LATS1 Knockdown with SiRNA Induces Pyroptosis via the Activation of TNF- α /NF- κ B Signaling in Bladder Cancer Cells

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Research

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Abstract

Background: Traditionally, it is believed that large tumor suppressor 1 (LATS1) is a negative regulator of oncogene. But the latest research showed that LATS1 has an opposite effect in some tumors. We found that LATS1 has a cancer-promoting effect in BLCA, but the specific mechanism is unknown.

Methods: RNAi method was used for the related genetic functional analysis. CCK-8 method and colony formation assay were used to explore the cellular viabilities and proliferation of BLCA cells transfected with LATS1 siRNAs (si-LATS1), in vitro. Flow Cytometry (FCM) was used to analyze the cell cycle and cellular apoptosis of the BLCA cells or the expression of CD68, CD86, CD11b, and CD163 in PMA-treated THP-1 macrophages incubated with conditioned medium (CM) from the si-LATS1 cells or in the THP-1 macrophages cultured directly with BLCA cells. RT-qPCR method was used to detect the mRNA levels of IL-1 β , IL-2/4/6/10/18, TNF- α , and IFN- γ in the BLCA cells. Western Blot was performed to detect the expressions of LATS1, Yap1, Bcl-2, Bax, caspase-1/3, GSDMD, TNF- α IL-1 β , IL-18, NF- κ B, AIM2, NLRC4 and NLRP3 in the BLCA cell lines. For in vivo experiments, a xenograft model was used to investigate the inhibitory effects of LATS1 knockdown on BLCA cells in nude mice.

Results: LATS1 knockdown via siRNA inhibited the proliferation of the BLCA cells, neither changing cell cycle distribution nor inducing apoptosis. Via further analysis, we found that the expressions of TNF- α , p-NF- κ B/RelA, NLRP3, NLRC4, and AIM2 in si-LATS1 BLCA cells were significantly increased. The activity of caspase-1 and the expressions of IL-1 β , IL-18 and GSDMD were obviously increased in the si-LATS1 BLCA cells, which was restored by the addition of NF- κ B inhibitor PS341. In the LATS1 over-expression cells (OE-LATS1), the expressions of TNF- α , p-NF- κ B/p65, NLRP3, NLRC4 and AIM2 were notably inhibited, and the expressions of IL-1 β , IL-18 and GSDMD were significantly decreased. THP-1 macrophages exhibited an M1 phenotype polarization in the presence of si-LATS1 BUC-87 cell supernatant or when cocultured with the BLCA cells transfected with LATS1 siRNA, represented by an increase in the surface expression of CD86. Furthermore, we observed that LATS1 knockdown inhibited the cell proliferation in xenograft model.

Conclusion: Our findings showed that LATS1 knockdown via siRNA induces BLCA cell pyrolysis due to the enhanced formation of inflammasomes by activation of TNF- α /NF- κ B pathway; and inflammatory factors released by the pyrolytic cells promote M1 polarization of THP-1-derived macrophages in vitro, providing a therapeutic target for BLCA and a brand-new idea for the development of BLCA immunotherapy drugs.

Background

Bladder cancer (BLCA) is recognized as one of the most common malignant tumors in the urological system, and the number of new cases and deaths of BLCA ranks first in China (1). And more than 90% of bladder cancer is urothelial carcinoma. Although it can undergo traditional surgical resection followed by subsequent chemotherapy or immunotherapy, the recurrence rate and mortality of BLCA have not been

significantly improved(2, 3).At present, there are no clear and effective therapeutic targets for the treatment of BLCA. Therefore, it is necessary and urgent to identify specific molecular signatures that better predict the clinical prognosis and molecular markers that serve as better therapeutic targets of BLCA.

Large tumor suppressor 1 (LATS1) is a highly conserved serine/threonine kinase and a key player of the Hippo pathway in mammals. As a tumor suppressor, LATS1 loss or mutation is most closely associated with tumorigenesis and progression of various human cancers, including bladder cancer(4-10). But the latest studies showed that deletion of LATS1/2 in tumors thus enhances anti-tumor immune responses via the activation of TLRs-MYD88/TRIF pathway, stimulating anti-tumor responses of an immune-competent host(11). And the loss of LATS1/2 prevents the growth of murine colon adenocarcinoma MC38 cells(12). In addition, it has been reported that LATS1 has a pro-tumorigenic role in hepatocellular carcinoma (HCC) and high levels of LATS1 mRNA correlates with poor overall and disease-free HCC patient survival(13). These findings suggest that LATS1 does not always act as a tumor suppressor and LATS1 inhibition can be a potential therapeutic approach for some types of cancers.

Pyroptosis is an inflammatory form of lytic programmed cell death in a caspase-dependent manner, triggered by formation of a large supramolecular complex termed the inflammasome(14). The inflammasome is a multi-protein complex that typically consists of the sensor molecule - a pattern recognition receptor (PRR), the adaptor protein ASC and Pro-caspase 1. The pro-caspase 1 is activated by the inflammasome complexes, leading to the N-terminal cleavage of gasdermin D (GSDMD) which results in its oligomerization to form a lytic pore in the plasma membrane(15-17). And the active caspase 1 cleaves the pro-inflammatory cytokines like pro-IL-18 and pro-IL-1 β their mature bioactive forms, IL-1 β and IL-18, which are released from the necrotic cells inducing widespread inflammatory responses(18). The PRR family consists of various members, including nucleotide-binding and oligomerization domain-like receptors (NLRs), membrane-bound Toll-like receptors (TLRs), absent in melanoma (AIM) -like receptors (ALRs) and RIG-I-like receptors. Of these, NLRs and ALRs have the ability to form an inflammasome(19). PRRs are unique in the inflammasome formation, so each inflammasome is named after the specific PRR involved. Several distinct types of inflammasomes have been well established and characterized, including NLRP1, NLRP3, NLRC4, AIM2, and Pyrin inflammasomes (19, 20).

It is now clear that nuclear factor κ B (NF- κ B) induces the expression of a number of pro-inflammatory genes, and proteins that is a part of the inflammasome(21). The activation of NF- κ B pathway induces the pyroptosis in cervical cancer, melanoma, colorectal cancer, and nasopharyngeal cancer by enhancing the expression of NLRP3(22-25). And in the cervical cancer cells, the upregulated NF- κ B/RelA by the SIRT1 loss increased AIM2 inflammasome-related genes, leading to AIM2 inflammasome-regulated pyroptosis(26). It's well known that tumor necrosis factor alpha (TNF- α) is one of the most potent physiological inducers of NF- κ B pathway once interacting with receptors on the cell membrane. Studies have shown that TNF- α induces pyroptosis of HepG2 cells(27). And TNF- α together with IFN- γ induce pyroptosis of bone marrow-derived macrophages(28).

One of the major problems in the failure of cancer treatments is the immunosuppressive property of the tumor micro-environments (TME), within which regulatory T cells and tumor-associated macrophages (TAMs) constitute the main population of immune cells (29). In BLCA, the tumor cells recruited the regulatory T cells (Treg) and TAMs, resulting in an immune-suppressive TME (30-32). At the same time, the infiltrated monocytes undergo M2 polarization in the tumor region, also promoting the formation of an immunosuppressive TME, which has been confirmed by single-cell RNA sequencing on BLCA tumor samples (33). The M2-polarized TAMs affect angiogenesis, tumor grade and invasiveness (34) and associate with poor prognosis after intravesical instillation of BCG (35). M2-like TAMs are potential targets for the BLCA immunotherapy. Thus, re-education of M2 macrophages toward M1 is a strategy against tumor progression (36, 37).

At present, the role of LATS1 in the occurrence and development of BLCA is still unclear. We found that BLCA patients with high expression of LATS1 have a poor prognosis through the analysis using TCGA data, which gives us an interesting hint that LATS1 may have a totally distinct role in BLCA. This study reported for the first time that the significant LATS1 knockdown via siRNA in BLCA cells induce the pyroptotic cell death, which is associated with the inflammasome formation. Meanwhile, the cellular pyroptosis contributes to M1-like phenotype polarization of THP-1 cells. Our data demonstrate an important role of LATS1 in the development of BLCA and provide a new molecular target for the treatment of BLCA.

Methods

Cell culture and Transfection

The BLCA cell lines including BIU-87, 5637, T24, RT-4 and normal bladder cells SV-HUC-1, and THP-1 cells were purchased from the American Type Culture Collection (ATCC) and maintained in RPMI-1640 medium or McCoy's 5A (Procell Co., Ltd) supplemented with 10% fetal bovine serum (Biological Industries, Israel) at 37°C in a humidified atmosphere of 5% CO₂. Cells were seeded in 6-well plates before transfection with gene-specific small interfering RNAs (siRNAs). The siRNA was purchased from Sigma-Aldrich, and the sequence used in this experiment is provided in the attached Table S1 and Table S2. jetPRIME® transfection reagent (Ployplus-transfection, France) was used as transfection reagent according to the manufacturer's protocol.

GO and KEGG Analysis

Total RNAs from BIU-87 cells transfected with NC or LATS1 siRNAs were collected for Bulk RNA-Seq (Novogene, Beijing, China). sva R packages were used to remove batch effects on the sequencing results, and the GenomicFeatures and biomaRtR packages were used to convert counts data into TPM data. DESeq2 package was used to extract differential genes (DEGs) directly based on the count data. Then, GO

and KEGG enrichment analysis on the DEGs were performed via the online DAVID database V6.8 (<http://david.abcc.ncifcrf.gov/>), and the enrichment results were visualized with the ggplot2 package.

Gene Set Enrichment Analysis (GSEA)

GSEA was performed using GSEA v4.1.0 software. Hallmarks (h.all.v7.2.symbols.gmt), Reactome (c2.cp.reactome.v7.2.symbols.gmt) and KEGG (c2.cp.kegg.v7.2.symbols.gmt) 3 gene sets were downloaded from the GSEA website (<http://www.gsea-msigdb.org/gsea/index.jsp>) for analysis. The LATS1 siRNA group versus NC group (si-LATS1 vs. Control) was used as the gene set label and the number of permutations was set to 1000. All other parameters are set to the default values.

Cell viability assay

At 24h post-transfection, the 5637 or Bui-87 cells were seeded in a 96-well plate (0.25×10^4 cells/well). Before measurement, add 10 μ L CCK-8 solution into each well and then placed 96-well plates in incubator for 2h. Finally, the OD value of each well was read by a microplate reader.

Colony formation assay

The 5637/Bui-87 cells were seeded into 6-well plates with 500 cells per well. After 2 weeks, the medium was discarded and then the wells were washed three times with cold phosphate-buffered saline (PBS) solution. Then, the cells were fixed with 4% paraformaldehyde for 30 mins and stained with 1% crystal violet for 20 mins. Finally, use a digital camera to take pictures, and colonies with more than 50 cells per well were counted using Image J software.

Flow cytometric analysis

BLCA cells were harvested and washed three times with precooled PBS then fixed in 75% precooled ethanol. Before analyzing cell cycle, the propidium iodide (PI) stain was added to each tube, then incubate at 4 °C for 15 minutes and analyze by flow cytometry. Before analyzing cell apoptosis, the cells were resuspended in 300 μ L binding buffer, then add 5 μ L PI/FITC to each tube. Finally, incubate at 4 °C for 15 minutes and analyze by flow cytometry.

For M1 (CD11b+CD68+CD86+) or M2 (CD11b+CD68+CD163+) cell surface markers staining, THP-1 cells were stimulated with phorbol myristate acetate (PMA, 25 ng/ml; Sigma-Aldrich) for 24h at 37 °C. After cultured with the conditioned medium, the cells were harvested and stained with CD11b, CD68, CD86, CD163 mAbs for 30 min at 4 °C. Finally, cells were washed, resuspended, and analyzed with a FACSCelesta Flow Cytometer. The data were analyzed using BD FACSDiva 8 (BD Biosciences).

Lactate dehydrogenase (LDH) assay

The supernatant from the cell culture medium was collected for LDH level measurement using LDH Cytotoxicity Test Kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The microplate reader was applied to acquire the absorbance of each well at 450 nm.

Quantitative real-time PCR

Total RNA was extracted from the cultured cells using the Trizol reagent (TaKaRa, Japan). 1 µg of quantified RNA was used for reverse transcription using PrimeScript RT reagent Kit (TaKaRa, Japan). Quantitative PCR was performed with the TB Green Premix Ex Taq II (Takara, Japan) using the LightCycler® 480 II system (Roche, Basel, Switzerland). The comparative expression of LATS1 was calculated according to the $2^{-\Delta\Delta C_t}$ method. Primer sequences are provided in Table S1.

Western blotting

All cells were lysed with RIPA lysis buffer (Beyotime, Shanghai, China) containing protease and phosphatases inhibitors (1 µg/ml). The protein concentration was determined using BCA Protein Quantification Kit (Vazyme, Nanjing, China). Denatured proteins were separated via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (0.45 µm). The membrane was incubated with the corresponding antibodies against LATS1 (1:1,000, Cell Signaling Technology), Yap1 (1:1,000, Cell Signaling Technology), P-YAP1 (1:1000, Cell Signaling Technology), Bcl2 (1:1000, Cell Signaling Technology), Bax (1:1000, Cell Signaling Technology), Cleaved-caspase3 (1:1000, Cell Signaling Technology), Cleaved-caspase7 (1:1000, Cell Signaling Technology), Cleaved-caspase-1 (1:1000, Immunoway), TNF-α (1:1000, Cell Signaling Technology), IL-1β (1:1000, Bioss), IL-18 (1:1000, ABclonal), P-NF-κB/P65 (1:2000, Immunoway), NF-κB (1:1000, Cell Signaling Technology), GSDMD (1:1000, Abclonal), NLRP3 (1:1000, proteintech) and NLRC4 (1:1000, ABclonal). β-actin (1:10000, Immunoway) was used as an endogenous reference.

Caspase 1 / Caspase 4 Activity Assay

According to the manufacturer's instructions, Caspase 1 Assay Kit/Caspase 4 Assay Kit (Abbkine, Wuhan, China) was used to detect the Caspase-1/Caspase 4 activity in 5637 cells and Biu-87 cells. The treated cells were harvested and lysed with chilled Cell Lysis Buffer, then centrifuge at 16,000 g for 15 minutes and the protein concentration was determined by using the Bradford method. Prepare the pNA calibration curve by diluting the pNA standard solution with reaction buffer. Then measure the absorbance at 405 nm by a microtiter plate reader and determine the amount of pNA according to the standard curve. Finally, the Caspase 1 /Caspase 4 activity is calculated by the OD value.

DNA Ladder Assay

Cell Apoptosis DNA Ladder Detection Kit (keygen BioTECH, Jiangsu, China) was used to detect cell apoptosis in both BIU-87 and 5637 cells. About the cells were cultured in 6-well plates at density of 5×10^5 per well. DNA was isolated from treated cells using Cell Apoptosis DNA Ladder Detection Kit (keygen BioTECH, Jiangsu, China). Isolated DNA samples were loaded onto a 1.5% agarose gel with 0.5 mg/mL GelGreen® Nucleic Acid Gel Stain (Biotium, USA) followed by electrophoresis (5 V/cm). Finally, DNA was visualized by an ultraviolet gel documentation system (Tanon 2500 R, Shanghai, China). DL2000 Plus DNA Marker (TaKaRa, Japan) was used as a DNA size standard.

In vivo studies

Four-to-five-week-old female nude mice (BLAB/c-nu) were provided by Beijing SIPEIFU Biotechnology Co., Ltd, and were raised in the Department of Laboratory Animal Science at China Medical University. All animal work was approved by the China Medical University Animal Care and Use Committee and was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. To assess cancer cell proliferation in vivo, 1×10^7 5637 cells in 200 μ L 1640 medium were subcutaneously injected into the axilla of nude mice. Six mice were assigned to each group. Tumor size was measured every 2 days to calculate the tumor volumes (volumes = width² $\times$ height $\times$ $\pi/6$). For subcutaneous tumor growth, the maximum single tumor cannot exceed 1.2 cm in diameter in mice and no experiments in this study generated tumor burden over this limit.

Statistical analysis

All in vitro experiments were performed at least three times in vitro, and all data were presented as mean $\pm$ standard deviation (SD). SPSS v23.0 software was used to conduct all statistical analyses. t-test is used to compare differences between any two groups. A value of $P < 0.05$ was considered statistically significant.

Results

LATS1 predicts poor clinical outcomes and knockdown of LATS1 inhibits the proliferation of BLCA cells in vitro and in vivo.

To explore the possible biological functions of LATS1 in BLCA, first we evaluated the association between LATS1 expression of and patients' prognosis in BLCA using TIMER database. Kaplan-Meier analysis showed that BLCA patients with high LATS1 expression had shorter overall survival (OS) (Figure 1A). According to multivariate Cox regression analyses using BLCA data from the TCGA bladder cancer data set, LATS1 expression might be a significant prognostic factor for reduced OS (HR = 1.89, $P = 0.001$) and

progression free survival (PFS) (HR = 1.75, P = 0.013) (Figure 1B and 1C), which suggests that high expression of LATS1 is a predictor of poor prognosis in BLCA patients.

To further confirm the function of LATS1 in BLCA, we examined LATS1 expression by western blot analysis in BLCA cell lines (Figure S1A). And then, BIU-87 and 5637 cells with higher LATS1 expression were selected to assess the effects of LATS1 changes on the cell proliferation in vitro for this study. Then, small interfering RNA (siRNA) was used to down-regulate LATS1 in BIU-87 and 5637 cells (Figure S1B and S1C). siRNA-mediated knockdown of LATS1 (si-LATS1) resulted in a statistically significant reduction of cell viability and a decrease of colony formation capacity in different BLCA cell lines (Figure 1D and 1E). Interestingly, analysis of apoptosis by Flow Cytometry showed that the number of apoptotic cells was not significantly increased in si-LATS1 cells compared with non-targeting control (NC) cells (Figure S1D), and the western blot analysis showed that the protein expressions of cleaved caspase 3, 7, BAX, BCL-2 were not significantly changed in si-LATS1 cells (Figure S1E). To confirm whether si-LATS1 affected its classic target YAP1, we examined the expression of YAP1 and phospho-YAP1 with Western Blot. We found that there were no significant changes of both YAP1 and phospho-YAP1 in the si-LATS1 cells than that in NC cells (Figure S1E). Moreover, si-LATS1 did not cause significant cell cycle change (Figure S1F), too.

For the in-vivo study, lentiviral-delivered shRNAs were used to inhibit LATS1 expression (Figure S1G) in 5637 cells to obtain LATS1 knockdown cell line after screened by neomycin. And then, cell-line-derived tumor xenograft (CDTX) model was established by subcutaneously injecting 5×10^6 5637 cells infected with empty lentivirus vector or lentivirus LATS1 shRNAs. The BLCA cell tumor began to be measurable 14 days after cell injection in the control group (LV-vector group), but there was no significant subcutaneous BLCA cell tumor found in LV-shLATS1 groups (Figure 1G).

Together, these findings demonstrated that LATS1 could serve as a prognostic predictor of BLCA survival, and si-LATS1 could inhibit the proliferation of BLCA cells in vitro and in-vivo, but without induction of cell apoptosis and cell cycle arrest. And Hippo signaling was not involved in the proliferation suppression induced by si-LATS1 in BLCA cells.

Gene enrichment analysis of LATS1 knockdown and wild-type BLCA cells

To elucidate the potential genes and possible pathways affected by knockdown of LATS1, BIU-87 cells were transfected with siRNA or siNC. After 24 hours, the RNA was isolated from the siRNA and siNC cells for use in Bulk RNA-sequencing (RNA-seq). Based on the R analysis, a total of 715 differentially expressed genes (DEGs), 501 upregulated genes and 214 downregulated genes, were identified in the cells of si-LATS1 groups compared with NC groups (Figure 2A). $P < 0.05$ and $|\log_2 FC| \geq 1$ were set as the threshold criteria.

To further explore the systematic characterization and biological functions of the identified DEGs, functional annotation and pathway analysis, including GO and KEGG, were performed using R packages. In this study, the two GO categories [biological process (BP) and molecular function (MF)] were

detected, respectively, using R packages. The GO analysis showed that DEGs were significantly enriched in different biological processes (BP) and molecular functions (MF), such as “response to lipopolysaccharide”(ontology: BP) and “DNA-binding transcription activator activity” (ontology: MF) (Figure 2B). Subsequently, KEGG pathway analysis showed that the DEGs were enriched in several key pathways, including ‘TNF signaling pathway’, ‘Rheumatoid arthritis’, and ‘NF-kappa B signaling pathway’ (Figure 2C).

Using the RNA-seq data, we also performed gene set enrichment analysis (GSEA) to extract cellular functions associated with LATS1 knockdown. GSEA revealed that the changes in gene expression caused by the knockdown of LATS1 correlated positively with high expression of inflammatory genes, especially HALLMARK_TNFA_SIGNALING_VIA_NFKB regulated genes (Figure 2D) (total 14 gene sets were meaningful, $P < 0.05$, $FDR < 0.05$) from ‘h.all.v7.4.symbols gene set collection’, and REACTOME_INFLAMMASOMES (total 16 gene sets were meaningful, $P < 0.05$, $FDR < 0.05$) from ‘c2.cp.reactome.v7.4.symbols gene set collection’.

Through the bioinformatic analyses above, we found that the alterations in gene expression caused by si-LATS1 were most related with inflammatory responses and pathways. Generally, all the BLCA cells in this study were harvested at 24h unless otherwise specified.

LATS1 knockdown increases the expressions of pro-inflammatory cytokines via activating TNF- α /NF- κ B signaling in BLCA cells

Based on the comprehensive bioinformatic analysis above, we first focused on the TNFA_SIGNALING_VIA_NFKB pathway (Figure 3A). To validate whether si-LATS1 induces the inflammatory responses in the BLCA cells, we used RT-qPCR to detect the mRNA expressions of some pro- and anti-inflammatory cytokine genes, most of which are the targets of NF- κ B. We found that si-LATS1 could significantly increase the mRNA expressions of TNF- α , IL-1 β , IL-18 (Figure 3B), IFN- γ , IL-2 and IL-6 (Figure S2A-2B) but decrease the expressions of IL-10, TGF- β and IL-4 (Figure S2C-2F). The Western Blot results demonstrated that the expression of phospho-NF- κ B/RelA and secreted TNF- α (sTNF- α) in si-LATS1 cells was significantly higher than that in their respective control (NC) cells, but without changing the expression of total NF- κ B/RelA (Figure 3C). Immunofluorescence (IF) results confirmed that si-LATS1 could promote the translocation of NF- κ B from cytoplasm to nucleus (Figure 3D).

To further determine if NF- κ B activation was required for inflammation responses in si-LATS1 cells, the NF- κ B inhibitor PS341 was used when the BLCA cells were transfected with LATS1 siRNAs. We found that the mRNA expressions of TNF- α , IL-1 β , IL-6 and IL-18 were obviously downregulated in the si-LATS1 cells co-treated with 200nM PS341 compared with those in the si-LATS1 cells (Figure S2G; Figure 3E).

All the results above proved that si-LATS1 could induce the activation TNF- α /NF- κ B signaling, increasing the expressions of pro-inflammatory cytokines in BLCA cells.

LATS1 knockdown induces cellular pyroptosis via TNF- α /NF- κ B signaling

To further confirm the non-apoptotic way of the cell death, DNA ladder assay was performed using agarose gel electrophoresis to assess DNA fragmentation in the BLCA cells transfected with LATS1 siRNA. We found that DNA cleavages were random and non-specific in the si-LATS1 cells (Figure 4A), which suggested that the cell death was predominantly non-apoptotic. Additionally, lactate dehydrogenase (LDH)-release into the culture medium was quantified to estimate the plasma membrane damage in si-LATS1 cells. And the results showed that the levels of LDH in culture medium were significantly elevated in the si-LATS1 cells compared with those in the NC cells (Figure 4B).

Based on the previous results and the bioinformatic analysis, we focused on the INFLAMMASOME pathway (Figure 4C and Figure 2E). The genes retrieved from the gene set (REACTOME_INFLAMMASOMES) were calculated by the cytoHubba in Cytoscape software according to the degree of connectivity to identify the possible hub genes that si-LATS1 affected, and the top 10 genes in protein-protein interaction (PPI) network were considered as hub genes (Figure 4C and 4D).

Up to date, several inflammasomes have been identified, including AIM2, NLRP3 and NLRC4, which have been reported to protect against tumor growth (38). We found that the expressions of AIM2, NLRP3 and NLRC4 were obviously elevated in the si-LATS1 cells at mRNA (Figure S3A) and protein (Figure 4F) levels. In the meantime, the activity of caspase 1 [but not caspase 4 (Figure S3B)] was significantly increased in the si-LATS1 cells than that in the NC cells (Figure 4E and 4F); and the expressions of IL-1 β and IL-18 were significantly increased; as the executioner of cell pyroptosis, GSDMD was cleaved and activated (Figure 4F). The addition of pan-caspase inhibitor Z-VAD-FMK markedly rescued the si-LATS1-induced cell viability reduction in the BLCA cells (Figure 4G). Furthermore, the addition of the NF- κ B inhibitor PS341 also reduced the upregulated expressions of AIM2, NLRP3 and NLRC4 in the si-LATS1 cells (Figure 4H).

Taken together, si-LATS1 contributed to the formation of AIM2, NLRP3 and NLRC4 inflammasomes via the activation of TNF- α /NF- κ B signaling, inducing the cellular pyroptosis in the BLCA cells.

LATS1 overexpression inhibits TNF- α /NF- κ B and the inflammasome pathways

To investigate roles of overexpressed LATS1 (OE-LATS1) in the activation of TNF- α /NF- κ B signaling and the formation of inflammasomes, we transfected LATS1-expressing plasmid into BIU-87 and 5637 cells. And the expression of LATS1 was obviously enhanced in BLCA cells after transfection with human the LATS1 plasmid at mRNA and protein levels (Figure 5A). And we found that the cellular viabilities of BIU-87 and 5637 cells were not evidently affected in the OE-LATS1 cells compared with NC cells (Figure 5B). But the expressions of sTNF- α and phospho-NF- κ B/RelA were markedly downregulated (Figure 5C). And the protein expressions of AIM2, NLRP3 and NLRC4, cleaved caspase 1, cleaved GSDMD were all clearly decreased in the OE-LATS1 cells (Figure 5D). In addition, the overexpression of the LATS1 has been shown to rescue the proliferation in the si-LATS1 cells (Figure 5E).

The results showed that LATS1 overexpression inhibited TNF- α /NF- κ B signaling to block the formation of AIM2, NLRP3 and NLRC4 inflammasomes.

LATS1 knockdown induced pyroptosis of BLCA cells promotes M1 polarization of THP1 derived macrophages

TAMs are important components of the TME, which can be used as the therapeutic targets of BLCA treatment. First, we used *xCell* R package to analyze the tumor-infiltrating immune cell in BLCA tissues using the BLCA data obtained from TCGA database. The results indicated that infiltration levels of CD8⁺ T cells, B cells, myocytes, NK cells, pDC, plasmacells, and smooth muscle cells were significantly associated with the overall survival of BLCA patients, including the M2 macrophages (Figure 6A). To validate the effects of si-LATS1 induced pyroptosis on the polarization of macrophages, THP-1 monocytes were polarized into macrophages using PMA and then PMA-treated THP-1 cells were co-cultured with si-LATS1 BUC-87 cells culture supernatant for 72h. Macrophage polarization was then assessed by measuring the expression of several classical M1 markers (CD68⁺CD11b⁺CD86⁺) and M2 markers (CD68⁺CD11b⁺CD163⁺).

In the absence of si-LATS1 BUC-87 cell supernatant, our flow cytometric analysis showed a low expression of the M1 marker CD86 and the M2 marker CD163. More importantly, in the presence of si-LATS1 BUC-87 cell supernatant, THP-1 macrophages exhibited an M1 phenotype, represented by an increase in the surface expression of CD86 (Figure 6B and 6C). These data suggested that the pyroptosis induced by si-LATS1 was involved in M1 polarization of macrophages. To further confirm these results, the THP-1 cells were directly co-cultured with BUC-87 cells transfected with LATS1 siRNA. Consistently, after 48h, the levels of CD86 were also upregulated in the THP-1 cells when compared with those co-cultured with the NC-transfected BUC-87 cells (Figure 6D and 6E). These results proved that si-LATS1 in BLCA cells could promote M1 polarization of THP-1 derived macrophages in vitro.

Discussions

Traditionally, LATS1 is considered as a tumor suppressor implicated in the pathogenesis of certain types of tumors, but recent studies have shown that it may have different biological functions in a context dependent manner. In human BLCA, how LATS1 is associated with the cancer progression remain unclear. In this study, using loss-of-function studies, we further investigated the function of LATS1 in BLCA. BUC-87 and 5637 cells were used as the in vitro surrogate for BLCA. Firstly, we found that high expression of LATS1 was associated with poor prognosis in BLCA patients through the bioinformatic analysis. This result agreed with the previous report that patients with higher LATS1 expression had a significantly shorter overall survival time than patients with lower LATS1 expression in HCC (13). Taken together, these results indicated LATS1 could be a candidate tumor prognostic marker and loss of LATS1 expression would be useful for predicting the poor prognosis of BLCA patients.

To examine the role of LATS1 played in proliferation of BLCA cells, we knocked down LATS1 gene by the LATS1 siRNAs. The expression of LATS1 in these knockout cells has been confirmed with RT-qPCR and Western blot. The proliferation assays revealed that LATS1 knockdown significantly reduced the proliferation ability of BUC-87 and 5637 cells both in vitro and in vivo. The results of the present study are

some similar with previous study carried out in the mice model(12), proving the tumor-promoting role of LATS1 in BLCA and the cell context dependent function of LATS1 in cell growth. Furthermore, we found that LATS1 knockdown did not significantly influence the apoptotic rate as well as the cell cycle of Biu-87 and 5637 cells. Then, we investigated the possible mechanisms of LATS1 knockdown on the proliferation of the Biu-87 and 5637 cells. As a core upstream kinase in Hippo pathway, LATS1 directly phosphorylates YAP for its proteasomal degradation, and depletion of LATS1 promotes the dephosphorylation and nuclear localization of YAP, thus enhancing its transcriptional activity. We found that LATS1 knockdown did not change the phosphorylation or expression levels of YAP in the BLCA cells, which suggested that the of inhibitory role of si-LATS1 on the proliferation was independent on its regulation on YAP. Although LATS1 deficiency obviously inhibited the tumor growth of B16-OVAz, SCC7 cells in vivo(11), and murine MC38 cells (12), the activity of YAP was significantly enhanced in these researches, which was totally different from our findings. The following RNA-seq analysis showed that the major changes at the level of transcription in the LATS1 knockdown cells were closely associated with TNF- α /NF- κ B and Inflammasomes pathways. Our results proved that si-LATS1 enhanced the mRNA levels of many pro-inflammatory cytokines, for example, IFN- γ , IL-1 β , TNF- α and IL-18, in BLCA cells. And at same time, the protein levels of sTNF- α and phosphorylated NF- κ B/RelA were also increased in the cells transfected with si-LATS1, indicating the activation of TNF- α /NF- κ B pathway. But LATS1 overexpression significantly reversed the activation of TNF- α /NF- κ B pathway.

The abnormal inflammasome activation is associated with the development of a variety of tumors, including colon cancer, liver cancer, lung cancer, and colorectal cancer (39–42). And the role of pyroptosis in the development of different cancers may be controversy and context dependent. On one hand, pyroptosis inhibits the occurrence and development of tumors; on the other hand, as a type of proinflammatory death, pyroptosis can form a suitable microenvironment for tumor cell growth and thus promote tumor growth(43). Currently, the role of pyrolysis in the development of BLCA has not been reported.

Here, we discovered that the expressions of NLRP3, NLRC4 and AIM2, the key components in inflammasomes, have been significantly increased in the BLCA cells transfected with si-LATS1. And pro-caspase 1 and GDSMD has also been cleaved into their active forms in the si-LATS1 cells, accompanied by the maturation of proIL-1 β and proIL-18. And the overexpression of LATS1 also decreased the protein levels of NLRP3, NLRC4 and AIM2. All these results demonstrated that si-LATS1 induced the pyroptosis via the activation of NLRP3, NLRC4 and AIM2 inflammasomes, finally inhibiting the proliferation of BLCA cells. Our findings also verified that cell pyroptosis blocked the tumor growth of BLCA cells, supporting those results in HCC (39), acute myeloid leukemia(44), and gastric cancer(45, 46).

Besides the direct killing effects, pyroptosis also alters the TME by the secretion of cell contents and inflammatory cytokines, thus deciding the fate of tumor cells. The latest study showed that even if the pyroptosis was only induced in a tiny minority of tumor cells, the pyroptosis-induced inflammation was enough to trigger robust anti-tumor immunity in TME (47). To elucidate possible the effects of si-LATS1 induced pyroptosis on the polarization of monocytes, we cocultured the THP-1 monocytes with the BLCA

cells transfected with/without LATS1 siRNAs for 48h. FACS analysis showed that knockdown of LATS1 in BLCA cells caused an increased frequency of CD11b⁺CD68⁺CD86⁺ in THP-1 cells, indicating that the si-LATS1 induced pyroptosis contributes to M1-like phenotype polarization of THP-1 cells. This finding suggested that si-LATS1 induced pyroptosis in BLCA cells could be a potential target for converting the noninflamed TME into an inflamed one, eliciting the antitumor immune responses. So, these suggest that re-polarization of TAMs from M2 to M1 anticancer phenotype by LATS1-induced pyroptosis will be providing another promising strategy for BLCA treatment in the future.

Conclusions

In summary, the results of the present study proved that knockdown of LATS1 inhibits the proliferation of BLCA cells, which further depends on the regulation of pyroptosis induced by the activation of NLRP3, NLRC4 and AIM2 inflammasomes via TNF- α /NF- κ B pathway. The results of this study not only revealed the molecular mechanisms by which LATS1 regulates the formation of inflammasomes but also proved that LATS1 is a therapeutic target for directly killing BLCA cells via pyroptosis, meantime, providing a novel strategy for BLCA immunotherapy by targeting TAMs of the TME.

Abbreviations

BLCA: bladder cancer;LATS1: large tumor suppressor 1; HCC: hepatocellular carcinoma; PPR: pattern recognition receptor; GSDMD: gasdermin D; NLR: nucleotide-binding and oligomerization domain-like receptors; TLR: Toll-like receptor, AIM:absent in melanoma;Yap1: Yes-associated protein 1; NLRP1: NLR Family Pyrin Domain Containing 1; NLRC4: NLR Family CARD Domain Containing 4; TME: tumor micro-environment;PFS: progression free survival; OS: overall survival; siNC: non-targeting control siRNA; FCM: Flow Cytometry;TAM: tumor-associated macrophage

Declarations

Acknowledgements

Not applicable.

Authors' contributions

SG and MC performed the experiments; XJ cultured the cells; YYperformed the experiments on the Nude mice; CL carried out the Western Blot analysis; YW performed the IF analysis; NLand BY performed the bioinformatic analysis; XMand ZY performed the statistical analysis of experimental data; XS performed the FACS analysis; BW conceived the study and participated in its design. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval

All animal protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the China Medical University Animal Care and Use Committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

LATS1 predicts poor clinical outcomes and knockdown of LATS1 inhibits the proliferation of BLCA cells in vitro and in vivo. A. Kaplan-Meier analysis to assess the effect of the mRNA expression of LATS1 on survival in BLCA cancer; B. Multivariate COX analysis to evaluate the relationship of LATS1 and other clinical and pathologic factors on overall survival (OS); C. Multivariate COX analysis to evaluate the relationship of LATS1 and other clinical and pathologic factors on progression-free survival (PFS); D. effects of LATS1 knockdown on the cell viabilities of BIU-87 and 5637 cells; E. Effects of LATS1 knockdown on the colony formation capacity of BIU-87 and 5637 cells; F. Effects of LATS1 knockdown on the growth of tumor xenografts in nude mice. Compared with NC or Control group, * means $P < 0.05$, ** means $P < 0.01$ and *** means $P < 0.001$.

Figure 2

Gene enrichment analysis of LATS1 knockdown in BLCA cell line BIU-87. A. Analysis of differentially expressed genes (DEGs); B. Gene Ontology (GO) analysis; C. Analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway; D. Hallmark Gene Set Enrichment Analysis; E. Analysis of Reactome pathways

Figure 3

LATS1 knockdown promotes pro-inflammatory cytokines release from BLCA cells via activating TNF- α /NF- κ B signaling A. Enrichment plot for the Hallmark TNFA_SIGNALING_VIA_NFKB; B. Effects of LATS1 knockdown on the mRNA expressions of TNF- α , IL-1 β and IL-18 in the BLCA cells; C. Western Blot analysis on the protein expressions of phospho-NF- κ B/RelA, total NF- κ B, membrane-bound TNF- α (mTNF- α) and secreted TNF- α (sTNF- α); D. Localization of the NF- κ B/RelA detected by Immunofluorescence Assay.

Figure 4

LATS1 knockdown promotes the formation of inflammasomes and induces cellular pyroptosis A. DNA ladder assay for detecting DNA fragmentation; B. LDH release assay to assess the level of plasma membrane damage in BLCA cells; C. Enrichment plot for the REACTOME_INFLAMMASOMES pathway; D. Hub genes prediction for the INFLAMMASOMES by PPI networks analysis; E. Caspase 1 activity determination in the BLCA cells; F. Western Blot analysis on the protein expressions of NLRP3, NLRC4, AIM2, GSDMD, Cleaved GSDMD, Caspase 1, Cleaved Caspase 1, IL-1 β and IL-18; G. Effect of caspase inhibitor (Z-vad-fmk) on BLCA cell viability.

Figure 5

LATS1 overexpression inhibits TNF- α /NF- κ B signaling and the inflammasome pathways A. The expression profiling of LATS1 in BLCA cells transfected with human LATS1 plasmid at mRNA and protein levels; B. the effect of LATS1 overexpression on the cell viability; C. the effect of LATS1 overexpression on the protein expressions of phospho-NF κ B/RelA and TNF- α ; Western Blot analysis on the protein expressions of NLRP3, NLRC4, AIM2, GSDMD, Cleaved GSDMD, Caspase 1 and cleaved caspase 1 in the cells of LATS1 overexpression.

Figure 6

LATS1 knockdown in BLCA cells promotes M1 polarization of THP1 derived macrophages in vitro A. The profiles of the tumor infiltrating immune cells and prognostic values associated with the microenvironment of BLCA; B. The expression of CD86 and CD163 in the CD11b⁺ and CD68⁺ cell subsets cultured in the conditioned medium for 3 days; C. The percentage of CD11b⁺ CD68⁺CD86⁺ and CD11b⁺ CD68⁺ CD163⁺ cells cultured in the conditioned medium; D. The expression of CD86 and CD163 in the CD11b⁺ and CD68⁺ cell subsets co-cultured with the BLCA cells for 2 days; E. The percentage of CD11b⁺ CD68⁺CD86⁺ and CD11b⁺ CD68⁺ CD163⁺ cells co-cultured with the BLCA cells.

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