LukS-PV Inhibits Hepatocellular Carcinoma Cells Migration via the TNNC1/PI3K/AKT Axis

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Purpose: Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. LukS-PV is the S component of Pantone-Valentine leucocidin (PVL), a toxin secreted by *Staphylococcus aureus*. We aimed to investigate the role of LukS-PV in HCC cell migration and the specific molecular mechanism involved.

Methods: We used scratch assays to detect the mobility of liver cancer cells treated with LukS-PV. Quantitative real-time PCR and Western blot analysis were performed to detect the expression levels of related genes. RNA sequencing and quantitative proteomics sequencing were used to assess the transcriptional and proteomic alterations of target genes. RNA sequencing and Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA) pathway analyses revealed the downstream signaling pathway targets of LukS-PV.

Results: Our results demonstrated that LukS-PV could inhibit HCC cell migration in a concentration-dependent manner. LukS-PV could also downregulate the expression of TNNC1, which was highly expressed in HCC cells. Additionally, the study showed that LukS-PV inhibited HCC cell migration by downregulating TNNC1. Further studies showed that LukS-PV inhibited the phosphorylation of PI3K/AKT pathway by targeting TNNC1, thereby inhibiting HCC cell migration.

Conclusion: Our study demonstrated that LukS-PV has an inhibitory role in the migration of liver cancer cells through the TNNC1/PI3K/AKT axis.

Keywords: hepatocellular carcinoma, LukS-PV, migration, TNNC1/PI3K/AKT axis

Introduction

Hepatocellular carcinoma (HCC) is a clinically common malignant tumor with an incidence rate that ranks it as the sixth highest solid tumor worldwide.¹ HCC is prone to metastasis² and the 5-year survival rate of patients is low.³ Both surgery and chemotherapy have certain limitations, with the latter exerting notable toxicity and side effects.⁴ Moreover, relapse frequently occurs after surgical treatment, and thus it is urgent to identify new targeted drugs.

In recent years, because of their specificity and cytotoxicity in binding to target cells, bacterial toxins have become a hot spot in the research and development of antitumor drugs, such as dCD133KDEL, a recombinant *Pseudomonas* exotoxin that can specifically kill CD133⁺ tumor-initiating cells and inhibit the proliferation of head and neck carcinoma cells.⁵ A bispecific ligand-directed toxin (BLT), another type of recombinant deimmunized *Pseudomonas* exotoxin (dEGF4KDEL), can have striking effects against systemic human pancreatic cancer.⁶ Listeriolysin O toxin produced by *Listeria monocytogenes* can exhibit anticancer activities in

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leukemia⁷ and melanoma,⁸ and diphtheria toxin produced by the bacterium *Corynebacterium diphtheriae* can treat cutaneous T-cell lymphoma.⁹ LukS-PV is the S component of Pantan-Valentine leucocidin (PVL) secreted by *Staphylococcus aureus*. We previously found that LukS-PV can induce the apoptosis and differentiation of human acute myeloid leukemia cells.¹⁰ LukS-PV can also inhibit HCC progression.¹¹

TNNC1, which is known as slow troponin C, contains a calcium-binding subunit and facilitates the interaction between actin and myosin in muscle cells.¹² However, in non-muscle cells, TNNC1 may act as a regulatory protein for cellular locomotion. For example, in ovarian cancer, TNNC1 overexpression can promote tumor cell invasion and metastasis.¹³ In early-stage tongue cancer, TNNC1 can predict the prognosis of TSCC and its occult cervical lymphatic metastasis.¹⁴ In liver cancer, we identified several tumor-related proteins and related metabolic pathways in HepG2 cells treated with LukS-PV altered using quantitative proteomic sequencing, some forms of which demonstrated antitumor activity.¹⁵ High-throughput transcriptomic and proteomic analyses of HepG2 cells treated with LukS-PV indicated that LukS-PV could downregulate the expression of TNNC1 at both the mRNA and protein level. Thus, TNNC1 may also be a target for regulating HCC cell movement.

Our previous studies have shown that LukS-PV could inhibit the proliferation and promote the apoptosis of liver cancer cells,¹¹ but its effect on the migration of HCC cells is still unclear. In this study, the role of LukS-PV in the migration of HCC cells and its molecular basis were further explored. The study results revealed that LukS-PV inhibited HCC cell migration by targeting TNNC1 and activating the PI3K/AKT signaling pathway. These results indicated that LukS-PV harbors great potential significance as a therapeutic drug for HCC.

Materials and Methods

Cell Culture

The human normal liver cell line L02 and liver cancer cell lines HepG2, Hep3B and Huh-7 were purchased from the Shanghai Cell Bank (Chinese Academy of Sciences, Shanghai, China). L02 cell were cultured in RPMI-1640 (Gibco, USA) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO₂. HepG2, Hep3B and Huh-7 cells were cultured in DMEM (Gibco, USA) containing 10% fetal bovine

serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO₂. Our study was approved by the Ethics Committee and Institutional Review Board of University of Science and Technology of China, Anhui, China.

Production, Purification, and Quantification of LukS-PV

The sequence of LukS-PV was amplified from PVL-positive *Staphylococcus aureus* isolates by PCR, and recombinant LukS-PV was generated as described previously.¹⁶ Recombinant LukS-PV was purified using a His-Bind Purification Kit (Millipore, USA) and quantified using a BCA Kit (Beyotime, China).

Quantitative Real-Time PCR Analysis

Total RNA from HCC cells was extracted using Trizol reagent (Invitrogen, USA) and cDNA from total RNA was prepared by reverse transcription. PCR was performed using SYBR Green Master Mix (Takara, China) according to the manufacturer's instructions. The primers were as follows: TNNC1 (5'-ATG ATG GTT CGG TGC ATG AAG GAC-3' and 5'-TCC GTG ATG GTC TCG CCT GTA G-3'); and GAPDH (5'-GGA GCG AGA TCC CTC CAA AAT-3' and 5'-GGC TGT TGT CAT ACT TCT CAT GG-3'). The relative mRNA expression levels of target genes were quantified using the $2^{-\Delta\Delta Ct}$ method, and GAPDH was used as an internal reference. All experiments were performed in triplicate.

Western Blot Analysis

Proteins were lysed with RIPA lysis buffer and quantified with a BCA kit. The soluble protein was separated on 10% and 12.5% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk for 1.5 h and then incubated with anti-GAPDH (Abclonal, China), anti-TNNC1 (Proteintech, China), anti-MMP2 (Abclonal), anti-MMP9 (Abclonal), anti-PI3K (Proteintech), anti-p-PI3K (Proteintech), anti-AKT (Cell Signaling Technology, USA), and anti-p-AKT (Cell Signaling Technology) antibodies at 4°C overnight. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1.5 h at room temperature.

Transfection

TNNC1 plasmid and corresponding empty vector were purchased from GenePharma Biotechnology (Shanghai,

China). siRNA-TNNC1 and the corresponding negative control were purchased from General Biotechnology (Anhui, China). HCC cells were transfected with the expression plasmid at a final concentration of 20 nmol/L using Lipofectamine 2000 (Invitrogen, USA) and siRNA at a final concentration of 70 nmol/L using Lipofectamine 2000 (Invitrogen, USA). Transfection efficiency was detected by qRT-PCR and Western blotting. The target sequence for siRNA-TNNC1 was as follows: sense, 5'-GCA CCA AGG AGC UGG GCA ATT-3'; antisense, 5'-UUG CCC AGC UCC UUG GUG CTT-3'.

Cell Scratch Assay

An appropriate number of HCC cells was cultured to full confluence in 12-well plates. After culturing for 12 h in complete medium, wounds were made using 10- μ L pipette tips and the cells were cultured in fresh serum-free medium. After 0 h, 48 h from scratching, the wounds were photographed with an inverted microscope and the width changes were recorded to enable the scratch heal rate of HCC cells in the control and experimental groups to be calculated. Three wells were used in each group and three independent experiments were performed.

RNA Sequencing

Total RNA of the samples was isolated using an RNeasy Mini kit (Qiagen, Germany). Paired-end libraries were synthesized using a TruSeq™ RNA sample preparation kit (Illumina, USA) according to the manufacturer's instructions. Purified libraries were quantified by a Qubit®2.0 Fluorometer (Life Technologies, USA) and validated by an Agilent 2100 bioanalyzer (Agilent Technologies, USA) to confirm the insert size and calculate the molar concentration. The library construction and sequencing were performed at the Shanghai Sinomics Corporation.

Quantitative Proteomic Sequencing

HepG2 cells treated with various concentrations of LukS-PV and PBS were sonicated three times on ice using a high intensity ultrasonic processor (Scientz, China) in lysis buffer, then the supernatant was collected and the protein concentration was quantified with a BCA kit. For digestion, the protein solution was reduced with 5 mM dithiothreitol and alkylated with 11 mM iodoacetamide, and the sample was diluted by adding 100 mM TEAB to a urea concentration of less than 2M. Finally, trypsin was added for overnight digestion.

After digestion, the peptide was reconstituted in 0.5 M TEAB and treated with a TMT kit/iTRAQ kit according

to the manufacturer's protocol. The peptide was fractionated using an Agilent 300Extend C18 column by high pH reverse-phase HPLC. Tryptic peptides were dissolved in 0.1% formic acid, directly loaded onto a home-made reversed-phase analytical column, and subjected to a NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive™ Plus (Thermo) coupled online to the UPLC. Finally, the resulting MS/MS data were analyzed using the Maxquant search engine (v.1.5.2.8).

Statistical Analysis

Differences between the two groups were analyzed by the unpaired Student's *t*-test, and differences between multiple groups were assessed by ANOVA. A *P*-value of *P*<0.05 (*), *P*<0.01 (**), or *P*<0.001 (***) was considered statistically significant.

Results

LukS-PV Inhibited the Migration of HCC Cells

To investigate whether LukS-PV has a role in HCC cell migration, we treated HepG2 and Huh-7 cells with different concentrations of LukS-PV for 48 h. Then, scratch assays were undertaken to measure the migration rate of the HCC cells. Compared with the control group, the migration capacity of HepG2 and Huh-7 cells treated with LukS-PV was reduced after 48 h of wounding. Furthermore, the reduction rate was positively correlated with the concentration of LukS-PV (Figure 1A and B). Additionally, Western blot results also showed that LukS-PV could reduce the expression of the migration-related proteins MMP-2 and MMP-9 in HepG2 and Huh-7 cells (Figure 1C). These results indicated that LukS-PV could inhibit the migration of HCC cells in a concentration-dependent manner.

LukS-PV Downregulated the High Expression of TNNC1 in HCC Cells

To further investigate the potential mechanism of LukS-PV inhibition in HCC cell migration, we used RNA sequencing and quantitative proteomic sequencing to analyze HepG2 cells treated with LukS-PV and PBS. Among a series of downregulated tumor-related genes, we identified TNNC1 (Figure 2A and B). TNNC1 has also been reported to be associated with the invasion and metastasis of ovarian cancer,¹³ and therefore we chose to study it further. QRT-PCR and Western blotting were used to further verify that

Figure 1 LukS-PV inhibited the migration of HCC cells. (A) Scratch assays were performed to detect the mobility of HCC cells treated with different concentrations of LukS-PV. (B) Relative migration percentage was calculated in HCC cells treated with different concentrations of LukS-PV for 48 h. * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$ compared with the control cells. (C) Western blot was applied to detect the protein levels of MMP-2 and MMP-9 in HCC cells treated with LukS-PV.

LukS-PV could downregulate *TNNC1* expression in HCC cells (Figure 2C and D). To further confirm the role of *TNNC1* in HCC, qRT-PCR and Western blotting were performed to detect the mRNA and protein expression levels of *TNNC1* in the normal liver cell line L02 and liver cancer cell lines HepG2, Hep3B, and Huh-7, proving that *TNNC1* was highly expressed in HCC cells (Figure 2E and F). We also used GEPIA¹⁷ to analyze *TNNC1* expression in liver cancer in cases from The Cancer Genome Atlas (TCGA) database. These data showed *TNNC1* was highly expressed in tumor samples compared with normal tissues (Figure 2G). Further analysis of TCGA database revealed that high *TNNC1* expression was associated with poor overall survival (Figure 2H). These results showed that LukS-PV could decrease *TNNC1* expression in HCC cells and *TNNC1* could be a prognostic marker for liver cancer.

LukS-PV Inhibited HCC Cell Migration by Downregulating *TNNC1*

To define the role of *TNNC1* in HCC cell migration, we first knocked down *TNNC1* in HepG2 and Huh-7 cells.

SiRNA-mediated knockdown of *TNNC1* resulted in a dramatic decrease in the migration of HepG2 and Huh-7 cells (Figure 3A and B). In contrast, scratch assays under ectopic *TNNC1* expression revealed that the *TNNC1*-overexpressing cells displayed a marked increase in migration (Figure 3C–E). Moreover, ectopic expression of *TNNC1* significantly attenuated the inhibitory effects of LukS-PV on the migration of HepG2 and Huh-7 cells compared with that of the LukS-PV treatment group (Figure 3C–E). These results indicated that LukS-PV inhibited HCC cell migration by downregulating *TNNC1*.

LukS-PV Inhibited HCC Cell Migration via the *TNNC1*/PI3K/AKT Axis

To further explore the molecular mechanism of LukS-PV inhibition of HCC cell migration by downregulating *TNNC1*, RNA sequencing was performed. LukS-PV treatment promoted the upregulation of 1678 genes and the downregulation of 1008 genes compared with the PBS group; the downregulated genes included *TNNC1* (Figure 4A). After running the differentially expressed genes

Figure 2 LukS-PV downregulated the high expression of TNNC1 in HCC cells. (**A** and **B**) RNA sequencing and quantitative proteomic sequencing were used to identify the changes in TNNC1 expression level in HepG2 cells treated with LukS-PV or PBS. (**C** and **D**) qRT-PCR and Western blotting were performed to detect the mRNA and protein expression levels of TNNC1 in HepG2 and Huh-7 cells treated with different concentrations of LukS-PV. *P<0.05, **P<0.01, or ***P<0.001 compared with the control cells. (**E**) qRT-PCR was performed to detect the mRNA expression levels of TNNC1 in L02, HepG2, Hep3B, and Huh-7 cells. *P<0.05, ***P<0.001 compared with L02 cells. (**F**) Western blotting was performed to detect the protein expression levels of TNNC1 in L02, HepG2, Hep3B, and Huh-7 cells. (**G**) TCGA database was used to analyze the expression levels of TNNC1 in liver cancer tissue and normal tissue. ***P<0.001 compared with normal tissue. (**H**) TCGA database analysis demonstrating the correlation between TNNC1 and the overall survival of liver cancer patients.

Figure 3 LukS-PV inhibited HCC cell migration by downregulating TNNC1. **(A)** The metastatic ability of HCC cells was analyzed by scratch assays after knocking down TNNC1. **(B)** The relative migration rate was calculated after knocking down TNNC1 in HCC cells. * $P < 0.05$ compared with the control cells. Western blot was performed to detect the protein expression levels of MMP-2 and MMP-9 after knocking down TNNC1 in HCC cells. **(C–E)** After HCC cells were treated with EV (empty vector), TNNC1 OE (TNNC1 plasmid), LukS-PV, or both LukS-PV and TNNC1 OE, scratch assays were performed to detect the metastatic ability of HCC cells. The relative migration rate was calculated, and Western blotting was used to detect the protein expression levels of MMP-2 and MMP-9 in HCC cells. * $P < 0.05$, ** $P < 0.01$ compared with the control cells.

through Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA) pathway analyses, the PI3K/AKT signaling pathway attracted our attention (Figure 4B and C). The PI3K/AKT signaling pathway has been reported to be involved in the migration and invasion of various tumor types.^{18,19} Therefore, we

hypothesized that LukS-PV might inhibit the activation of the PI3K/AKT signaling pathway by downregulating TNNC1. To verify this hypothesis, Western blot analysis was undertaken to detect the expression of these pathway proteins. The results revealed that PI3K and AKT phosphorylation was decreased compared with the control

Figure 4 LukS-PV inhibited HCC cell migration via the TNNC1/PI3K/AKT axis. **(A)** Volcano map showing differences in gene expression in HepG2 cells treated with LukS-PV or PBS by RNA sequencing. TNNC1 is labeled in the map. **(B and C)** KEGG and GSEA pathway enrichment analysis revealed that the PI3K/AKT signaling pathway is involved in LukS-PV function. **(D)** Western blotting was used to detect PI3K/AKT pathway protein levels in HCC cells treated with LukS-PV or PBS. **(E)** PI3K/AKT pathway protein levels in HCC cells were detected by Western blotting after knocking down TNNC1. **(F)** After HCC cells were treated with EV (empty vector), TNNC1 OE (TNNC1 plasmid), LukS-PV, or both LukS-PV and TNNC1 OE, Western blotting was performed to detect PI3K/AKT pathway protein levels in HCC cells. **(G–I)** After HCC cells were treated with PBS, SC79 (AKT phosphorylation agonist), LukS-PV, or both LukS-PV and SC79, scratch assays were undertaken to detect the metastatic ability of HCC cells. The relative migration rate was calculated, and Western blotting was performed to detect the protein expression levels of MMP-2 and MMP-9 in HCC cells. * $P < 0.05$, ** $P < 0.01$ compared with the control cells. **(J)** Schematic diagram of LukS-PV inhibition of HCC cell migration.

group after LukS-PV treatment for 24 h (Figure 4D). The role of TNNC1 in the PI3K/AKT pathway was further investigated and revealed that the phosphorylation levels of PI3K and AKT were significantly reduced in the TNNC1 knockdown group (Figure 4E). Conversely, when TNNC1 was overexpressed, the phosphorylation levels of PI3K and AKT were increased (Figure 4F). Furthermore, the effect of LukS-PV on p-PI3K and p-AKT could be reversed by TNNC1 overexpression (Figure 4F). These results demonstrated that LukS-PV could inhibit the phosphorylation of PI3K/AKT signaling molecules by downregulating TNNC1. To further prove that LukS-PV inhibited the migration of HCC cells by inhibiting the activation of the PI3K/AKT pathway, we performed scratch assays and Western blot analysis to detect the migration rates and migration-associated proteins of HCC cells treated in different ways. Rescue experiments revealed that the inhibitory effect of LukS-PV on the migration of HCC cells could be reversed by AKT phosphorylation agonist (Figure 4G–I). This study demonstrated that LukS-PV inhibited the phosphorylation of the PI3K/AKT pathway by downregulating TNNC1, thereby inhibiting the migration of liver cancer cells (Figure 4J).

Discussion

Hepatocellular carcinoma is the most common primary liver cancer with high morbidity and mortality.¹ The treatment of liver cancer includes surgical resection, liver transplantation, liver-directed therapy, and systemic therapy. However, only surgical resection and liver transplantation are considered as potentially curative approaches.²⁰ The therapeutic effect of hepatocellular carcinoma is affected by multiple factors, including liver function, performance status, and tumor stage. Hence, only 30% of patients can receive curative therapies (surgery or ablation).²¹ In recent years, bacterial toxins have played an increasing role in tumor therapy.⁹ LukS-PV is the S component of PVL secreted by *Staphylococcus aureus*. Our previous study found that LukS-PV could inhibit leukemia cell proliferation and induce cell cycle arrest and apoptosis.^{22,23} Further studies have shown that LukS-PV could also inhibit HCC cell proliferation and induce cell cycle arrest and apoptosis,¹¹ but the role of LukS-PV in HCC cell migration and its molecular mechanism were unclear. To investigate the role of LukS-PV in HCC cell migration, we performed scratch assays by stimulating HCC HepG2 and Huh-7 cells with different concentrations

of LukS-PV. Compared with the control groups, LukS-PV could inhibit HCC cell migration in a concentration-dependent manner. To further explore the molecular mechanism of LukS-PV inhibition of HCC cell migration, we performed transcriptome and proteomic sequencing to explore the downstream target genes of LukS-PV. Sequencing results showed TNNC1 ranked higher in the degree of downregulation in both “omic” studies, and therefore we chose TNNC1 for further study. A literature review revealed that TNNC1 could promote ovarian cancer cell migration and invasion,¹³ as well as serve as a potential marker for the early occult cervical lymph node metastasis and prognosis of tongue cancer.¹⁴ Further analysis of TCGA database showed that TNNC1 was highly expressed in HCC and was associated with poor overall survival. Therefore, we hypothesized that TNNC1 was a downstream target gene of LukS-PV that inhibited HCC cell migration. First, we used qRT-PCR and Western blot analysis to verify that LukS-PV could downregulate TNNC1 in HCC cells. Knockdown of TNNC1 could reduce the migration ability of liver cancer cells, while TNNC1 overexpression could increase the migration ability of liver cancer cells and reverse the inhibitory effect of LukS-PV on liver cancer cell migration. These results demonstrated that LukS-PV inhibited HCC cell migration by downregulating TNNC1. To further explore the molecular mechanism of LukS-PV inhibition of HCC cell migration, RNA sequencing was undertaken. KEGG and GSEA pathway analysis identified the PI3K/AKT signaling pathway as being significant. The PI3K/AKT pathway is an important signaling cascade that is implicated in multiple oncogenic processes, including cell proliferation, apoptosis, differentiation, epithelial–mesenchymal transition, migration, and invasion.^{24–31} We first used Western blot analysis to detect the phosphorylation of PI3K/AKT proteins in liver cells treated with LukS-PV as well as TNNC1 knockdown and overexpression. LukS-PV could inhibit the phosphorylation of PI3K/AKT pathway proteins by downregulating TNNC1. Finally, scratch assays demonstrated that AKT phosphorylation agonist could reverse the inhibitory effect of LukS-PV on HCC cell migration. These results ultimately proved that LukS-PV inhibited liver cancer cell migration via the TNNC1/PI3K/AKT axis.

There are some limitations in our study. We first proved that LukS-PV inhibited HCC cell migration via the TNNC1/PI3K/AKT axis, but the specific molecular mechanism of LukS-PV regulating TNNC1 has not been

fully clarified. We will continue to investigate this in future studies. Second, the expression and prognosis of TNNC1 in liver cancer were analyzed only with TCGA database. In future experiments, clinical samples will be collected and analyzed to verify the expression of TNNC1 in HCC and its correlation with the prognosis and clinicopathological features of liver cancer. Finally, we undertook cell experiments to prove that LukS-PV could inhibit HCC cell migration in vitro, but whether LukS-PV can inhibit HCC cell migration in vivo remains to be elucidated. In the future, we will also construct a patient-derived tumor xenograft nude mouse model for verification.

In summary, we first proved that LukS-PV could inhibit HCC cell migration in vitro. Second, there have been few reports on TNNC1 in liver cancer. Our results proved that TNNC1 could promote the migration of liver cancer cells and LukS-PV inhibited HCC cell migration by downregulating TNNC1. Finally, our study results showed that LukS-PV inhibited HCC cell migration through the TNNC1/PI3K/AKT axis.

Conclusion

We showed that LukS-PV inhibited HCC cell migration, probably by downregulating TNNC1 and inhibiting the PI3K/AKT signaling pathway. Our results confirm a tumor-suppressive role for LukS-PV in HCC cells, suggesting its use as a therapeutic drug.

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Disclosure

The authors report no conflicts of interest in this work.

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