STEAP1 facilitates metastasis and epithelial-mesenchymal transition of lung adenocarcinoma via the JAK2/STAT3 signaling pathway.

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Abbreviation

STEAP1 (Six-transmembrane epithelial antigen of prostate-1)

EMT (epithelial to mesenchymal transition)

LUAD (lung adenocarcinoma)

JAK2 (Janus kinase 2)

STAT3 (signal transducer and activator of transcription 3)

GAPDH (glyceraldehyde-3-phosphate dehydrogenase)
Six-transmembrane epithelial antigen prostate-1 (STEAP1) is a relatively newly identified gene target from prostate cancer, breast cancer and gastric cancer. However, functions of STEAP1 in lung adenocarcinoma are still unknown. In this study, we explored the molecular and cellular mechanisms of STEAP1 in lung adenocarcinoma. Western blot and Q-PCR were conducted to detect the protein and mRNA expressions respectively. The cell proliferation was tested by CCK8 assay. The effects of STEAP1 on the metastasis and epithelial-mesenchymal transition (EMT) of lung adenocarcinoma were evaluated by EdU assay, wound healing assay and transwell migratory assay. H1650, H358, HCC827, H1299, H23, A549, H1693 were selected as Human LUAD cell lines in the study. Results
have shown that STEAP1 expression was upregulated in lung adenocarcinoma cells compared with normal lung epithelial cells. Knockdowning of STEAP1 suppressed the proliferation, migration and invasion of lung adenocarcinoma epithelial cells. Importantly, after comparing the proliferation, migration and invasion of lung adenocarcinoma to the corresponding control groups treated in STAT3 inhibitor ADZ1480, we found that STEAP1 regulates epithelial to mesenchymal transition (EMT) via JAK2/STAT3 signaling pathway. In conclusion, STEAP1 can serve as a therapeutic target, and it may have important clinical implications for lung adenocarcinoma treatment.

**KEYWORDS** STEAP1; JAK2/STAT3 signaling; EMT; Lung adenocarcinaoma

1 Introduction

Nowadays, lung cancer is one of the major causes of cancer-related deaths[1]. According to the related publications, lung cancer can be generally divided into 2 subtypes: small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC), each of them accounts for 15% and 85% of all lung cancer cases, respectively[2]. Lung adenocarcinoma (LUAD) is likely to occur in women, asian people and non-smokers[3]. LUAD is often metastatic and has a relatively poor prognosis[4]. Chemotherapy, surgery and radiotherapy are traditional treatments for LUAD[5,6], but the recurrence of early post-operative lung cancer patients are still very high[7]. Meanwhile, chemotherapy and targeted therapies have extensive drug resistance[8,9]. Thus, to explore the molecular mechanism of target genes are important strategy for searching of LUAD treatment.

Six-transmembrane epithelial antigen of prostate-1 (STEAP1) is a novel 339 amino acids cell
surface protein. It appears to be an ion channel or transporter, plays a role in cell adhesion and may be related to tumor proliferation and invasion\textsuperscript{[10]}. The relationship between STEAP1 and LUAD is barely reported.

The Janus kinase (JAK)2/signal transducer and activator of transcription (STAT)3 signaling pathway are known to be often excessive and they are in relevant with various of physiological processes, and a wide range of genes are affected by them. In this report, we will focus on the regulatory functions of STEAP1 in proliferation, metastasis and invasion of LUAD, and how the JAK2/STAT3 signaling pathway is involved in these processes.

2 Material and Methods

2.1 Reagents and Cells

Human LUAD cell lines, including H1650, H358, HCC827, H23, A549, H1693, H1299 were purchased from American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (KeyGen Biotech Co. Ltd., Nanjing, China) with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin and streptomycin (KeyGen Biotech Co. Ltd., Nanjing, China) in a humidified incubator at 37°C containing 5% CO\textsubscript{2}.

Real-time quantitative PCR primers were provided by Bioengineering. In addition, FastQuant RT Kit (WithgDNase) (Cat:#KR106-02), RNA Extraction Kit (Cat:#RK123) and SuperReal PreMix Plus (SYBR Green) Kit (Cat:#FP205-02) were purchased from TIANGEN. Lipofectamine 2000 transfection reagents were purchased from Invitrogen (Cat:#1854323). Antibodies against GAPDH (Cat:#60004-1-Ig) was purchased from Proteintech and STEAP1 antibody (Cat#88677) was purchased
from Cell Signaling Technology. EdU cell proliferation test kit was bought from Solarbio (Cat:#1170).

2.2 Western blot assay

Lung adenocarcinoma cells were washed with cold PBS for 3 times to prepare whole-cell protein extracts. Then, 8% or 10% sodium dodecylsulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins according to the molecular weight. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were probed with the primary antibodies after washing 3 times with PBST, the membranes then probed with appropriate secondary antibodies (Cell Signaling Technology). Enhanced chemiluminescence (ECL) plus detection kit was applied for detecting the immunoreactive bands. The primary antibodies used were as follows:

- GAPDH (cat:#5174,CST), STEAP1 (cat:#PA5-20404, Invitrogen).

2.3 Real-time quantitative PCR (Q)-PCR assay

The RNA extraction kit was used to extract total RNA. Then the FastQuant RT Kit (With gDNase) was applied to reverse transcribe RNA into cDNA. The PCR mixtures were prepared according to the instructions of SuperReal PreMix Plus (SYBR Green) Kit,. PCR amplification and analysis were conducted on the ABI 7300 real-time PCR machine. All Ct values were normalized by the Ct value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer lists were as follows: GAPDH F: 5’-TGCACCACCAACTGCTTAGC-3’, R: 5’-GGCATGGACTGTGGTCATGAG-3’, STEAP1 F: 5’-AATTCAGATCTACAGATACAAGCTACTCTA-3’, R: 5’-AGCTTAGAGTAGCTTGTATCTGTAGGATCTG-3’.

2.4 Immunohistochemical (IHC) analysis
Paraffin-embedded tissues were sectioned at 5µm thickness. Slides were baked at 60 °C for 1 h, then deparaffinized, finally rehydrated for 10 min in sodium citrate buffer. Sections were cooled to room temperature, treated with 3% H2O2 for 10 min and blocked with 5% goat serum for 40 min at room temperature. Primary STEAP1 antibodies were used for incubation for 2-3 hours at 37°C (STEAP1 polyclonal antibody (Cat# PA5-20404) with a concentration of 25 µg/mL (Thermo Fisher) Then, sections were washed in phosphate-buffered saline (PBS) and incubated with the secondary antibody (biotinylated goat anti-rabbit, diluted 1:200) for 30 min. After washing again with PBS, the sections were dehydrated with ethanol for 2 min, followed by xylene transparency for 5 min. then tablets were eventually quickly sealed with neutral gum and ultra-thin cover glass. Sections were observed under ordinary optical microscope.

2.5 Wound healing test

equal numbers of H1299NC, H1299shRNA, A549vector and A549STEAP1 cells were seeded in 6-cm dishes wells, the next day when the cell were attached, vertical wounds were made on the cell monolayers cells by using 200 µL pipette tips. Then scratched cells were washed off with phosphate buffer saline (PBS). An optical microscope was used to photograph changes of wounds after being scratched for 0 and 48 h.

2.6 CCK8 assay

The cell counting kit-8 (CCK-8) was implemented to measure cell proliferation. The cells from each groups were seeded at a density of 2,000 cells/well in 100 µL media in the triplicate groups in 96-well plates. Starting from the following day, 10 µL CCK-8 reagent were add into each well and
incubated at 37°C for two to four hours, and the OD value at 450 nm wavelength was measured using a microplate reader.

2.7 Transwell invasion assay and transwell migration assay

Transwell invasion assay was performed using the 24-well invasion chambers (Corning, BioCoat, USA) with an 8 micron pore size PET membrane with Matrigel Matrix being treated. Cells were trypsinized and resuspended, then inoculated into the upper Matrigel chamber in 500 μL of serum-free DMEM medium at a concentration of 1×10^5 cells/well. DMEM medium containing 30% fetal bovine serum in the lower chamber served as the chemoattractant. At the end of incubation, the noninvading cells on the upper membrane surface were erased with cotton swabs. The invaded cells on the lower surface of the membrane were fixed and stained with Giemsa for 5 min. Nine visual fields of each chamber were randomly chosen, and observed under a X71 microscope (Olympus, Tokyo, Japan), and the number of invading cells from pictures was counted by cell counter.

2.8 Cell transfection and reagents used

Transfection of shRNA was carried out using lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Briefly, cells were seeded at a concentration of 2×10^5 cells/dish (6cm) and grown to 70% confluence. Lipofectamine 2000 and shRNA were then mixed and the mixture was incubated in Opti-MEM at room temperature for 15 min. Subsequently, the cells were incubated in
medium for 24 h, and then harvested for assays. The recombinant vector was validated by Sanger sequencing. The empty vector pEnter (Vigene) was used as the negative control. The efficiency of transfection was verified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot.

2.9 Statistical analysis

Q-PCR data was analyzed by GraphPad Prism 7 software. T-test was used for the comparison of samples within two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1 STEAP1 expression in lung adenocarcinoma tissues

We investigated the expression of STEAP1 in lung adenocarcinoma (LUAD) tissues and para-carcinoma normal tissues, results have shown that STEAP1 expression level in LUAD tissues was significantly higher than para-carcinoma normal tissues (Fig. 1 A-C). The Relative STEAP1 expression of lung adenocarcinoma (LUAD) tissues and para-carcinoma normal tissues in the GEPIA dataset were shown in Fig. 1 D. Datas obtained from the Gene Expression Profiling Interactive Analysis (GEPIA) revealed that overall survival in the low STEAP1 group was higher than that in high STEAP1 group (Fig. 1 E). The correlation between STEAP1 expression and clinical variables (age, gender, differentiation, tumor size, lymph node metastasis, distant metastasis, tumor stage) showed that lymph node metastasis, distant metastasis and tumor stage can significantly affect STEAP1 expression ($P < 0.05$, Table 1).
3.2 STEAP1 promotes proliferation of lung adenocarcinoma cells

To further validate the results listed above, we firstly investigated the protein and mRNA expression levels of STEAP1 in seven common lung adenocarcinoma cell lines (H1650, H358, HCC827, H1299, H23, A549, and H1693). Western blot and Q-PCR showed that among seven lung adenocarcinoma cell lines, H1299 was mostly expressed and A549 was least expression (Fig. 2A).

Secondly, to investigate the effects of knocking down and overexpression of STEAP1, we designed shRNA targeting at STEAP1, and its random control shRNA (NS) and transfected them in H1299 cell line. We develop STEAP1 expression plasmid and empty vector control and did over expression experiment in A549 cells (Fig. 2B). The cell proliferation of each group of cells (H1299NC, H1299shRNA, A549vector and A549STEAP1) were measured with CCK8 reagent for five consecutive days, and the results showed that the OD450 of A549STEAP1 and H1299shRNA were significantly lower than that of A549vector and H1299NC groups on days 2-5 (both P < 0.01, Fig. 2C). Ratio of EdU positive cells in H1299NC was significantly higher than that in H1299shRNA group, ratio of EdU positive cells of A549STEAP1 was significantly higher than that of A549vector (Fig. 2D). We also estimated the expression of proliferation-related proteins, PCNA and cyclinD1, in H1299NC, H1299shRNA, A549vector and A549STEAP1 groups, western blot and Q-PCR assays results showed that the expressions of cyclinD1 in H1299NC were significantly higher than that in H1299shRNA, meanwhile, A549STEAP1 was significantly higher than A549vector. Besides, the expression of PCNA in H1299NC was significantly higher than that in H1299shRNA, A549STEAP1 was significantly higher than A549vector (Fig. 2E).

3.3 STEAP1 promotes migration and invasion of lung adenocarcinoma cells
Next, wound healing assay was used to examine the migration of cells with each treatment. Results showed that the cell migration of H1299NC group was significantly slower compared with H1299shRNA, the migration of A549STEAP1 group cells was significantly slower compared with A549vector group (Fig. 3A). Moreover, cell numbers of H1299shRNA group was significantly greater than that of H1299NC group, and A549vector group was significantly greater than that of A549STEAP1 group (Fig. 3B). Cell numbers in H1299shRNA group was significantly greater than that in H1299NC group, in addition, A549vector group was significantly greater than that of A549STEAP1 group (Fig. 3C). Furthermore, we analyzed the expression of migration related proteins, mmp9, mmp2 and snail1, Western blot and Q-PCR results indicated that the expression of snail1 in H1299NC group was significantly higher than H1299shRNA group, the A549STEAP1 group was significantly higher than A549vector group. The expression of mmp2 in H1299NC group was significantly higher than H1299shRNA group, A549STEAP1 group was significantly higher than A549vector group. The expression of mmp9 in H1299NC group was significantly higher than H1299shRNA group, A549STEAP1 group was significantly higher than A549vector group (Fig. 3D).

3.4 STEAP1 regulates epithelial to mesenchymal transition (EMT) via JAK2/STAT3 signaling pathway

Previous studies have showed that proteins in key signaling pathways can regulate tumor growth. We hypothesis that STEAP1 can regulate EMT via JAK2/STAT3 signaling pathway. To verify, western blot analysis and Q-PCR were employed to detecte the protein and mRNA expressions of N-cadherin, E-cadherin and Vimentin in H1299NC, H1299shRNA, A549vector and A549STEAP1 groups. Our results showed that E-cadherin expression was markedly increased, however, vimentin and N-cadherin expression were decreased after STEAP1 was silenced in A1299 cells (Fig. 4A-B). Besides,
the protein and mRNA levels of JAK2, p-JAK2, STAT3 and p-STAT3 in H1299NC, H1299shRNA, A549 vector and A549STEAP1 groups were analyzed through western blotting and Q-PCR. The results showed JAK2, p-JAK2, STAT3 and p-STAT3 were markedly depleted by H1299shRNA at both protein and mRNA levels. The protein and mRNA levels of JAK2, p-JAK2, STAT3 and p-STAT3 were markedly increased in A549STEAP1 group (Fig. 4 C-D). The protein and mRNA expression of JAK2/STAT3 signaling pathway proteins, LCAM1, CCL12 and survivin were markedly decreased in H1299shRNA group. The protein and mRNA levels of LCAM1, CCL12 and survivin were markedly increased in A549STEAP1 group. (Fig. 4 E, G). ADZ 1480, a specific STAT3 inhibitor, we investigated the influences of ADZ 1480 on the EMT in A549 cells, which can be shown in Fig. 4F. ADZ 1480 increase E-cadherin expression and lower the expression level of Vimentin and N-cadherin.

3.5 STEAP1 facilitates metastasis and epithelial-mesenchymal transition of lung adenocarcinoma via the JAK2/STAT3 signaling pathway

To determine if STAT3 is involved in STEAP1 regulatory net works, STAT3 inhibitor ADZ1480 was using in our experiments. Western blot analysis indicated that the protein expression of p-STAT3 in NC+ADZ1480 was significantly decreased as compared to NC in H1299 cells (Fig. 5 A). Cell proliferation in NC+ADZ1480, NC+DMSO, shRNA+DMSO, shRNA+ADZ1480, vector+DMSO, vector+ADZ1480, STEAP1+DMSO, STEAP1+ADZ1480 groups were measured using CCK8 reagent for five consecutive days, results showed that the OD450 of shRNA+ADZ1480 group was significantly lower than NC+DMSO group in H1299 cell, the OD450 of STEAP1+DMSO group was significantly higher than vector+DMSO group in A549 cell (Fig. 5 B). The migration assay showed that cell numbers of NC+ADZ1480 group was markedly higher than that of shRNA+ADZ1480 group, cell numbers of
shRNA+DMSO group was markedly lower than that of NC+DMSO group in H1299 cell. The invasion assay result was consistent with the migration assay. (Fig.5 C-D). Cell numbers of vector+DMSO group was markedly lower than that of STEAP1+DMSO group, cell numbers of vector+ADZ1480 group was markedly lower than that of STEAP1+ADZ1480 group in A549 cell (Fig.5 E-F).

4. Discussion

In this study, we concluded that STEAP1 expression was correlated with metastasis and epithelial-mesenchymal transition of lung adenocarcinoma. Knocking down of STEAP1 significantly inhibited the proliferation and migration of lung adenocarcinoma cells. In addition, we confirmed that the regulation of STEAP1 in lung adenocarcinoma is associated with the JAK2/STAT3 signaling pathway. According to relevant literatures, STEAP1 had been studied in several types of cancers, such as breast cancer, prostate cancer and gastric cancer[11-13]. However, our results revealed that STEAP1 was expressed more in H1299 cells and less in A549 cells. The in vitro studies showed that knocking down of STEAP1 inhibited proliferation and migration of the lung adenocarcinoma cells. Thus, STEAP1 plays an crucial role in progression of human lung adenocarcinoma.

Furthermore, we found that STEAP1 regulates epithelial to mesenchymal transition (EMT) via JAK2/STAT3 signaling pathway. To our knowledge, this is the first time to report the role of STEAP1 in JAK2/STAT3 signaling pathway in lung adenocarcinoma.

Based on results of the study, STEAP1 can serve as a therapeutic target. Thus, the strategies targeting STEAP1 via JAK2/STAT3 signaling pathway may worth further researches.
The authors declare that they have no conflict of interest.

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**Authors’ contribution**

TIAN Ying-xuan and HUO Shu-fen were mainly responsible for putting forward hypothesis, collecting research background, study implementation and manuscript revision. SHANG Wen-li, YU Min, REN Xiao-ping, WEN Hong-xia, CHAI Chun-yan, SUN Li and HUI Ke were mainly conduct study implementation and data statistical analysis. LIU Ling-hua, WEI Sheng-hong, WANG Xiao-xiao and WANG Yi were mainly work on writing the draft of the study and correcting research equipments.
**FIGURE LEGENDS**

**Fig. 1** The expression level of STEAP1 in lung adenocarcinoma (LUAD) tissues and para-carcinoma normal tissues. A. Western blot analysis of lung adenocarcinoma (LUAD) tissues and para-carcinoma normal tissues. B. Western blot analysis of STEAP1 and GAPDH. C. Detection of mRNA expression in lung adenocarcinoma (LUAD) tissues and para-carcinoma normal tissues by Q-PCR. D. Relative STEAP1 expression among lung adenocarcinoma (LUAD) tissues and para-carcinoma normal tissues in the GEPIA dataset. E. Survival percentage of the high and low STEAP1 group in the GEPIA dataset.

**Fig. 2** STEAP1 promotes the proliferation of lung adenocarcinoma cells. A. Western blot and Q-PCR assay of H1650, H358, HCC827, H1299, H23, A549, H1693. B. Western blot and Q-PCR assay of H1299NC, H1299shRNA, A549vector and A549STEAP1. C. Cell proliferation in H1299NC, H1299shRNA, A549vector and A549STEAP1 were measured using CCK8 reagent for five consecutive days. D. Relative ratio of EdU positive cells of H1299NC, H1299shRNA, A549vector and A549STEAP1. E. Western blot and Q-PCR assay of PCNA and cyclinD1.

**Fig. 3** STEAP1 promotes migration and invasion of lung adenocarcinoma cells. A. Wound healing test of H1299NC, H1299shRNA, A549vector and A549STEAP1. The images were obtained at 0 h and 48 h after scratching. B. The migration assay of H1299NC, H1299shRNA, A549vector and A549STEAP1. C. The invasion assay of H1299NC, H1299shRNA, A549vector and A549STEAP1. D. Western blot and Q-PCR assay of migration related protein (mmp9, mmp2 and snail1).
Fig. 4 STEAP1 regulates EMT via JAK2/STAT3 signaling pathway. A. Western blot analysis of N-cadherin, E-cadherin and vimentin in H1299NC, H1299shRNA, A549vector and A549STEAP1. B. Q-PCR assay of N-cadherin, E-cadherin and vimentin in H1299NC, H1299shRNA, A549vector and A549STEAP1. C. Western blot analysis of JAK2, p-JAK2, STAT3 and p-STAT3 in H1299NC, H1299shRNA, A549vector and A549STEAP1. D. Q-PCR assay of JAK2, p-JAK2, STAT3 and p-STAT3 in H1299NC, H1299shRNA, A549vector and A549STEAP1. E. Western blot analysis of JAK2/STAT3 signaling pathway protein (LCAM1, CCL12 and survivin) in H1299NC, H1299shRNA, A549vector and A549STEAP1. F. Influence of ADZ1480 on EMT in A549 cells tested by the Western blot analysis. G. Q-PCR assay of LCAM1, CCL12 and survivin in H1299NC, H1299shRNA, A549vector and A549STEAP1.

Fig. 5 STEAP1 facilitates metastasis and epithelial-mesenchymal transition of lung adenocarcinoma via the JAK2/STAT3 signaling pathway. A. Western blot analysis of p-STAT3 and STAT3 in NC+ADZ1480, NC, shRNA, shRNA+ADZ1480 in H1299NC and H1299shRNA. B. Cell proliferation in NC+ADZ1480, NC+DMSO, shRNA+DMSO, shRNA+ADZ1480, vector+DMSO, vector+ADZ1480, STEAP1+DMSO, STEAP1+ADZ1480 were measured using CCK8 reagent for five consecutive days in H1299NC, H1299shRNA, A549vector and A549STEAP1. C-D. The migration and invasion assay of NC+ADZ1480, NC+DMSO, shRNA+DMSO, shRNA+ADZ1480 in H1299NC, H1299shRNA. E-F. The migration and invasion assay of vector+DMSO, vector+ADZ1480, STEAP1+DMSO, STEAP1+ADZ1480 in A549vector and A549STEAP1.
