Silencing Akt1 Inhibits Starvation Induced Lung Metastasis Through Epithelial Mesenchymal Transition Independent of E-cadherin in Prostate Cancer

Mei Yang
Department of Anatomy, Institue of Neuroscience

Hui Liu
Department of Anatomy, Institute of Neuroscience

Guo Ping Qiu
Department of Anatomy, Institute of Neuroscience

Fei Gao (✉ gaofei667@yahoo.com)
The first affiliated hospital of chongqing medical university

Research

Keywords: Epithelial to mesenchymal transition, starvation resistance, cell invasion

DOI: https://doi.org/10.21203/rs.3.rs-42759/v1

License: ☺ ☁ This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

Background

Epithelial to mesenchymal transition (EMT) of prostate cancer (PCa) cells facilitates their progress to metastasis. We have recently reported Akt1 is an important EMT regulator, and silencing Akt1 induced EMT and inhibited the growth of PCa cells. These results imply Akt1 silencing may increase starvation resistance of PCa cells. However, little is known about the role of Akt1 of PCa cells in starvation.

Methods

Apoptosis was detected by TUNEL and flow cytometry, cell invasion was detected by transwells and ECIS, lung metastasis was evaluated by tail vein injection animal model, variation of EMT markers was detected by Western-blot.

Results

We found starvation slowed down the growth and proliferation, and increased apoptosis of PCa cells; Silencing Akt1 gene inhibited these effects of starvation and decreased E-cadherin. Akt1 silencing enhanced invasion induced by starvation of PCa cells in vitro; however, it inhibited lung metastasis induced by starvation of PCa cells in vivo unexpectedly.

Conclusion

Starvation or silencing Akt1 alone can promote lung metastasis of PCa cells, however, silencing Akt1 while starving PCa cells can significantly inhibit this function via EMT independent of E-cadherin.

Background

Prostate cancer (PCa) is the most common cancer in men; it is the second leading cancer related death among men in United States[1]. In the process of tumor proliferation, nutrients are required for solid tumors to grow. Without nutrition supporting, tumors can’t grow to particular size, this phenomenon is also known as Warburg effect[2]. Many therapeutic strategies aimed to cut off the nutritional supply of cancer cells, such as anti-angiogenesis and metabolism strategy, which have achieved good results at the beginning, and once brought hope to human, but finally scientists found that starvation didn’t kill all cancer cells, eventually cancer will still progress.

On one hand, starvation is a cellular stress that induces apoptosis or death of cancer cells[3], on another hand, cancer cells adapt to harsh environments through a series of changes. Through these processes, the invasion of cancer cells enhanced, resulting in increased cell invasion[4]. After starvation exposure, cancer cells obtain the invasion and metastasis ability through Epithelial mesenchymal transition (EMT) process[5]. By EMT process, epithelial cells transformed into fibroblast-like mesenchymal cells in some physiological and pathological process, which significantly enhanced the invasion and metastatic ability
of cancer cells[6]. Through EMT, cancer cells acquire stem cell like characteristics, reduce proliferation and demand for nutrients[7, 8]. These changes reduced nutrition consume and facility cancer cells survive in starvation.

For many years, our researches focus on EMT regulatory mechanism. We found that Akt1 gene, main subtype of Akt, is not only an important regulator of cell growth and proliferation, but also an important regulator of EMT[9]. Akt1 regulates the growth and metabolism of cancer cells, Akt1 silencing could inhibit the growth and proliferation of PCa cells[10], and enhance cancer cells invasion via EMT[9]. Because the cells proliferation and growth inhibited by Akt1 silencing, the demand for nutrients may be greatly reduced. These results implied Akt1 silencing have potential ability to enhance the starvation resistance of cancer cells. Although there are some studies on the role of Akt1 or starvation of PCa cells, little is known about the role of Akt1 of PCa cells in starvation.

E-cadherin is a cell-cell adhesion protein, and belongs to type-I classical cadherins alongside N-cadherin, P-cadherin, R-cadherin and M-cadherin. Down regulation of E-cadherin is often found in malignant epithelial cancers, thus E-cadherin was considered as a potent tumor suppressor[11, 12]. Most studies show that the decrease of E-cadherin expression is the characteristic of EMT. Loss of E-cadherin in cancer cells leads to cancer metastasis and activation of many EMT transcription factors[13]. However, some researchers believe that loss of E-cadherin is not the cause of EMT, nor the necessity of EMT, and the recovery of E-cadherin expression cannot reverse EMT[14]. The role of E-cadherin for metastasis is also different in different cancer cells. For some cell lines, the absence of E-Cadherin is not conductive to the formation of distant metastases in cancer cells[15]. For some cell lines, cancer cells can cause metastases independent of E-cadherin[16]. As we know, E-cadherin is regulated by Akt1 in PCa; silencing Akt1 induced E-cadherin decreased to very low level. Based on this, the present study used DU145 (E-cadherin negative) and PC3 (E-cadherin positive) PCa cells lines, aimed to investigate the role of E-cadherin in PCa metastasis regulated by Akt1 in starvation.

In our study, we found PCa cells induce lung metastasis doesn’t depend on E-cadherin; PCa cells can form lung metastasis without E-cadherin. In addition, Akt1 silencing enhanced invasion induced by starvation of PCa cells in vitro; however, it inhibits lung metastasis induced by starvation of PCa cells in vivo unexpectedly. This contradictory result is related to the different mechanism of metastasis regulation of cancer cells in vivo and that in vitro. Starvation may maintain the phenotype of mesenchymal cells of Akt1 silencing PCa cells; although this change can enhance the invasion of cells in vitro, it is not facility to cause lung metastasis in vivo. These findings give us new information about cancer metastasis mechanisms; which further elucidate the role of Akt1 in the starvation of PCa cells. Our results may be helpful for the treatment of PCa based on starvation theory, such as anti-angiogenesis or cancer metabolism strategy.

Materials And Methods

Cell culture and Animals
Human PCa cell lines DU145 and PC3 were purchased from ATCC (Manassas, VA). Primary HUVECs was obtained from Lonza (CC-2527; Lonza, Allendale, NJ). PC3 and DU145 cells were maintained in DMEM medium (Hyclone, Logan, UT) with 10% FBS, 100 U/ml penicillin and streptomycin. Primary HUVECs (CC-2527; Lonza, Allendale, NJ) were maintained in EBM-2 medium with a GM-2 Bullet Kit (Lonza; Walkersville, MD). The cells were maintained at 37°C in a humidified incubator containing 5% CO2, and were passed on routinely at 80-90% confluence.

Eight-week old male nude mice (22-26g) necessary for the lung metastasis experiments were purchased from Animal experiment management center of Chongqing Medical University (BALB/c background, Chongqing, China). All animal experiments were carried out in accordance with guideline set by Chongqing Medical University (Chongqing, China). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals. Carbon dioxide asphyxiation followed by cervical dislocation was performed for killing. Isoflurane inhalation was used for anaesthesia. This study was approved by the ethics board of our university.

**Generated ShAkt1 stable cell lines**

PC3 and DU145 cells with stable knock-down of Akt1 ShRNA Akt1 deficient, Sh Akt1, and respective control cells (control) were generated using hU6-MCS-EGFP lentiviral vector (10^9 p.f.u.) (JiKai, Shanghai China). The stable ShAkt1 cell line generated as previous study[9]. Briefly, PC3 and DU145 cells were cultured into 6-well plates, and then infected by lent virus solution with polybrene (Sigma-Aldrich) for 16 hours, and then fresh medium was changed. Three days later, transfection efficiency was tested through GFP expression and subjected to 4 µg/ml purinomycin (Life Technologies, Grand Island, NY) selection until GFP was expressed in all cells. After selection, the cells were maintained in complete DMEM medium containing 0.6 ng/ml purinomycin.

**Cell viability Assay**

DU145 and PC3 cells were plated at a density of 1000 cells/well in 96-well plate with 100 ul growth medium at each well. At the end of indicated time-point, fresh media containing 20ul of MTT (5 mg/ml stock) was added, and incubated for another 4 h in a CO2 incubator. At the end, media was removed and 150 µl of DMSO was added to each well. Color intensity was measured by taking absorbance at 540 nm. Four times independent experiments were performed.

**Foci (colony) formation assay**

The cells were seeded into 6-well plates at a concentration of 500 cells per well. After 14 days of standardized culture, colony counts and rates were calculated after the colonies were fixed with absolute methanol and stained with 0.1 % crystal violet. Colonies of >50 cells were counted.

**Detection of apoptosis by flow cytometry**
The cells were simultaneously stained with Alexa Fluor 488-conjugated Annexin-V and PI, using the Vybrant Apoptosis Assay kit (Molecular Probes, USA), according to the manufacturer's instructions. Samples were analyzed by FacsCalibur flow cytometer. The percentage of apoptotic cells estimated by means of the Cell Quest Pro software. The simultaneous staining of cells with Annexin-V and PI allowed the resolution of viable cells (A-/PI-), early apoptotic cells (A+/PI-), necrotic cells (A-/PI+) and late apoptotic cells (A+/PI+).

**Cancer cells invasion detected by Transwells**

Invasion assay was performed using matrigel invasion chambers from BD Biosciences as previous study[17]. Briefly, we used 24 wells invasion chamber with 8 um pores coated with Matrigel to detect the invasion. DU145 and PC3 cells were seeded on the top chambers, after 10 hours of incubation, the cells in the upper chamber were wiped off with cotton swab. The invaded cells in the lower membrane surface were fixed with eosin staining. After three washes with PBS, the cells were counted under a microscope (Olympus, Japan).

**ECIS assay for cancer cell micro-invasion**

The ability of cancer cells to penetrate the endothelial cell monolayer was used to study the migration (micro invasion) of cancer cells across the endothelial cell monolayer, thus destroying the integrity of the endothelial barrier (measured as the resistance of the endothelial cell monolayer), which was measured by cell matrix impedance sensing (ECIS) technology (Applied Biophysics, Troy, NY). We performed as previous study[18]. Briefly, HMEC was seeded on gelatin coated ECIS array (8w10e) + with a density of 1:1. Each array contains 8 holes and each hole has 16 gold electrodes. The dish was supplemented with fresh medium 24 hours after sowing, and the experiment began when the cells reached the monolayer. PC3 and DU145 cells with concentration of 0.5x10^5/well were added to HMEC monolayer. After that, measure the resistance in multi-frequency mode.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay**

The TUNEL assay for the in situ detection of apoptosis was performed using an apoptosis detection kit (Roche, California, USA) following the manufacturer's instructions. Briefly, cells were first fixed with 4 % paraformaldehyde for 30 minutes at room temperature, washed twice with PBS, and then permeabilized with 0.1 % Triton X-100. The cells were then fixed in 20 μg / ml protease K for 20 minutes, washed twice with PBS, and then stained in sequence with TUNEL reaction mixture. TUNEL positive cells are brown in nucleus. In order to show other normal nuclei, we used hematoxylin to re-dye the nuclei for counting.

**Lung metastasis analysis in vivo**

Nude mice were randomly divided into four groups; each group has 10 mice, 5 mice for 2 weeks model study and 5 mice for 3 days model study for the administration of DU145 cells. Cells (0.5x10^6) suspended in sterile normal saline were injected (i.v.) to each group of mice separately via the tail vein.
all experiments, mice were assessed for metastasis at 3 days and 2 weeks after DU145 cells were administered. 3 days model designed for detecting early metastasis stage and 2 weeks model for late stage. For 2 weeks mouse model, 1.5 ml of 15 % India-ink solution was injected intratracheally to stain the lungs and visualize non-stained the tumor nodules. Then lung were then harvested and put in Fekete’s solution(300 ml 70 % ethanol, 30 ml 37 % formaldehyde, 5 ml glacial acetic acid), then put in fresh Fekete’s solution overnight. The metastasis tumor nodules are white. For 3 days mouse model, mice lung tissue was made into frozen sections, then sections mounted with coverslips with Vectashield mounting solution containing DAPI (Vector Laboratories, Burlingame, CA). The number of GFP containing cancer cells was viewed under confocal imaging microscope (LSM510, Carl Zeiss, Germany).

**Western blot analysis**

Western blotting was performed as described previously[19]. Briefly, Cells were collected and washed three times with a phosphate buffer (PBS). Extraction of cells were lysated by Ripa buffer containing protease and phosphatase inhibitor cocktail (Sigma, St Louis, MO). The homogenate was centrifuged at 4 °C at 10000 × g for 20 minutes. The supernatant was collected and the protein concentration was determined by Bradford protein test kit (Bio-Rad, Hercules, USA). Samples were separated on 8-12 % SDS-PAGE gels, and then transferred onto PVDF membrane. According to the primary antibody, the imprinted membrane was sealed with 5 % milk or 5 % bovine serum albumin for 30 minutes, followed by incubation with primary antibodies against Caspase-3, E-cadherin, ZO-1, Claudin-5 or N-cadherin etc. (1: 1000 dilution), and β-actin (1: 5000 dilution), respectively. After that, the membrane was incubated horse radish peroxidase (HRP)-conjugated secondary antibodies (1: 5000, Abcam, Cambridge, MA). After developing the membrane with ECL reagent (Life Technologies, Grand Island, NY), the expression of the protein was observed.

**Statistical Analysis**

All the data are expressed as Mean + SD and were calculated from multiple independent experiments. The Student's two-tailed *t* test or ANOVA were used to determine significant differences among groups using SPSS 11.0 software. Data with *p*< 0.05 were considered to have significant differences.

**Results**

1. **Starvation significantly reduces the growth of PCa cells; silencing Akt1 gene significantly reduces the response of PCa cells to starvation.**

To clarify the response of PCa cells to Nutrient starvation (NST), we used prostate cancer cell line DU145 and PC3 cell lines. E-Cadherin is an important intercellular junction protein between epithelial cells. The decrease of E-Cadherin level is considered as a marker of EMT.DU145 cells do not contain E-Cadherin,
while PC3 cells contain this molecule. Based on this, it is facilitated to study the EMT mechanisms of PCa using PC3 and DU145 cells in starvation. In the present study, we developed a 10 cycles of NST of PCa cells to establish cell starvation model to mimic the environment in clinic, in which Pca cells were continuously repeated exposed to NST.

As previous studies, Akt1 is an important regulator for cell growth and proliferation\[20, 21\]. In order to explore the function of Akt1 gene on NST of PCa cells, we developed stable Akt1 silencing Pca cells. We used MTT and Cell number methods to detect cell growth. Our study found that starvation decreased cell growth of PCa cells in control PCa cells (Fig.1 A-D, p<0.01). Interestingly, in ShAkt1 cells, the cell growth was not significant different after starvation (Fig.1 A-D, p>0.05). These results indicated the response to NST was inhibited in ShAkt1 PCa cells.

Then, we used colony formation to study the effects of Akt1 gene silencing on cell proliferation in starvation. We found that starvation significantly reduced the number of colony in control cells (Fig.1 E-H, p<0.01). In ShAkt1 cells, starvation didn’t significantly reduced the number of colony, the colony number was not significantly different between ShAkt1 cells and ShAkt1+NST cells (Fig.1 E-H, p>0.05), but the number of Sh Akt1 cells was still significantly lower than that of control cells (Fig.1 E-H, p<0.01). These results demonstrated silencing Akt1 and starvation decreased proliferation of PCa cells.

2. Starvation induces apoptosis of PCa cells; silencing Akt1 gene significantly inhibits starvation-induced apoptosis of PCa cells.

In this study, TUNNEL staining and flow cytometry were used to detect the number of apoptotic cells in each group, and Western blotting was used to detect the changes of Caspase-3, a key apoptotic regulate protein in each group. Both endogenous and exogenous apoptotic pathways require caspase-3\[22\].

Our study found that starvation significantly increased the number of apoptotic cells compared with the control group, manifested by an increase in TUNNEL-positive cells and an increase in PI-/Annexin-V+ cells by flow cytometry\[Fig.2 A,2C,E-G p<0.01\]. In ShAkt1 cells, starvation didn’t increased apoptosis, there was no significant difference in the number of TUNEL positive cells and PI-/Annexin-V+ cells between ShAkt1 cells and ShAkt1+NST cells\[Fig.2 A,2C, E-G p>0.05\]. These results indicated Akt1 silencing inhibit apoptosis of PCa cells induced by starvation.

Because the main purpose of the present study is not to explore the apoptotic mechanism of PCa cells in starvation, we only detected the level of Caspase-3 which is the main executor of apoptosis. We found that the level of Caspase-3 in the Control+NST group was the highest among the four groups\[Fig.2 B, 2D p<0.01\], and there was no statistical difference for the level of Caspase-3 among the other three groups\[Fig.2 B,2D p>0.05\].

3 Silencing Akt1 gene significantly enhanced PCa cells invasion induced by starvation in vitro.

In this study, Transwells method was used to detect cancer cells invasion of each group. Our study found starvation enhanced the invasion of PC3 and DU145 cells, which showed that the number of Transwells
positive cells was significantly increased in Control+NST groups compared with that of Control group in Fig.3 A-D, *p*<0.01. Silencing Akt1 significantly enhanced PCa cells invasion induced by starvation, which showed the number of Transwells positive cells in ShAkt1 + NST group was significantly increased compared with that in ShAkt1 group of PC3 and DU145 cells in Fig.3 A-D, *p*<0.01.

To verify the results of Transwells, we used ECIS to detect the invasion of PCa cells. In ECIS device, PCa cells penetrate vascular endothelial cells, leading to the destruction of vascular endothelial cell barrier, resulting in the decline of their resistance, the more invasions the cancer cells are, the stronger their ability to cause the decline of resistance[23]. As Transwells datas, ShAkt1 + NST group cells had the strongest reduction in electrical impedance among all the groups, indicated silencing Akt1 significantly enhanced PCa cells invasion induced by starvation in vitro in Fig.3 E-F, *p*<0.01.

4. Silencing Akt1 gene significantly inhibits lung metastasis induced by starvation.

As we reported above, starvation increased invasion of Pca cells in vitro. To investigate whether starvation increased lung metastasis of Pca cells in vivo, we generate two kinds’ animal metastasis models. One model is early stage lung metastasis model, which harvesting lung on 3 days after tail vein injection of Pca cells with GFP. Then we used influence microscopy to detected GFP labeled Pca cells. The other model is to harvest lung on 2 weeks after tail vein injection of Pca cells. We used india ink to detect the metastasis noodles in lung as previous study[17]. The 2 weeks model has longer time for cancer cells growth, so the cell growth and proliferation of cancer cells in lung tissues will affect the 2 weeks model more. And the ability of cancer cells to invade into lung tissues is more important for early stage lung metastasis model.

The present study found that starvation alone or Akt1 silencing alone significantly enhanced the ability of PCa cells to induce lung metastasis compared with the control group of 2 weeks model, manifested by the number of lung metastasis tumor nodules increased in Control+NST and ShAkt1 groups, compared with Control group in Fig.4 A-B, *p*<0.01. Surprisingly, if the PCa cells are starved on the basis of silencing Akt1 gene, the ability of inducing lung metastasis will be severely weakened, and the number of lung metastases nodules will be slightly less than that of control group in Fig.4 A-B, *p*<0.01. To explore whether these results related to cell growth or proliferation of cancer cells in lung tissues, we detected positive GFP PCa cells of lung tissue section by fluorescence of 3 days models. We got the similar results as 2 weeks model, GFP positive cancer cells of ShAkt1 + NST group was significantly lower than that in the other three groups in Fig.4 C-D, *p*<0.01.

5. Silencing Akt1 enhanced EMT induced by starvation, and inhibited the lung metastasis induced by starvation independent of E-cadherin.

E-Cadherin is an important epithelial cell marker molecule, and N-Cadherin is a typical mesenchymal cell marker molecule. In general, when EMT occurs in cancer cells, there is a decrease in E-cadherin levels and an increase in N-Cadherin levels. This pairwise change in E-Cadherin and N-cadherin is also known as E-Cadherin and N-Cadherin switch[24]. Some studies have found that E-Cadherin and N-Cadherin switch is
not necessary for EMT\cite{14, 24}. In the present study, we used PC3 and DU145 PCa cells, in which DU145 cells do not contain E-Cadherin, while PC3 cells contain this molecule.

The results of E-Cadherin in the control group, ShAkt1 and ShAkt1+NST group of DU145 cells was very low, and it was difficult to detect using WB, however, E-Cadherin levels of Control +NST group were increased significantly compared with the other three groups of PC3 and DU145(Fig.5 A-D, p<0.01). Consistent with the results of lung metastasis, PCa cells still induced lung metastasis with very low level E-cadherin. Silencing Akt1 decreased E-cadherin to a very low level and could hardly be detected by WB in DU145. At this time, silencing Akt1 still significantly inhibited the lung metastasis caused by starvation. These results indicated the function of Akt1 silencing in lung metastasis after starvation is independent of E-cadherin.

The results of other EMT markers showed that, similar to our previous studies, silencing of Aktl gene significantly reduced the levels of E-Cadherin and Claudin-5 in the epithelial cell markers of PC3 cells, and increased the level of the mesenchymal marker N-Cadherin. These results indicated silencing Akt1 induced EMT of PCa cells. Since the content of another epithelial cell marker ZO-1 was very low in PC3 cells, the difference could not be detected. After silencing the Aktl gene, N-Cadherin was significantly elevated compared to control cells. And other epithelial cell marker molecules, ZO-1 and Claudin-5 levels, were significantly reduced after silencing the Aktl gene in DU145 and PC3 cells (Fig.5 A-B, E-G, p<0.01).

Snail is an important regulator of EMT, and its elevated level suggests the occurrence of EMT. This study confirms that silencing Akt1 gene lead to increase of Snail molecular level compared with Control cells. The levels of Snail indicated the status of EMT, its levels increased indicted further mesenchymal cells status. Compared with other three groups, snail levels of ShAkt1 + NST group is the highest in DU145 and PC3 cells (Fig.5 A-B, H, p<0.01).

**Discussion**

Nutrient starvation (NST) is the basic for anti-angiogenesis and cancer metabolism strategy\cite{25}. However, research has confirmed that NST could not cure cancer. On the contrary, starvation makes cancer cells adapt to adverse conditions, and the surviving cells become more aggressive via EMT\cite{5, 26}. Our study confirmed that NST and silencing Akt1 promoted the EMT process of PCa cells. More interestingly, we found Akt1 silencing enhanced invasion induced by starvation of PCa cells in vitro; it inhibits starvation induced lung metastasis of PCa cells in vivo unexpectedly. This lung metastasis regulated by Akt1 is independent of E-cadherin. Together with the EMT mechanism, these results have deepened our understanding of PCa metastasis and have important clinical and scientific value.

In starvation, the growth rate of cancer cells slows down and apoptotic cells increase\cite{27, 28}. In this study, we confirmed starvation induced apoptosis and grow slowly of control PCa cells. However, starvation could not induce apoptosis and inhibit growth of ShAkt1 cells. These results indicated the response of ShAkt1 cells to starvation were inhibited, ShAkt1 cells growth slowly and apoptosis resistance. The results of Caspase-3 indicated that only in NST group, the results of Caspase-3 increased,
while in other groups, the level was very low. The increased Caspase-3 in NST group could cause apoptosis of PCa cells.

From the literature and our previous study, we know cancer cells increased invasion after starvation exposure and silencing of Akt1 gene [9]. Till now, little is known about the function of silencing Akt1 on starvation cells. The present study used Transwell and ECIS to detect PCa cells invasion. Our results found the PCa cells invasion increased in ShAkt1+NST group compared with ShAkt1 group, this indicated silencing Akt1 gene enhanced the invasion of PCa cells induced by starvation in vitro.

The tail-vein injection lung metastasis model has been confirmed by many studies since it is a reliable animal model for studying the metastasis of cancer cells. Because cancer cells directly enter the blood circulation system, the process of growth and invasion of cancer cells in situ is omitted. As the results of in vitro experiments, silencing Akt1 gene or NST alone significantly enhanced the ability of cancer cells to induce lung metastasis. However, the ability of lung metastasis will be severely weakened in silencing Akt1 gene + NST group, which is different from the in vitro data Fig.4 A-B, p<0.01.

In general, the increased invasion of cancer cells in vitro often suggests that they are more capable of causing metastasis in vivo. But it is not absolute, as known to us all, the mechanisms of invasion ability in vitro and the ability to induce metastasis in vivo is essentially different, and the mechanism of cancer cell metastasis in vivo is much more complex. With the further study of the mechanism of cancer cell metastasis, a few high-quality articles reported that in special circumstances, there may be inconsistencies for invasion and metastasis of cancer cells between in vivo and in vitro data, which may be related to different EMT status of cancer cells[29].

In the process of metastasis, the epithelial and mesenchymal phenotypes of cancer cells will change to adapt to different microenvironments, which is conducive to the metastasis of cancer cells. This ability of cancer cells to transform between epithelium and mesenchymal is called EMT plasticity of cancer cells. Even if EMT can enhance the invasion of cancer cells, it requires MET to develop metastasis, which ultimately forms epithelial metastasis similar to the original site[30, 31]. In the present study, the results showed that silencing Akt1 gene could further enhance the effect of starvation on EMT of PCa cells, which was manifested by the further decrease of epithelial markers and the increase of mesenchymal markers. And under the dual effects of starvation and silencing of Akt1 gene, Snail, an important EMT-TFs molecule, increased of ShAkt1+NST group compared with ShAkt1 or NST cells. These results suggest that NST may further promote EMT in ShAkt1 PCa cells than ShAkt1 cells or NST cells alone. Recent studies have found overexpression of Twist1, an EMT-TFs molecule, will maintain the cancer cells in mesenchymal state, at this time, the ability of cancer cells to cause distant metastasis will be significantly reduced[29, 32]. Based on these results we believe that Snail expression induced by dual silencing of Akt1 gene and NST may maintain the phenotype of mesenchymal cells through a similar mechanism, which is a not facility to cause lung metastasis. This may be the reason why the invasion of ShAkt1 + NST cells increased in vitro compared with ShAkt1 cells and NST cells, but lung metastasis of ShAkt1 + NST cells inhibited in vivo.
To investigate whether NST induced EMT of Pca cells, we detected EMT markers of Pca cells by Western-blot. Our study confirms that NST significantly reduce the levels of epithelial marker ZO-1 and Claudin-5 and significantly increase the levels of mesenchymal marker N-Cadherin in DU145 and PC3 cells. These results suggested that NST could induce EMT in PCa cells. Similar to previous studies, we also found that silencing Akt1 gene caused epithelial cells markers E-Cadherin, ZO-1 and Claudin-5 decreased and mesenchymal marker N-Cadherin levels increased compared with control cells. The above results confirmed silencing Akt1 gene induced EMT of PCa cells [9]. Taken together, we speculated silencing Akt1 gene, cell growth and proliferation slowed down, and its dependent nutrients also decreased. At the same time, cancer cells acquired stronger invasion through EMT process. These changes not only helped PCa cells survive in starvation environment, but also facilitate PCa cells metastasis.

E-Cadherin is an epithelial marker and is considered to be an important marker of EMT in cancer cells. The E-Cadherin level decreases indicated EMT occurs in cancer cells. Because of the cancer cells invasion increased via EMT, most scholars believe that the level of E-Cadherin should be reduced, so as to facilitate the metastasis of cancer cells. However, recent studies have confirmed that metastasis of cancer cells requires the involvement of this molecule[15]. For some cell lines, the absence of E-Cadherin is not conducive to the formation of distant metastases in cancer cells[15]. For some cell lines, cancer cells can cause metastases independent of E-Cadherin[16]. These studies suggest the complexity of this molecule's role in the metastasis of cancer cells. To further understand the effect of E-Cadherin on metastasis of PCa cells. We used DU145 cells without E-Cadherin and PC3 cell lines with rich E-Cadherin molecule in this study. Our results found the level of E-cadherin decreased significantly after silencing Akt1 gene, but silencing Akt1 gene alone could not block the metastasis of PCa cells, or even slightly increase lung metastasis, suggesting Akt1 regulated lung metastasis of PCa cells is E-cadherin independent. The role of E-Cadherin in metastasis needs further studies.

In addition, the present study found that starvation caused a significant increase in E-Cadherin level in PC3 cells. Even for DU145 cells, E-Cadherin was difficult to detect by WB method in control cells, which levels was also significant increased after starvation. Although it is rarely reported that starvation can increase the E-cadherin of PCa cells PC3 and DU145. However, similar reports have been found in other cancer cell studies[33]. Silencing Akt1 suppresses the increased of E-cadherin caused by starvation. These results imply Akt1 is an important E-cadherin regulator.

Based on the above, the present study further elucidates the role of Akt1 gene silencing in NST of PCa. Our results may be helpful for the treatment of PCa based on NST theory, such as anti-angiogenesis or cancer metabolism strategy. In this study, we found Akt1 silencing enhanced invasion induced by starvation of PCa cells in vitro, however, it inhibits starvation induced lung metastasis of PCa cells in vivo, which enriched our understanding of the mechanism of cancer cell metastasis. In the future work, we need to further study the mechanism of this inconsistency, and further clarify the role of E-cadherin in cancer cell metastasis.

**Conclusion**
Starvation and silencing Akt1 alone increased lung metastasis; however, silencing Akt1 inhibits the lung metastasis induced by starvation via EMT independent of E-cadherin in prostate cancer. These results further elucidate the role of AKT1 in starvation of PCa. It is helpful for the treatment of PCa based on starvation, such as anti-angiogenesis or cancer metabolism strategy.

**Abbreviations**

Akt1: protein kinase B α; PCa: Prostate Cancer; NST: Nutrient starvation; EMT: Epithelial mesenchymal transition; ShAkt1: ShRNA Akt1 deficient.

**Declarations**

**Ethical approval and consent to participate**

This study was approved by the Ethics Committee of Chongqing Medical University (Chongqing, China). All animal experiments were performed in accordance with animal protocols approved by the Chongqing Medical University, which also accordance with the guideline and regulations for Animal Health and Use (National Standardization Administration of China, 2016).

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

Funds were provided by the National Nature Science Foundation of China to Fei Gao (No.81672893) and Mei Yang (NO.81971230 and 81671312). This work has been accomplished using the resources of Senior Young-middle-aged Medical Talents Program of Chongqing Health Committee and Reserve Talents Program for academic Leaders of the First Affiliated Hospital of Chongqing Medical University to Fei Gao.

**Authors’ contributions**

Conception and design: Fei Gao; Data production, analysis and interpretation: Fei Gao, Hui Liu and Guo Ping Qiu; writing the manuscript: Fei Gao and Mei Yang. All authors reviewed the manuscript and accepted the content.
Acknowledgments

This work has been accomplished using the resources and facilities at Chongqing Medical University.

References

1. Benke IN, Leitzmann MF, Behrens G, Schmid D: Physical activity in relation to risk of prostate cancer: a systematic review and meta-analysis. Annals of oncology : official journal of the European Society for Medical Oncology 2018, 29(5):1154-1179.

2. Vander Heiden MG, Cantley LC, Thompson CB: Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 2009, 324(5930):1029-1033.

3. Marchesi F, Monti P, Leone BE, Zerbi A, Vecchi A, Piemonti L, Mantovani A, Allavena P: Increased survival, proliferation, and migration in metastatic human pancreatic tumor cells expressing functional CXCR4. Cancer research 2004, 64(22):8420-8427.

4. Garcia-Jimenez C, Goding CR: Starvation and Pseudo-Starvation as Drivers of Cancer Metastasis through Translation Reprogramming. Cell metabolism 2019, 29(2):254-267.

5. Zada S, Hwang JS, Ahmed M, Lai TH, Pham TM, Kim DR: Control of the Epithelial-to-Mesenchymal Transition and Cancer Metastasis by Autophagy-Dependent SNAIL1 Degradation. Cells 2019, 8(2).

6. Mittal V: Epithelial Mesenchymal Transition in Tumor Metastasis. Annual review of pathology 2018, 13:395-412.

7. Sakunrangsit N, Ketchart W: Plumbagin inhibits cancer stem-like cells, angiogenesis and suppresses cell proliferation and invasion by targeting Wnt/beta-catenin pathway in endocrine resistant breast cancer. Pharmacological research 2019:104517.

8. Aigner K, Dampier B, Descovich L, Mikula M, Sultan A, Schreiber M, Mikulits W, Brabletz T, Strand D, Obrist P et al: The transcription factor ZEB1 (deltaEF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity. Oncogene 2007, 26(49):6979-6988.

9. Gao F, Alwhaibi A, Sabbineni H, Verma A, Eldahshon W, Somanath PR: Suppression of Akt1-beta-catenin pathway in advanced prostate cancer promotes TGFbeta1-mediated epithelial to mesenchymal transition and metastasis. Cancer letters 2017, 402:177-189.

10. Wang Q, Chen X, Hay N: Akt as a target for cancer therapy: more is not always better (lessons from studies in mice). Br J Cancer 2017, 117(2):159-163.

11. Rosso M, Majem B, Devis L, Lapuykkjy L, Besso MJ, Llaurvedo M, Abascal MF, Matos ML, Lanau L, Castellvi J et al: E-cadherin: A determinant molecule associated with ovarian cancer progression, dissemination and aggressiveness. Plos one 2017, 12(9):e0184439.
12. Schneider MR, Kolligs FT: E-cadherin's role in development, tissue homeostasis and disease: Insights from mouse models: Tissue-specific inactivation of the adhesion protein E-cadherin in mice reveals its functions in health and disease. *BioEssays* : news and reviews in molecular, cellular and developmental biology 2015, **37**(3):294-304.

13. Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA: Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer research* 2008, **68**(10):3645-3654.

14. Hollestelle A, Peeters JK, Smid M, Timmermans M, Verhoog LC, Westenend PJ, Heine AA, Chan A, Sieuwerts AM, Wiemer EA et al: Loss of E-cadherin is not a necessity for epithelial to mesenchymal transition in human breast cancer. *Breast cancer research and treatment* 2013, **138**(1):47-57.

15. Padmanaban V, Krol I, Suhail Y, Szczерba BM, Aceto N, Bader JS, Ewald AJ: E-cadherin is required for metastasis in multiple models of breast cancer. *Nature* 2019, **573**(7774):439-444.

16. Reichert M, Bakir B, Moreira L, Pitarresi JR, Feldmann K, Simon L, Suzuki K, Maddipati R, Rhim AD, Schlitter AM et al: Regulation of Epithelial Plasticity Determines Metastatic Organotropism in Pancreatic Cancer. *Developmental cell* 2018, **45**(6):696-711 e698.

17. Gao F, Alwhaibi A, Artham S, Verma A, Somanath PR: Endothelial Akt1 loss promotes prostate cancer metastasis via beta-catenin-regulated tight-junction protein turnover. *Brit J Cancer* 2018, **118**(11):1464-1475.

18. Gao F, Al-Azayzih A, Somanath PR: Discrete functions of GSK3alpha and GSK3beta isoforms in prostate tumor growth and micrometastasis. *Oncotarget* 2015, **6**(8):5947-5962.

19. Gao F, Artham S, Sabbineni H, Al-Azayzih A, Peng XD, Hay N, Adams RH, Byzova TV, Somanath PR: Akt1 promotes stimuli-induced endothelial-barrier protection through FoxO-mediated tight-junction protein turnover. *Cellular and molecular life sciences : CMLS* 2016.

20. Reyes-Gordillo K, Shah R, Arellanes-Robledo J, Cheng Y, Ibrahim J, Tuma PL: Akt1 and Akt2 Isoforms Play Distinct Roles in Regulating the Development of Inflammation and Fibrosis Associated with Alcoholic Liver Disease. *Cells* 2019, **8**(11).

21. Chen R, Malagola E, Dietrich M, Zuellig R, Tschopp O, Bombardo M, Saponara E, Reding T, Myers S, Hills AP et al: Akt1 signalling supports acinar proliferation and limits acinar-to-ductal metaplasia formation upon induction of acute pancreatitis. *The Journal of pathology* 2019.

22. Lossi L, Castagna C, Merighi A: Caspase-3 Mediated Cell Death in the Normal Development of the Mammalian Cerebellum. *International journal of molecular sciences* 2018, **19**(12).

23. Amatschek S, Lucas R, Eger A, Pflueger M, Hundsberger H, Knoll C, Grosse-Kracht S, Schuett W, Koszik F, Maurer D et al: CXCL9 induces chemotaxis, chemorepulsion and endothelial barrier disruption through CXCR3-mediated activation of melanoma cells. *Br J Cancer* 2011, **104**(3):469-479.
24. Loh CY, Chai JY, Tang TF, Wong WF, Sethi G, Shanmugam MK, Chong PP, Looi CY: The E-Cadherin and N-Cadherin Switch in Epithelial-to-Mesenchymal Transition: Signaling, Therapeutic Implications, and Challenges. *Cells* 2019, 8(10).

25. Ma J, Waxman DJ: Dominant effect of antiangiogenesis in combination therapy involving cyclophosphamide and axitinib. *Clinical cancer research: an official journal of the American Association for Cancer Research* 2009, 15(2):578-588.

26. Yang Z, Sun Q, Guo J, Wang S, Song G, Liu W, Liu M, Tang H: GRSF1-mediated MIR-G-1 promotes malignant behavior and nuclear autophagy by directly upregulating TMED5 and LMNB1 in cervical cancer cells. *Autophagy* 2019, 15(4):668-685.

27. Fu Y, Li J, Lee AS: GRP78/BiP inhibits endoplasmic reticulum BIK and protects human breast cancer cells against estrogen starvation-induced apoptosis. *Cancer research* 2007, 67(8):3734-3740.

28. Tomas-Hernandez S, Blanco J, Rojas C, Roca-Martinez J, Ojeda-Montes MJ, Beltran-Debon R, Garcia-Vallve S, Pujadas G, Arola L, Mulero M: Resveratrol Potently Counteracts Quercetin Starvation-Induced Autophagy and Sensitizes HepG2 Cancer Cells to Apoptosis. *Molecular nutrition & food research* 2018, 62(5).

29. Ocana OH, Corcoles R, Fabra A, Moreno-Bueno G, Acloque H, Vega S, Barrallo-Gimeno A, Cano A, Nieto MA: Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer Prrx1. *Cancer cell* 2012, 22(6):709-724.

30. Shen M, Zhao X, Zhao L, Shi L, An S, Huang G, Liu J: Met is involved in TIGAR-regulated metastasis of non-small-cell lung cancer. *Molecular cancer* 2018, 17(1):88.

31. Brabletz T: EMT and MET in metastasis: where are the cancer stem cells? *Cancer cell* 2012, 22(6):699-701.

32. Tsuji T, Ibaragi S, Shima K, Hu MG, Katsurano M, Sasaki A, Hu GF: Epithelial-mesenchymal transition induced by growth suppressor p12CDK2-AP1 promotes tumor cell local invasion but suppresses distant colony growth. *Cancer research* 2008, 68(24):10377-10386.

33. Dong S, Khoo A, Wei J, Bowser RK, Weathington NM, Xiao S, Zhang L, Ma H, Zhao Y, Zhao J: Serum starvation regulates E-cadherin upregulation via activation of c-Src in non-small-cell lung cancer A549 cells. *American journal of physiology Cell physiology* 2014, 307(9):C893-899.

**Figures**
Figure 1

Starvation significantly reduces the growth of PCa cells; silencing Akt1 gene significantly reduces the response of PCa cells to starvation. A-B, Cell number assays were performed with PC3 and DU145 cells. To avoid the influence of the initial cell number on the results, we normalize the cell number at each time point to the initial cell number to get the relative cell number at each time point. C-D, MTT assays were performed to detect cell growth of PCa cells. The blue line indicated control cells and red line indicated ShAkt1 cells. E-F: Colony formation assays were used to detect the proliferation of PCa cells. All data are shown as the mean ±SD, *indicated p<0.05, and indicated p>0.05.
Figure 1

Starvation significantly reduces the growth of PCa cells; silencing Akt1 gene significantly reduces the response of PCa cells to starvation. A-B, Cell number assays were performed with PC3 and DU145 cells. To avoid the influence of the initial cell number on the results, we normalize the cell number at each time point to the initial cell number to get the relative cell number at each time point. C-D, MTT assays were performed to detect cell growth of PCa cells. The blue line indicated control cells and red line indicated ShAkt1 cells. E-F: Colony formation assays were used to detect the proliferation of PCa cells. All data are shown as the mean ±SD, *indicated p<0.05, and indicated p>0.05.
Figure 2

Silencing Akt1 gene significantly inhibits starvation-induced apoptosis of PCa cells. A,C: Apoptosis cells of PCa detected by TUNNEL. The apoptosis cells were brown cells in nuclear. Red arrow indicated apoptosis cells, Bar indicated 20um. B,D: The western-blot analysis was performed to detect the expression of Caspase-3 of DU145 and PC3 cells (n=3). E-G: Apoptosis cells of PCa detected by flow cytometry (n=5). PI-/Annexin-V+ cells were early stage apoptosis cells, All apoptosis cells were PI-/Annexin-V+ cells and PI+/Annexin-V+ cells. All data are shown as the mean ±SD, *indicated compared with control group, p<0.01, indicated between the two groups, p>0.05. Red arrow indicated the apoptosis cells detected by TUNNEL.
Figure 2

Silencing Akt1 gene significantly inhibits starvation-induced apoptosis of PCa cells. A, C: Apoptosis cells of PCa detected by TUNNEL. The apoptosis cells were brown cells in nucleus. Red arrow indicated apoptosis cells, Bar indicated 20um. B, D: The western-blot analysis was performed to detect the expression of Caspase-3 of DU145 and PC3 cells (n=3). E-G: Apoptosis cells of PCa detected by flow cytometry (n=5). PI-/Annexin-V+ cells were early stage apoptosis cells, All apoptosis cells were PI-/Annexin-V+ cells and PI+/Annexin-V+ cells. All data are shown as the mean ±SD, *indicated compared with control group, p<0.01, indicated between the two groups, p>0.05. Red arrow indicated the apoptosis cells detected by TUNNEL.
Figure 3

Silencing Akt1 gene enhanced invasion induced by starvation in vitro. A-D: The transwells assays were performed to detect the invasion of PCa cells (n=5). E-F: The invasion of PCa cells were detected by ECIS (n=3). * indicated during the time range, there is significant different between ShAkt1+NST group and ShAkt1 group, p<0.05; indicated during the time range, there is significant different between Control group and ShAkt1 group, p<0.05. Bar indicated 20μm.
Figure 3

Silencing Akt1 gene enhanced invasion induced by starvation in vitro. A-D: The transwells assays were performed to detect the invasion of PCa cells (n=5). E-F: The invasion of PCa cells were detected by ECIS (n=3). * indicated during the time range, There is significant different between ShAkt1+NST group and ShAkt1 group, p<0.05; indicated during the time range, There is significant different between Control group and ShAkt1 group, p<0.05. Bar indicated 20um.
Figure 4

Silencing Akt1 gene significantly inhibits lung metastasis induced by starvation. A-B: The lung metastasis of nude mice after 2 weeks tail vein injection of DU145 cells detected by india-ink (n=5). The tumor nodules were the white dots, and the black tissues are normal lung tissues. Arrow indicated tumor nodules. C-D: The lung metastasis of nude mice after 3 days tail vein injection of DU145 cells detected by ICC. The metastasis PCa cells are GFP positive cells, which are green in the photos (red arrow indicated metastasis cells, Bar indicated 20um.). We counted these GFP positive cells, then calculated the percentage of metastasis cells (GFP positive cells/total cells). *indicated between the two groups, p<0.01.
Figure 4

Silencing Akt1 gene significantly inhibits lung metastasis induced by starvation. A-B: The lung metastasis of nude mice after 2 weeks tail vein injection of DU145 cells detected by india-ink (n=5). The tumor nodules were the white dots, and the black tissues are normal lung tissues; arrow indicated tumor nodules. C-D: The lung metastasis of nude mice after 3 days tail vein injection of DU145 cells detected by ICC. The metastasis PCa cells are GFP positive cells, which are green in the photos (red arrow indicated metastasis cells; Bar indicated 20um.). We counted these GFP positive cells, then calculated the percentage of metastasis cells (GFP positive cells/total cells). * indicated between the two groups, p<0.01.
Figure 5

Silencing Akt1 enhanced EMT induced by starvation, and inhibited the lung metastasis induced by starvation independent of E-cadherin. A-B: Western blot assays were performed to detect EMT markers of PC3 and DU145 cells. Fig.5A is for PC3 and Fig.5B is for DU145 (n=3). C-H: The analyzed results of WB in PC3 and DU145 cells. Most WB results are normalized to control. However, for very low molecules in the control group, such as the E-cadherin level of DU145, we use the method of ratio between the target protein and β-actin. The ZO-1 levels of PC3 are very low in each group, so we didn’t show its analyzed results. * indicated for PC3 cells, compared with control group, p<0.05. indicated for DU145 cells, compared with control group, p<0.05.
Figure 5

Silencing Akt1 enhanced EMT induced by starvation, and inhibited the lung metastasis induced by starvation independent of E-cadherin. A-B: Western blot assays were performed to detect EMT markers of PC3 and DU145 cells. Fig. 5A is for PC3 and Fig. 5B is for DU145 (n=3). C-H: The analyzed results of WB in PC3 and DU145 cells. Most WB results are normalized to control. However, for very low molecules in the control group, such as the E-cadherin level of DU145, we use the method of ratio between the target protein and β-actin. The ZO-1 levels of PC3 are very low in each group, so we didn’t show its analyzed results.* indicated for PC3 cells, compared with control group, p<0.05. indicated for DU145 cells, compared with control group, p<0.05.