/	To compare the expression of SIRT5 in lung cancer and normal tissues.
Kaplan–Meier Plotter	http://www.kmplot.com/	To analyze the prognostic role of SIRT5 in lung cancer patients.
HPA	https://www.proteinatlas.org/	To observe the protein expression of SIRT5 by Immunohistochemical staining and its RNA expression in cell lines used in the study.
GEPIA	http://gepia.cancer-pku.cn/	To analyze the correlation between SIRT5 and ACAT1 and NRF2.
TISIDB	http://cis.hku.hk/TISIDB/	To analyze the correlation between SIRT5 and CD8+T cell abundance.
String	https://string-db.org/	To predict functional associations between proteins.
UniProt	https://www.uniprot.org/	To predict the sequence and possible posttranslational modification site of ACAT1



cell line	Mutation type	cell line	Mutation type
A549	wild type	H1975	T790M L858R
CALU-1	wild type	PC-9	19DEL
H2170	wild type	H3255	L858R
H358	wild type	HCC827	19DEL
H157	wild type	HCC4011	L858R

Fig. 1. SIRT5 was highly expressed in LUAD and *EGFR*-mutant cell lines. The mRNA expressions of SIRT5 in LUAD and normal tissues are based on **(A)** Oncomine, **(B)** UALCAN, and **(C)** GEPIA databases. **(D)** The protein expressions of SIRT5 in LUAD and LUSC tissues by immunohistochemistry from the HPA database. The red arrow marks the representative staining of SIRT5. Scale bar = 50 μm. The results from the Kaplan-Meier Plotter database of LUAD patients' **(E)** overall survival (OS) and **(F)** disease-free survival (DFS) with high or low SIRT5 expression. **(G)** The mutation characters of ten typical and representative LUAD cell lines were identified through the CCLE database. **(H)** The mRNA expressions of SIRT5 in the typical LUAD cell lines are based on the HPA database. **(I)** The protein expressions of SIRT5 in cell lines used in this study were determined by western blotting and the relative protein expressions were analyzed by ImageJ software. *$P < 0.05$, ns: not significant.

3.3. *The NRF2 pathway mediated the suppressive effect of SIRT5 on chemokines*

Our previous study demonstrated that the NRF2 pathway could be activated by SIRT5 in ovarian cancer [21], and it was also reported that NRF2 and its downstream target genes are closely related to the synthesis and secretion of CCL5 and CXCL10 [16–19]. We speculated that the NRF2 pathway can also be regulated by SIRT5 in LUAD and may be a potential regulatory factor for these chemokines. As shown in Fig. 3A, the results from the STRING database showed potential interactions among SIRT5, NRF2, CCL5, and CXCL10. In addition, a significant positive correlation between SIRT5 and NRF2 was confirmed by the GEPIA database (Fig. 3B). Furthermore, NRF2 and its downstream HO-1 proteins were upregulated upon SIRT5 overexpression in *EGFR*-wild-type cells and downregulated upon SIRT5 knockdown in *EGFR*-mutant cells (Fig. 3C). To provide further support, a functional rescue experiment was designed. A549 cells were treated with ML385, a specific inhibitor of NRF2, for 12, 24, or 48 h. NRF2 expression was significantly suppressed at 24 h (Fig. 3D). Hence, this was selected as the suitable condition for subsequent study. The levels of secreted CCL5 and CXCL10 in A549 cells overexpressing SIRT5 were then tested by ELISA after the cells were treated with or without ML385 for 24 h. The

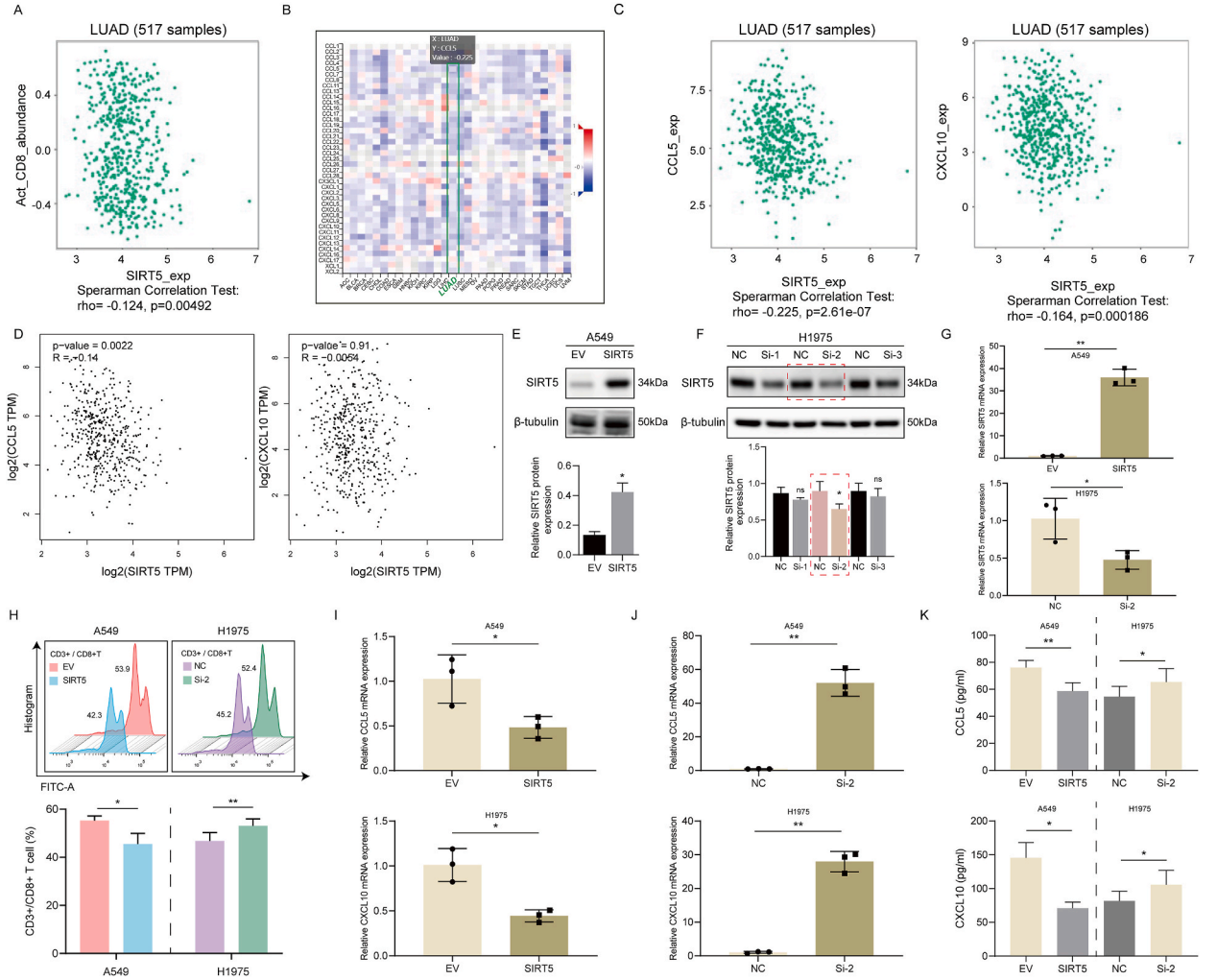


Fig. 2. SIRT5 was related to suppressive TIME and regulated chemokines of $CD8^+$ T cells negatively. **(A)** The expression of SIRT5 was negatively correlated with $CD8^+$ T cell abundance based on the TISIDB database. **(B)** The expression of SIRT5 was negatively correlated with most chemokines in LUAD based on the TISIDB database. **(C, D)** The negative relativity between SIRT5 expression and CCL5 and CXCL10 in LUAD through TISIDB and GEPIA database, respectively. SIRT5 was **(E)** upregulated in *EGFR* wild-type cells and **(F)** knocked down in *EGFR* mutant cells. The interference efficiency was detected by western blotting. No.2 siRNA sequence exerted the highest transfection efficiency and was highlighted by red dotted frames. **(G)** The interference efficiency was detected by qRT-PCR. **(H)** CD3+/CD8+ T cells recruitment was evaluated by the chemotaxis assay; cells that migrated to the lower chamber were stained and recognized by a flow cytometer, and analyzed by Flowjo software. The mRNA levels of CCL5 and CXCL10 were detected after SIRT5 was **(I)** overexpressed in *EGFR* wild-type cells and **(J)** downregulated in *EGFR* mutant cells by qRT-PCR. **(K)** ELISA was performed to determine the secreted levels of CCL5 and CXCL10 after regulating SIRT5. EV, empty vector; NC, negative control; Si-2, No.2 of small interfering RNA that targets SIRT5. $*P < 0.05$, $**P < 0.01$, ns: not significant.

results showed that the inhibition of these chemokines by SIRT5 was reversed upon ML385 treatment (Fig. 3E). Taken together, these results revealed that SIRT5 inhibits these chemokines by regulating the NRF2 pathway.

3.4. SIRT5 functioned as a desuccinylase of ACAT1 in EGFR-mutant LUAD

To further investigate the underlying role of SIRT5 in LUAD, we compared the succinylation levels of LUAD cell lines by Western blotting since SIRT5 is reported to possess weaker deacetylase activity but stronger desuccinylase activity. Consistent with our hypothesis, the succinylation levels of *EGFR*-mutant cells were lower than those of *EGFR*-wild-type cells, while the acetylation or malonylation levels were similar (Fig. 4A). When the expression of SIRT5 was upregulated, the levels of succinylation in the *EGFR*-wild-type cells decreased, while the opposite effect was observed in the *EGFR*-mutant cells (Fig. 4B). Then, mass spectrometry analyses were performed to further investigate the substrate of SIRT5. The results showed that acetyl-CoA acetyltransferase 1 (ACAT1), the abnormal elevated enzyme activity of which was recently reported in diverse human cancer cell lines [30], could interact with SIRT5 (Fig. 4C). The prediction results from the UniProt database also confirmed that ACAT1 could be desuccinylated by SIRT5 (Fig. 4D).

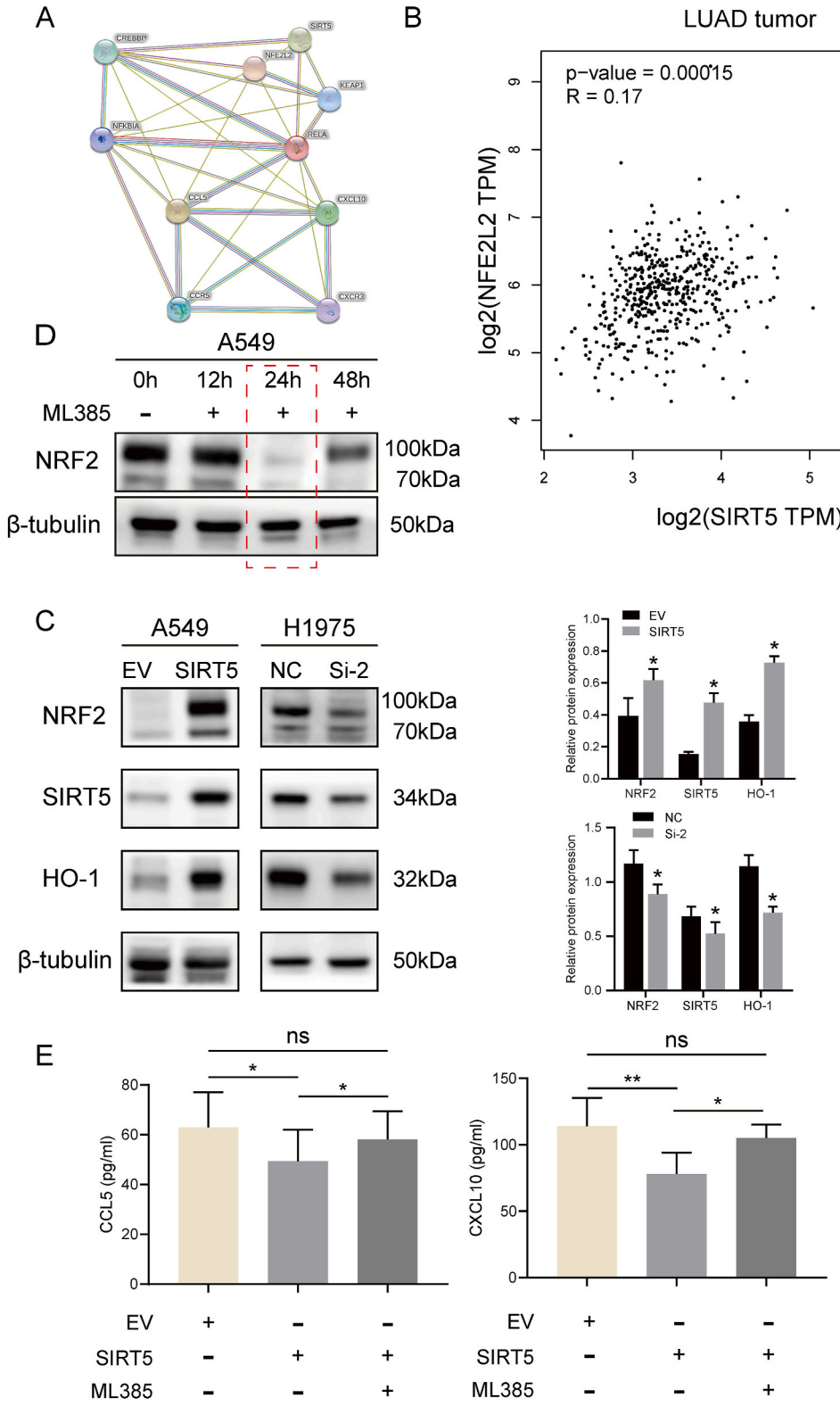
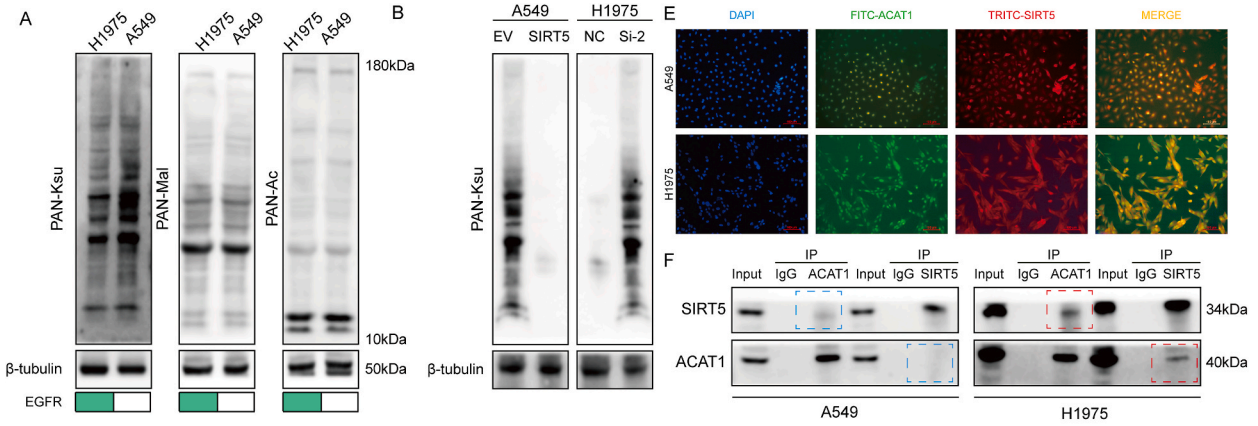


Fig. 3. NRF2 pathway mediated the suppressor role of SIRT5 on chemokines. **(A)** The interactions among SIRT5, NRF2, CCL5, and CXCL10 were analyzed by the String database. **(B)** The positive relationship between mRNA expression of SIRT5 and NRF2 in LUAD was observed through the GEPIA database. **(C)** Changes in key protein levels in the NRF2 pathway were tested by western blotting after up- or down-regulating SIRT5. The relative western blot gray values were shown in the histogram. **(D)** The inhibitory efficacy of ML385 on NRF2 in A549 cells was tested by western

blotting. The expression of NRF2 was significantly inhibited after exposure to 5 μM ML385 for 24h and was highlighted by red dotted frames. **(E)** The secreted levels of CCL5 and CXCL10 were tested by ELISA after overexpressed SIRT5 and the addition of ML385 in A549 cells. EV, empty vector; NC, negative control; Si-2, No.2 of small interfering RNA that targets SIRT5. *$P < 0.05$, **$P < 0.01$, ns: not significant.



C

Accession	Protein name	Description	Positions	Modified sequence	Mass	Score	Matches	Sequences	emPAI
P24752	ACAT1	Acetyl-CoA acetyltransferase, mitochondrial OS=Homo sapiens OX=9606 GN=ACAT1 PE=1 SV=1	268	_FSKVPKLK(su) TV_	45456	93	1(1)	1(1)	0.07

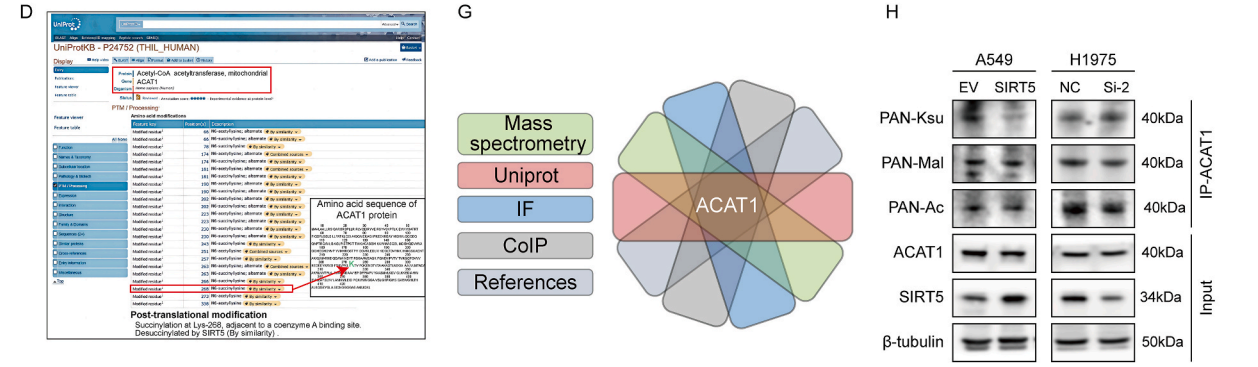


Fig. 4. SIRT5 functioned as a desuccinylase of ACAT1. **(A)** The succinylation (PAN-Ksu), malonylation (PAN-Mal), and acetylation (PAN-Ac) levels of *EGFR*-mutant cells and *EGFR*-wild cells were compared by western blotting. **(B)** Changes in succinylation levels in both *EGFR*-mutant cells and *EGFR*-wild cells after regulating SIRT5 were tested by western blotting. Results from **(C)** mass spectrometry analysis and **(D)** UniProt database recognized that ACAT1 could interact with the SIRT5 protein. **(E)**Immunofluorescence staining was performed to detect SIRT5 and ACAT1 localization in both *EGFR*-mutant cells and *EGFR*-wild cell lines (10 ×). Scale bar = 100 μm. **(F)** Co-IP results of SIRT5 and ACAT1 proteins in both *EGFR*-mutant cells and *EGFR*-wild cells. The positive IP results were highlighted by red dotted frames while negative ones were blue dotted frames**. (G)** The Venn diagram of results from mass spectrometry, UniProt database, Immunofluorescence staining (IF), Co-IP, and references showed that ACAT1 may be a potential enzyme target of SIRT5. **(H)** *EGFR*-wild cells and *EGFR*-mutant cells transfected with SIRT5 vector or siRNA respectively were immunoprecipitated with an anti-ACAT1 antibody. The succinylation, malonylation, and acetylation levels were tested by western blotting. EV, empty vector; NC, negative control; Si-2, No.2 of small interfering RNA that targets SIRT5.

Interestingly, the immunofluorescence staining results showed that SIRT5 and ACAT1 were both located in the cytoplasm of *EGFR*-mutant cells, while SIRT5 was located in the cytoplasm, and ACAT1 was located in the nucleus in *EGFR*-wild-type cells (Fig. 4E). Similar results were observed in the Co-IP assay; that is, in *EGFR*-mutant cells, SIRT5 and ACAT1 could interact with each other, while combinations of both were not observed in *EGFR*-wild-type cells (Fig. 4F). Based on the above findings and our previous results in prostate cancer [23], we suspected that ACAT1 may be a potential target of SIRT5, especially in *EGFR*-mutant cells (Fig. 4G). Then, the variation in the succinylation level of ACAT1 in response to SIRT5 expression regulation was tested by Co-IP and Western blotting. The results showed that the succinylation level of ACAT1 increased when SIRT5 was upregulated but decreased when SIRT5 was downregulated. However, the acetylation or malonylation levels of ACAT1 were not related to variations in SIRT5 expression (Fig. 4H). Overall, these results revealed that SIRT5 functioned as a desuccinylase of ACAT1 in *EGFR*-mutant LUAD.

3.5. ACAT1 activity was regulated by SIRT5 to activate the NRF2 pathway

Next, the relationships among SIRT5, ACAT1, and the NRF2 pathway were further investigated. Using the GEPIA database, we first found that the mRNA expression of SIRT5 and ACAT1 was positively correlated (Fig. 5A). However, the western blotting results showed that the protein expression of ACAT1 remained regardless of whether SIRT5 expression was up- or downregulated (Fig. 5B). Besides, based on the Kaplan–Meier Plotter, the high expression of ACAT1 was associated with better disease-free survival and overall survival (Fig. 5C). We suspected that the enzyme activity of ACAT1, rather than the protein itself, may function as an anti-tumor target. It has been reported that succinylation can regulate enzyme activity [31]; hence, we tested whether the activity of ACAT1 was affected by SIRT5. As shown in Fig. 5C, the enzyme activity of ACAT1 increased significantly when SIRT5 was overexpressed but decreased upon SIRT5 knockdown, suggesting that ACAT1 activity could be regulated by SIRT5. Next, we investigated the mechanism underlying the interaction between ACAT1 and the NRF2 pathway. We retrospectively collected tumor tissues of *EGFR* mutant and *EGFR* wild-type LUAD patients and conducted immunohistochemistry to explore the protein expression levels of NRF2 and ACAT1. Similar to our findings at the cellular level, as shown in Fig. 5D, the protein expression of NRF2 was higher in EGFR mutant tissues than in wild-type tumors whereas the expression of ACAT1 was comparable in both types of tumors. Then, a specific ACAT1 enzyme inhibitor, avasimibe, was used to perform a rescue experiment. The efficiency of the inhibitor was confirmed first, and it was clear that 48 h was suitable for subsequent observation (Fig. 5E). The results showed that the activation of the NRF2 pathway by SIRT5 was reversed when ACAT1 activity was inhibited (Fig. 5F), suggesting that ACAT1 played an essential role in regulating the NRF2 pathway by SIRT5. Overall, the enzyme activity of ACAT1, rather than the protein itself, was an intermediate bridge between the NRF2 pathway and SIRT5.

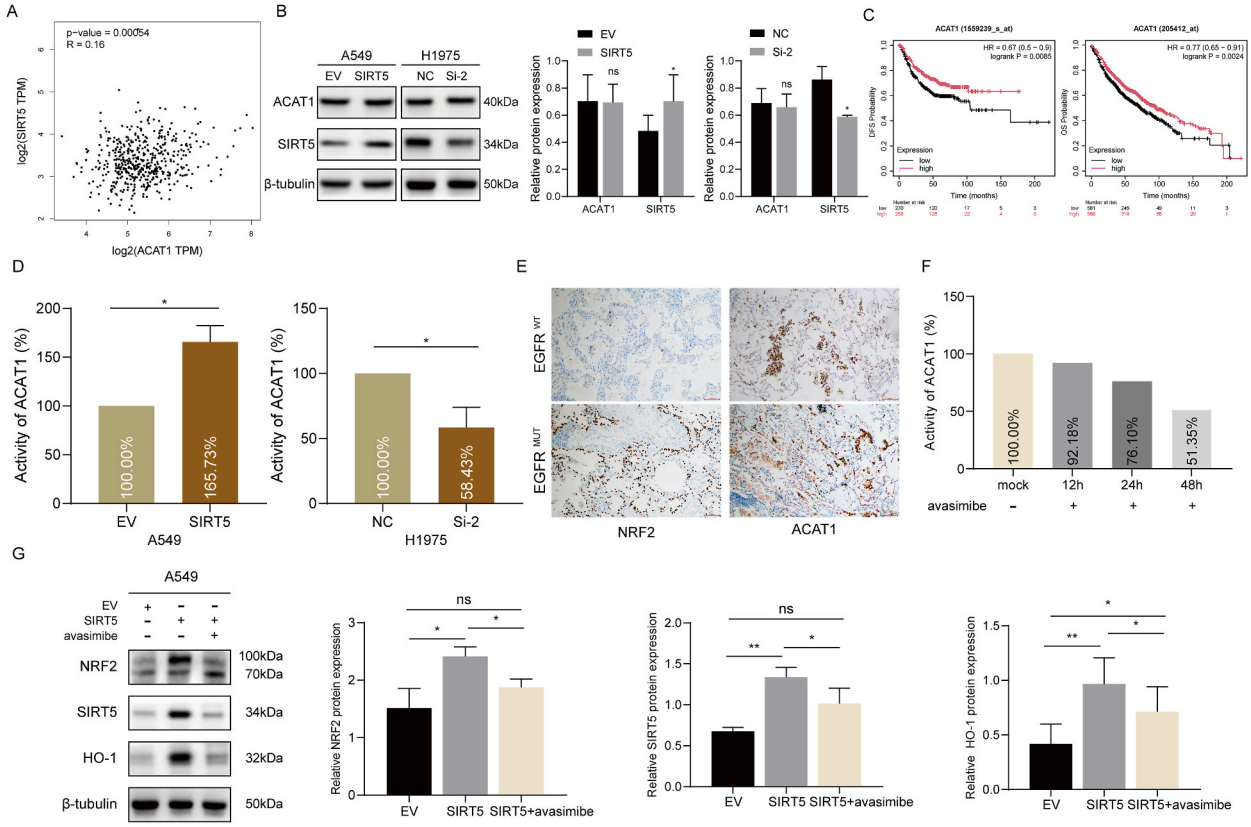


Fig. 5. ACAT1 activity was regulated by SIRT5 to active NRF2 pathway. **(A)** The mRNA expression relationship between SIRT5 and ACAT1 was analyzed by the GEPIA database. **(B)** The protein expression of SIRT5 and ACAT1 were determined by western blotting after *EGFR*-wild cells and *EGFR*-mutant cells were transfected with SIRT5 vector or siRNA, respectively. The relative gray values are shown in the histogram. **(C)** The results from the Kaplan-Meier Plotter database of LUAD patients' disease-free survival (DFS) and overall survival (OS) with high or low ACAT1 expression. **(D)** The activity of ACAT1 was tested after up- or down-regulating SIRT5 in *EGFR*-wild cells and *EGFR*-mutant cells. **(E)** The representative images of immunohistochemistry present the protein expressions of NRF2 and ACAT1 in *EGFR* mutant and wild-type tumors. **(F)** The inhibitory efficacy of avasimibe on ACAT1 activity was tested after A549 cells exposure to 7.5 μM avasimibe for 12, 24, and 48h. **(G)** After being transfected with the SIRT5 vector and treated with avasimibe in A549 cells, the protein expressions of the NRF2 pathway were tested by western blotting, and the relative western blot gray values were shown in the histogram. EV, empty vector; NC, negative control; Si-2, No.2 of small interfering RNA that targets SIRT5. $^{*}P < 0.05$, $^{**}P < 0.01$, ns: not significant.

4. Discussion

EGFR-mutant LUAD exhibits a poor response to ICIs by shaping a suppressive TIME, the mechanism of which remains unclear. Here, we demonstrated that SIRT5 participated in the suppressive TIME of *EGFR*-mutant LUAD by desuccinylating ACAT1 to enhance its enzymatic activity and subsequently activate NRF2 axis-mediated chemokine regulation (Fig. 6).

SIRT5 is involved in cell metabolism, including glycolysis, the tricarboxylic acid cycle, fatty acid oxidation (FAO), nitrogen metabolism, the pentose phosphate pathway, antioxidant defense, and apoptosis [32]. SIRT5 has important regulatory roles in tumor progression and is highly expressed in many solid tumors, including NSCLC [20]. Similarly, we also observed that SIRT5 was highly expressed in both LUAD tissues and cell lines, especially in *EGFR*-mutant cells, suggesting a potential role for SIRT5 in the development of *EGFR*-mutant LUAD. In addition, it was recently reported that SIRT5 mRNA expression was negatively correlated with the level of immune infiltration in several pan-cancer cell types, especially in antitumor immune cells, including $CD8^+$ T cells [33]. Here, we also confirmed that SIRT5 was significantly negatively correlated with $CD8^+$ T-cell abundance in 517 patients with LUAD in the TISIDB database, suggesting its positive relationship with a suppressive TIME. CCL5 and CXCL10 are two essential chemokines that can induce $CD8^+$ T cells to enter the TIME, and we observed that both the mRNA and secreted levels of these two chemokines were negatively regulated by SIRT5. However, there have been no reports of this novel role of SIRT5 in directly regulating chemokines, there should be a mediating factor that needs to be explored.

Tumors take advantage of the NRF2/HO-1 axis, which is an important antioxidative pathway under normal conditions, to maintain malignant behavior. Previously, we reported that SIRT5 can activate this pathway to promote the malignant phenotype of ovarian cancer cells [21]. Similarly, in NSCLC and even cerebral ischemia-reperfusion, the NRF2/HO-1 axis is an important pathway that mediates the multiple functions of SIRT5 [34,35]. In addition, recent studies have shown that this pathway is involved in immune cell infiltration by regulating chemokines, such as CCL5 [36], and that when this axis is suppressed, the $CD8^+$ T-cell response is enhanced [37], suggesting a novel role for the NRF2/HO-1 axis in the TIME. Here, we also observed a potential relationship between SIRT5, NRF2, CCL5, and CXCL10 based on the STRING database. The modulatory effect of SIRT5 on the NRF2/HO-1 axis was confirmed by western blotting, and rescue experiments verified that this pathway mediated the suppressive effect of SIRT5 on the levels of the abovementioned chemokines. However, the combined score between SIRT5 and NFE2L2 was just 0.298 by textmining based on the STRING database, which was not very convincing evidence to predict the strong correlation from the protein-protein interaction between them. We suspected that there may be a molecule to functions as a linker between them instead of combined directly. Previously, we have found that NRF2 was able to bind to the promoter of BRCA1, which is an important DNA damage repair gene, to inhibit the STING pathway to participate in the suppressive TIME of KRAS/KEAP1 co-mutant NSCLC [27]. Besides, we have also found that BRCA1 was an upstream factor of SIRT5 in ovarian cancer [21]. Hence, BRCA1 or STING pathway may be the linkers between SIRT5 and NRF2, further studies are needed to provide more mechanistic insights.

Succinylation is a newly discovered protein posttranslational modification that occurs widely in cells and can participate in a variety of life activities by regulating protease activity and gene expression. Abnormal regulation of lysine succinylation occurs in

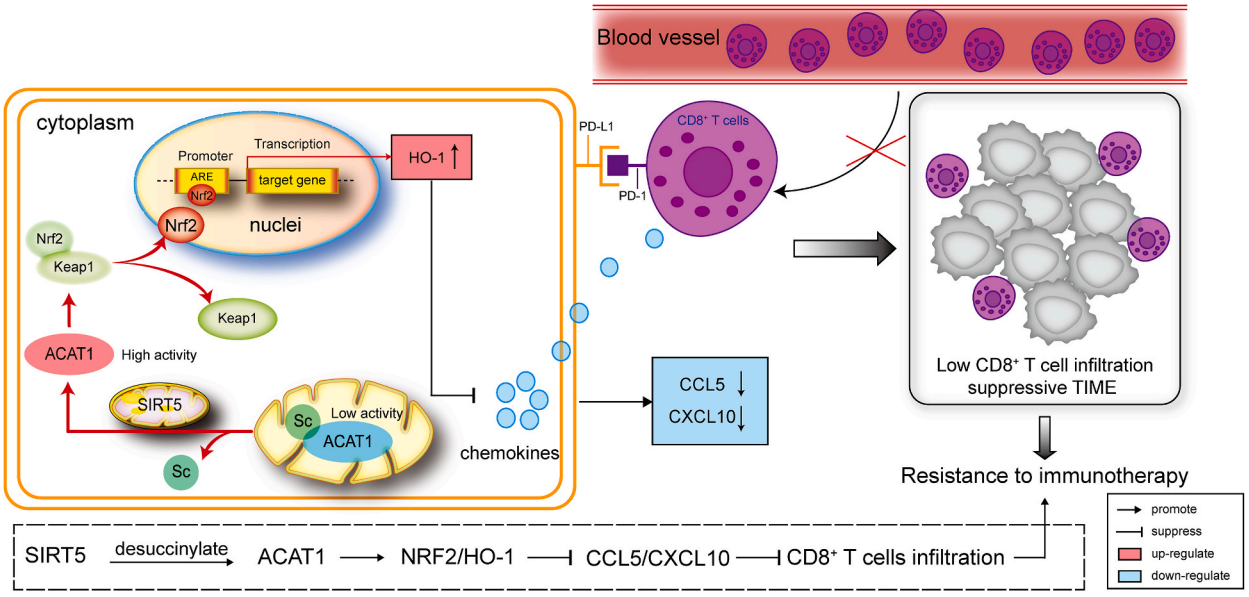


Fig. 6. Schematic of SIRT5 participates in the suppressive tumor immune microenvironment of *EGFR*-mutant lung adenocarcinoma by regulating the succinylation of ACAT1. The left box represents EGFR-mut LUAD cells. SIRT5 desuccinylates ACAT1, enhances its enzyme activity, and promotes the dissociation of NRF2 from KEAP1 in the cytoplasm. NRF2 binds to antioxidant response elements (ARE) in the promoter region of target genes after nuclear entry, promotes the transcription and expression of target genes (HO-1), inhibits the synthesis and secretion of chemokines CCL5 and CXCL10, hinders the migration and infiltration of CD8+T cells, and forms inhibitory TME (right box), leading to immunotherapy tolerance.

different cancers [26]. Compared to other SIRT family members, SIRT5 is the only known mitochondrial desuccinylase with the ability to catalyze the removal of succinyl groups from lysine residues [32]. In this study, the succinylation levels of *EGFR*-mutant cells were lower than those of *EGFR*-wild-type cells, which was the opposite of the pattern observed for the expression of SIRT5. Interestingly, the levels of acetylation and malonylation, which are also the posttranslational modifications function of SIRT5, were similar in these cells. In addition, the succinylation levels exhibited opposite changes upon the regulation of SIRT5 expression, suggesting a potential role for SIRT5 as a desuccinylase. The target substrate of SIRT5, which may be the mechanism underlying its aforementioned effect, requires further investigation. ACAT1, which is believed to catalyze the reversible formation of acetoacetyl-CoA from two molecules of acetyl-CoA during FAO, has recently been identified as a potential new target for anticancer therapy [30]. It was reported to be widely participate in tumor initiation and progression, including lung cancer. Moreover, it is also essential for regulating anti-tumor immunity [38,39]. Similarly, we previously found that ACAT1 played an important role in the lncRNA-miRNA axis to promote NSCLC development [40]. Interestingly, the high expression of ACAT1 was associated with a better prognosis in patients with LUAD. Besides, it was also reported that Kras-specific antigenic peptides in combination with avasimibe could promote $CD8^+$ T cell infiltration and impair lung tumor progression [41]. Collectively, these findings were in accordance with our hypothesis, that the enzyme activity of ACAT1, rather than the protein itself, functions as an anti-tumor target. Several studies have indicated that SIRT5 is involved in the modulation of many enzymes involved in numerous metabolic pathways, including FAO, through its desuccinylase activity [32]. In a recent study that reanalyzed publicly available data, it was reported that the tumor promoter activity of SIRT5 was mediated through substrates such as ACAT1 [42]. Here, ACAT1 was identified as a substrate of SIRT5 in *EGFR*-mutant LUAD, and SIRT5 functions as a desuccinylase of ACAT1 to increase the enzyme activity of ACAT1, resulting in activation of the NRF2 pathway. These findings suggest a potential role for ACAT1 in cancer promotion and highlight its significance as a target for further anticancer investigations.

Overall, we clarified the effects of SIRT5 on the suppressive TIME of *EGFR*-mutant LUAD and revealed a novel regulatory mechanism underlying its effect on the NRF2 pathway and ACAT1 modification. Unfortunately, there is a lack of sufficiently detailed in vivo experiments to confirm our observations in vitro due to the limitations of our objective conditions. In addition, we failed to clarify the comprehensive mechanisms between the NRF2 pathway and SIRT5, how NRF2 activation influences the TIME, chemokine expression, and the specific site of ACAT1 modified by SIRT5. Thus, further studies are still warranted.

5. Conclusion

In conclusion, our study reveals a novel role of SIRT5 in the TIME and provides a new perspective for ICIs treatment of *EGFR*-mutant LUAD. The combination of SIRT5 inhibitors with ICIs may be a promising therapeutic approach for overcoming ICIs resistance. Our study provides the rationale for clinical trials of this combination strategy.

CRediT authorship contribution statement

Wang Shouhan: Writing – review & editing, Writing – original draft, Methodology. **Li Qingchang:** Writing – review & editing, Project administration, Conceptualization. **Sun Xiaodan:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Conceptualization.

Data availability statement

Data associated with the study has not been deposited into a publicly available repository. Data will be made available on request.

Ethics statement

The present study was approved by the Ethics Committee of the Jilin Cancer Hospital and carried out in accordance with the World Medical Association Declaration of Helsinki.

Ethics approval number:202202-003-01.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39743.

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