Acetylshikonin Suppresses Diffuse Large B-Cell Lymphoma Cell Growth By Targeting The TOPK Signaling Pathway

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Research

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Abstract

**Background:** Diffuse large B-cell lymphoma (DLBCL) is one of the most common causes of cancer death worldwide, and responds badly to the existing treatment. Thus, identifying the novel therapeutic targets of DLBCL are urgent.

**Methods and results:** In this study, we found that the T-lymphokine-activated killer cell-originated protein kinase (TOPK) was highly expressed in DLBCL cells and tissues. The TOPK expression were analyzed by bioinformatics analysis, immunohistochemistry (IHC) and western blot analysis. TOPK knockdown inhibited cell growth and induced apoptosis of DLBCL cells with MTS and flow cytometry. Further experiments demonstrated that acetylshikonin, the targeted compound of TOPK, could attenuate the cell growth and aggravate the cell apoptosis through TOPK/extra cellular signal-regulated kinase (ERK)-1/2 signaling using MTS, flow cytometry and western blot analysis. In addition, we demonstrated that TOPK overexpression significantly reduced the acetylshikonin effect on cell proliferation and apoptosis in U2932 and OCI-LY8 cells using MTS, flow cytometry and western blot analysis.

**Conclusions:** Taken together, the present study suggests that the targeted inhibition of TOPK by acetylshikonin may be a promising approach to the treatment of DLBCL.

Background

Diffuse large B cell lymphoma (DLBCL) is the most common type of pathology in non-Hodgkin lymphoma (NHL), representing about 30% of all diagnosed NHL cases, adding rituximab to cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) chemotherapy has been the standard treatment which significantly improved the survival rate of DLBCL patients\[^1\]–\[^3\]. However, there are still about 40% of patients with treatment failure, because of the biologic heterogeneity\[^4\],\[^5\]. Thus, Investigating novel biomarkers are important for the treatment of DLBCL patients.

The lymphokine-activated killer T (T-LAK)-cell-originated protein kinase (TOPK) is a member of the serine-threonine kinases of the mitogen-activated protein kinase (MAPKK) family and is highly expressed in many cancers, including adult T-cell leukemia/lymphoma (ATLL) and B cell lymphoma\[^6\]–\[^10\]. TOPK promotes cancer cell proliferation by its phosphorylation of ERKs\[^11\],\[^12\]. The knockdown of TOPK will activate cysteine proteases (caspase − 3 and caspase − 7) and finally kill the cancer cells\[^13\],\[^14\]. In primary central nervous system lymphoma (PCNSL), TOPK can be a potent novel biomarker, and its overexpression is associated with poor prognosis of PCNSL\[^15\]. However, the underlying role of TOPK in DLBCL remains unclear.

Studies have shown that the inhibition of TOPK could be the target in cancer chemoprevention and treatment\[^16\]. There have been some studies to report the function of TOPK inhibitors, such as OTS964, which could inhibit glioma stem cells survival, but the surviving glioma stem cells start to regrow at last\[^17\]. 3-DSC is found from the Caesalpinia sappan L. plant\[^18\], which binds to the ATP binding pocket of TOPK, could inhibit colon cancer growth by targeting the TOPK signaling pathway in vitro\[^14\].
Moreover, acetylshikonin, another TOPK inhibitor, reduces colorectal cancer cells proliferation and decreases the volume of patient-derived xenograft (PDX) tumors in mice[13]. These studies suggest that TOPK may be a promising target for anticancer therapy.

In this study, we report the crucial role of TOPK in DLBCL tumorigenesis. The knockdown of TOPK attenuated malignancy phenotypes of DLBCL cells, including cell proliferation and apoptosis induction. Furthermore, we demonstrate that acetylshikonin inhibits cell proliferation and induces cell apoptosis by through TOPK signaling pathway. These results suggest that TOPK may be a promising molecular target in DLBCL treatment.

Materials And Methods

Reagents

3-DSC (cat: JOT-10796) was purchased from Chengdu Pufei De Biotech Co., Ltd (Chengdu, China), acetylshikonin (cat: YRY134) was purchased from Chengdu Yirui Biotech Co., Ltd (Chengdu, China), Antibodies to detect total TOPK (cat: 4942), phosphorylated TOPK (cat: 4941), total ERKs (cat: 9102), phosphorylated ERKs (cat: 4370), caspase-3 (cat: 9662), caspase-7 (cat: 12827), cleaved caspase-3 (cat: 9664), cleaved caspase-7 (cat: 9491) and actin (cat: 3700) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

The human DLBCL cell lines (U2932, SUDHL-6 and OCI-Ly8) and normal B-cell line (WIL2S) were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS). WIL2S cells were maintained in Iscove's modified medium with 10% human serum (NABI Biopharmaceuticals, Boca Raton, FL, USA) and 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA). All cells were cultured with penicillin/streptomycin antibiotic mixture, penicillin (100 U.ml-1), streptomycin (100 µgml-1) at 37 °C in 5% CO₂.

Bioinformatics analysis

Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/) software was used to analyze the expression of TOPK in DLBCL. First, enter TOPK and select boxplots. Then, select DLBC under datasets selection (cancer name) and click plot button.

Immunohistochemical (IHC) Staining

DLBCL tissue array (cat: LY800b) was purchase from Alenabio biotechnology Co., Ltd, containing 62 cases of DLBCL samples and 20 cases of normal control tissue samples. IHC staining was detected followed by the previous protocol[19], using TOPK antibody (1:200).

Western blot analysis
The protein samples were separated with SDS-PAGE electrophoresis, then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Blocking with defatted milk, incubating with the first antibodies (1:1000), including TOPK, pTOPK, ERK, pERK, caspase 3, caspase 7, cleaved caspase 3, cleaved caspase 7 and actin, then incubating with the secondary antibodies. Images were captured with the Tanon-5200.

**Lentiviral infection**

Lentiviruses carrying TOPK-cDNA (accession number NM_018492.4), shTOPK.1 (ATTAGTGCATACAGAGAAGAGTT) and shTOPK.2 (GTCTGTGTCTTGCTATGGAAT) were from GenePharma Co. (Shanghai, China). For lentiviral infection, cells were infected with lentivirus (MOI=100), containing polybrene (5 μg/ml) for 1-3 days.

**Cell proliferation assay**

The cells were seeded (2 X 10^3 cells per well) in 96-well plates. After incubation for 24, 48, or 72h, cell proliferation was measured by MTS assay kit.

**Apoptosis Detection**

Flow cytometry was performed to observe the cell apoptosis with annexin V-FITC apoptosis detection kit (beyotime, C1062S). Cells were collected and washed with cold PBS. Added FITC annexin V and PI, incubated for 15 min at room temperature in the dark. Then analyzed by flow cytometry within 1 h.

**Statistical analysis**

Results were presented as the means ± standard deviation (SD) of 3 independent experiments, each dosage or treatment was tested in triplicate. Statistical analyses were performed with GraphPad Prism 6. Student’s t test were used to analyze the significant differences when the p value was less than 0.05.

**Results**

**TOPK is expressed at high levels in DLBCL**

To explore the role of TOPK in DLBCL, we analyzed TOPK expression in DLBCL cancer specimens and normal controls in the dataset of GEPIA. As shown in Fig. 1a, TOPK expression was obviously upregulated in DLBCL cancer specimens than normal samples. This result was further confirmed by testing TOPK expression using IHC in the DLBCL tumor microarray that included 62 cancer tissues and 20 control tissues (Fig. 1b). Similarly, TOPK expression was upregulated in DLBCL cell lines (U2932, OCI-LY8, SUDHL-6) compared with normal B-cell line (WIL2S) in protein levels by western blot (Fig. 1c). These results suggest that TOPK upregulation may promote DLBCL progression.

**Silencing TOPK reduces the tumorigenic properties of DLBCL**
To gain the insight into the potential role of TOPK as an oncogene whose overexpression was associated with DLBCL tumorigenesis, we generated the TOPK knockdown cells infected with lentivirous, the knockdown efficiency was verified in OCI-LY8 cells by western blot (Fig. 2a). The cell proliferation assays were performed by MTS to evaluate the effect of TOPK knockdown. Results showed that TOPK knockdown inhibited proliferation in U2932 and OCI-LY8 cells (Fig. 2b-c).

Studies have shown that TOPK knockdown could induced cell apoptosis[20, 21]. Here, the annexin V-FITC/PI double-staining assay results showed that TOPK knockdown increased apoptosis in U2932 and OCI-LY8 cells (Fig. 2d). We also observed that the cleavage of caspase-3 and caspase-7 were obviously enhanced by western blot (Fig. 2e, Figure. S1A). Overall, our data show that TOPK could modulate the cell proliferation and apoptosis in DLBCL.

**Acetylshikonin suppresses DLBCL cell growth by targeting TOPK signaling**

Previously studies have shown that acetylshikonin and 3-DSC could suppress colon cancer cell growth[13, 14]. To study the effects of acetylshikonin and 3-DSC on DLBCL cell growth, we first assessed the effect of acetylshikonin and 3-DSC on the proliferation of normal B-cell line. Incubation of WIL2S cells with acetylshikonin 3-DSC (0, 5, 10, 20μM) for 24, 48 or 72h did not show cytotoxicity up to 20μM at 72h (Fig. 3a), crucially, acetylshikonin inhibited the cell growth of DLBCL cell lines (U2932 and OCI-LY8) in a time- and dose-dependent manner (Fig. 3c-d). However, 3-DSC have shown cytotoxicity of WIL2S and U2932 cells to 10μM at 24h (Fig. 3b, 3e), but 3-DSC did not show cytotoxicity in OCI-LY8 for 10μM at 24h (Fig. 3f), so we estimated that acetylshikonin was more sensitive in DLBCL. Next, the role of acetylshikonin on cell apoptosis were examined. Treatment with 10μM acetylshikonin at 24h induced cell apoptosis in U2932 and OCI-LY8 cells (Fig. 4a). In addition, we determined whether acetylshikonin also affected TOPK signaling pathway, we determined the effects of acetylshikonin on the expression of phosphorylated TOPK (pTOPK), phosphorylated ERK (pERK). Treatment of U2932 and OCI-LY8 cells with 10μM acetylshikonin at 24h decreased the levels of pTOPK and pERK, and the total levels of TOPK, ERK were unchanged as compared to DMSO controls (Fig. 4b, Figure. S1B).

**TOPK overexpression attenuates the effect of acetylshikonin.**

To further determine whether TOPK could mediate the acetylshikonin induced cell growth inhibition, we infected TOPK cDNA and treated with acetylshikonin in U2932 and OCI-LY8 cells, followed by analysis of cell proliferation and apoptosis. The TOPK overexpression efficiency was shown in U2932 and OCI-LY8 cells by western blot (Fig. 5d, Figure. S1C). Results showed that the inhibition of cell proliferation treated by acetylshikonin was partly rescued upon overexpression of TOPK (Fig. 5a-b) and the induction of cell apoptosis treated by acetylshikonin was partly inhibited by the overexpression of TOPK (Fig. 5c). The cleavage of caspase-3 and caspase-7 showed coincident results (Fig. 5d, Figure. S1C).

**Discussion**
Despite the improvements in the treatments of DLBCL, it is still associated with a high mortality rate[22], thus, exploring the novel factors that affects DLBCL progression is significant for targeted therapy. This study reports that TOPK has a key role in DLBCL tumorigenic properties. Acetylshikonin suppresses DLBCL cell growth by targeting the TOPK signaling pathway and may be a potential treatment strategy for DLBCL.

TOPK is an active form of MEK1 which can phosphorylate ERK1/2[12]. Studies have shown the correlation between TOPK expression and poor prognosis in several cancers, such as breast cancer[23], lung cancer[24], leukemia[25], burkitt's lymphoma[9]. The overexpression expression of TOPK is associated with the tumorigenesis[26]. Although TOPK plays important roles in multiple cancer development, its function in DLBCL remains unclear. This work evaluates the function of TOPK in DLBCL.

Our results indicated that TOPK showed high levels in DLBCL, both in DLBCL cell lines and tissues (Fig. 1). For the functional study, we demonstrated that TOPK knockdown dramatically inhibited cell proliferation and induced cell apoptosis (Fig. 2, Figure. S1A) in U2932 and OCI-LY8 cells. These results suggest that TOPK knockdown obviously attenuates malignancy phenotypes of DLBCL cells, and thus, searching the appropriate inhibitors which could target TOPK is pregnant for DLBCL treatment.

An ideal anti-cancer compound should lack cytotoxicity against normal cells. In this study, we choose two native compound (acetylshikonin and 3-DSC) which were extracted from plants and showed anti-cancer effects by target TOPK[13, 14]. Our data indicated that acetylshikonin showed no cytotoxicity up to 20 µM at 72 h (Fig. 3a), but 3-DSC showed cytotoxicity of normal B-cells (WIL2S) up to 10 µM at 24 h (Fig. 3b). So we estimated that acetylshikonin was more suitable targeted compound for DLBCL. The observed anti-cancer effects of acetylshikonin was reported in human pancreatic panc-1 cancer cells and colon cancer cells[13, 27]. In our studies, we demonstrated that acetylshikonin inhibited cell proliferation (Fig. 3c-d) and induced cell apoptosis (Fig. 4a) in U2932 and OCI-LY8 cells. Papers have shown that TOPK is a major target of acetylshikonin to inhibit proliferation and induce apoptosis, several signalling molecules, including ERK, is involved in TOPK signalling and acetylshikonin decreased the phosphorylation of TOPK, ERK[13]. Our results confirm that acetylshikonin could decrease the phosphorylation of TOPK, ERK in U2932 and OCI-LY8 cells (Fig. 4b, Figure. S1B). In addition, TOPK overexpression abolished the cell proliferation inhibition, cell apoptosis induction and the increase of cleaved caspase-3 and caspase-7 that treatment with acetylshikonin, which further confirmed that TOPK could involved the acetylshikonin induced cell growth inhibition (Fig. 5, Figure. S1C).

**Conclusions**

In conclusion, the present study suggests that TOPK may mediate acetylshikonin induced cell growth inhibition in U2932 and OCI-LY8 cells. Acetylshikonin or its derivatives can be considered for the new target-based therapy for DLBCL and TOPK may be the candidate molecular target for DLBCL treatment.

**Abbreviations**
DLBCL: Diffuse large B-cell lymphoma; TOPK: T-lymphokine-activated killer cell-originated protein kinase; T-LAK: The lymphokine-activated killer T; IHC: immunohistochemistry; ERK: extra cellular signal-regulated kinase; NHL: non-Hodgkin lymphoma

Declarations

Acknowledgments

Not applicable

Author Contributions

J.W.L., J.K.C. and R.G. conceived and designed the experiments. J.K.C., R.G., Y.J.W., Y.S., X.W.S., C.Z. and H.W.L. performed the experiments. J.W.L., J.K.C. and R.G. analyzed the data. J.W.L. and C.Z. contributed the reagents. J.W.L. and J.K.C. wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and additional files.

Ethics approval and consent to participate

Not applicable

Consent for publication

Consent to publish has been obtained from the participants.

Conflict of interest

The authors declare that they have no conflicts of interest.

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**Figures**
Figure 1

TOPK is highly expressed in human DLBCL. (a) TOPK expression levels in the Cancer Genome Atlas dataset in DLBCL. (b) IHC staining on DLBCL tissues array, control (20 cases), cancer (62 cases). (c) TOPK protein levels were measured in DLBCL cell lines and normal B-cell line using western blot. The data represent the mean ± S.D. for three individual experiments (*p < 0.05, **p < 0.01, ***p < 0.001, n = 3).
TOPK knockdown inhibits cell growth and induces cell apoptosis in U2932 and OCI-LY8 cell lines. (a) OCI-LY8 cells were infected with shTOPK.1, shTOPK.2 or shNT, the TOPK knockdown efficiency was detected by western blot. (b and c) U2932 and OCI-LY8 cells were infected with shTOPK.1, shTOPK.2 or shNT, the cell proliferation was detected at 24, 48, and 72h with the MTS assay. (d) U2932 and OCI-LY8 cells were infected with shTOPK.1, shTOPK.2 or shNT, the cell apoptosis was detected staining with annexin V and PI.
PI. (e) U2932 cells were infected with shTOPK.1, shTOPK.2 or shNT, and the cleaved caspase 3 and caspase 7 protein levels were assessed with western blot. The data are the mean ± S.D. for three individual experiments (**p < 0.01, ***p < 0.001, n = 3).

Figure 4

Acetylshikonin induces DLBCL cells apoptosis and reduces the expression of proteins involved in TOPK signaling. (a) U2932 and OCI-LY8 cells were treated with 10µM acetylshikonin for 24h, the cell apoptosis was detected staining with annexin V and PI. (b) U2932 cells were treated with 10µM acetylshikonin for
24h, the protein levels of pTOPK, total TOPK, pERK, total ERK were detected by western blot. (**p < 0.001, n = 3).

Figure 5

TOPK overexpression attenuates the effect of acetylshikonin. (a-b) U2932 and OCI-LY8 cells were infected with a lentivirus carrying TOPK cDNA or treated with 10µM acetylshikonin, cell growth was determined at 72h using the MTS assay. (c) U2932 and OCI-LY8 cells were infected with a lentivirus carrying TOPK
cDNA or treated with 10µM acetylshikonin, the cell apoptosis was detected staining with annexin V and PI. (d) U2932 cells were infected with a lentivirus carrying TOPK cDNA or treated with 10µM acetylshikonin, the cleaved caspase 3 and caspase 7 protein levels were assessed with western blot. (*p < 0.05, **p < 0.01, ***p < 0.001, n = 3).

**Supplementary Files**

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