Cirsiliol targets tyrosine kinase 2 to inhibit esophageal squamous cell carcinoma growth in vitro and in vivo

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Abstract

Background: Esophageal squamous cell carcinoma (ESCC) is an aggressive and lethal cancer with a low 5 year survival rate. Identification of new therapeutic targets and its inhibitors remain essential for ESCC prevention and treatment.

Methods: TYK2 protein levels were checked by immunohistochemistry. The function of TYK2 in cell proliferation was investigated by MTT [(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and anchorage-independent cell growth. Computer docking, pull-down assay, surface plasmon resonance, and kinase assay were used to confirm the binding and inhibition of TYK2 by cirsiliol. Cell proliferation, western blot and patient-derived xenograft tumor model were used to determine the inhibitory effects and mechanism of cirsiliol in ESCC.

Results: TYK2 was overexpressed and served as an oncogene in ESCC. Cirsiliol could bind with TYK2 and inhibit its activity, thereby decreasing dimer formation and nucleus localization of signal transducer and activator of transcription 3 (STAT3). Cirsiliol could inhibit ESCC growth in vitro and in vivo.

Conclusions: TYK2 is a potential target in ESCC, and cirsiliol could inhibit ESCC by suppression of TYK2.

Keywords: Cirsiliol, TYK2, Esophageal squamous cell carcinoma, Surface plasmon resonance, Patient-derived xenograft

Background

Esophageal cancer is a malignant tumor worldwide, with the 7th and 6th highest incidence and mortality rates respectively [1]. Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) are two major histological types of esophageal cancer. ESCC occurred equally in the middle and lower esophagus while EAC occurred at approximately three-quarters of the distal esophagus respectively [2]. ESCC comprised over 90% of all esophageal cancer cases. Although chemotherapy, surgery, and radiotherapy are considered the most effective clinical treatments, the five-year survival rate of esophageal cancer is still less than 20% [3, 4]. Thus, the identification of effective molecular targets and their inhibitors are highly interested [5–8].

Tyrosine kinase 2 (TYK2) is a Janus kinase. It is extensively expressed in mammals. TYK2 is activated after stimulating by various cytokines [9]. After binding to its ligand, TYK2 is phosphorylated and activated at Tyr1054 or/and Tyr1055 [10]. After TYK2 activation, transcription factors such as signal transducer and activator of transcription 3 (STAT3) and STAT3, are dimerized and activated, thereby promoting the transcription
of related genes and causing abnormal tumor cell proliferation and differentiation [11, 12].

Nature compounds which were found in vegetables, fruits, as well as medicinal plants which were considered as potential inhibitor resources for cancer [13, 14]. Evidence has shown that many flavonoids which are found in plants exhibit anti-cancer effects through anti-proliferation, carcinogen inactivation, induction of apoptosis, and cell cycle arrest, etc. [15, 16]. Cirsiliol, a flavonoid found in many plants such as Artemisia, Salvia, and Leonotis nepetifolia, has anti-inflammatory, antioxidant, antibacterial, sedative, and hypnotic effects [17–20]. It has recently been shown to exert cancer inhibition effects against non-small cell lung cancer and other cancers [21, 22]. However, the potential anti-cancer activities and the underlying mechanisms of cirsiliol in ESCC have not been fully elucidated.

In this study, we found that TYK2 served as an oncogene in ESCC and its protein level was negatively associated with ESCC patients’ overall survival rates. Cirsiliol suppressed ESCC growth through targeting TYK2/STAT3 signaling pathways.

Materials and methods

Materials

ESCC tissue array (Cat#HEsoS180Su05) were purchased from OUTDO Biotech (Shanghai, China). Active TYK2 (Cat#T21-11G) and inactive STAT3 protein (Cat#S545-54BH) were bought from Signal Chem company (Richmond, BC, Canada). Cirsiliol (Cat#FD66719) was purchased from Carbosynth (Suzhou, China). Antibodies to detect p-STAT3 (Cat#9145), STAT3 (Cat#9139), Bcl-2 (Cat#15071), GAPDH (Cat#2118), myeloid cell leukemia-1 (Mcl-1, Cat#94296), PARP (Cat#9542) and β-actin (Cat#3700) were all purchased from CST antibody company (Beverly, MA, USA). TYK2 (Cat#ab223733) and Ki67 (Cat#ab16667) were purchased from abcam (Cambridge, MA, USA). NativePAGE™ Bis-Tris Gel (Cat#BN1002BOX) and NativePAGE™ Sample Buffer (Cat#BN2003) were purchased from ThermoFisher (Waltham, MA, USA). Cytoplasmic and Nuclear Protein Extraction Kit was bought from Beyotime (Cat#P0028, Shanghai, China). BCA kit was purchased from Solarbio (Cat#PC0020, Beijing, China).

Cell culture and proliferation assay

Human ESCC cell lines KYSE30, KYSE70, KYSE140, KYSE410, KYSE450 and KYSE510 were bought from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and the cell lines were mycoplasma-free and authenticated by STR analysis. The cancer cells were cultured in RPMI-1640 media with 10% fetal bovine serum. The immortalized normal human esophageal epithelial cell-SHEE was donated by Professor Enmin Li. Cells were seeded at 1.5–6 × 10^3 cells/well in 96-well plates, incubated no less than 12 h, and then added with a series concentrations of cirsiliol or vehicle. After incubation for an additional 24, 48, or 72 h, the proliferation of cell was detected using MTT (0.5 mg/mL) reagent.

Anchorage-independent cell growth and cell cycle analysis

After preparing the 0.5% base layer agar with vehicle, 5, 10 or 20 μM cirsiliol, KYSE140 and KYSE450 cells (8 × 10^3 cells/well) were seeded in a 0.3% top layer agar with vehicle, 5, 10 or 20 μM cirsiliol. The cells were cultured in an incubator for an additional 2 weeks. The colonies were photographed by a microscope and counted by Image-Pro Plus v6.0. To analyzing cell cycle, ESCC cells were cultured in 60 mm dishes and treated with 0, 5, 10 or 20 μM cirsiliol for 48 h. After fixation in 70% pre-cooled ethanol for 24 h and incubation with propidium iodide, the cells were detected using flow cytometer (BD Biosciences, San Jose, CA).

Cell migration

KYSE140 and KYSE450 cells (2 × 10^5 cells) resuspended in 200 μl RPMI-1640 media were seeded in the upper chamber of transwell plate insert (Cat#3422, Corning, USA). The down chamber part was added with 600 μl 10% FBS RPMI-1640 media. After culture for 24 h, the inserts were washed twice with PBS. After fixing with methanol, cells were stained with 500 μl 0.1% crystal violet, which, after imaging with an inverted microscope, were eluted with 33% acetic acid. The migration cells were quantified by measuring the absorbance at 570 nm.

Plasmid mutation and TYK2 overexpression in 293 T cell

The TYK2 pcDNA3.1–3 × Flag-C plasmid was purchased from Youbao Biotechnology Company (Changsha, China). The TYK2 mutation information showed as follow: 1A (Val 981 to Ala); 1A (Pro 982 to Ala); 2A (double amino mutation to Ala). The primers were showed in Table 1. The mutation was performed according the fast mutagenesis system kit (Cat#FM111, Trans, China). After transfected the wild type and mutant plasmid into 293 T cell for 48 h, the cells were harvested and prepared lysates for the pull down assay.

Lentiviral infection and transfection

The viral and packaging vectors (pMD2.G, pLKO.1-mock, psPAX2, and shTYK2) were co-transfected with Simple-Fect Transfection Reagent (Signaling Dawn Biotech, Wuhan, China) into 293 T cells. The shRNA sequences of TYK2 were shown as bellow: shTYK2#2-F: 5′CCGGGAAGATCCACCACITTAAGAAATCCTCGAGA TTCTTTAAGTGGTGGATCTCTTTTTG3′; R: 5′AATT
Circsiliol were added into the flow system to test the binding was equilibrated with PBS. A concentration series of cirsiolid across ligand flow channels. Then, the chip immobilized on a CM5 chip (Cat#BR-1005-30, GE Healthcare) across ligand flow channels. Then, the chip immobilized on a CM5 chip (Cat#BR-1005-30, GE Healthcare, MA, USA). TYK2 (20 μg/mL) was covalently activated Sepharose 4B (Cat#717086-00AF, GE Healthcare, MA) was covalently activated Sepharose 4B (Cat#717086-00AF, GE Healthcare, MA) specification [23]. Cell proteins (500 μg/mL) were rotated with 50% DMSO, 30 μg/mL puromycin and then heated for 10 min at 95°C. The proteins were detected using western blotting.

**In vitro kinase assay**

Inactive STAT3 proteins (1 μg) were incubated with 200 ng active TYK2 (442-end) for in vitro kinase assays. The reactions were conducted in kinase buffer II (Cat#K02–09, SignalChem, Canada) containing 200 μM adenosine triphosphate (ATP). Different concentrations of circsiliol were added (final concentration 0, 5, 10, 20 μM), and the samples were maintained at 30°C for 30 min. The kinase reactions were terminated using 5 μL × 6 loading buffer and heating for 10 min at 95°C. The proteins were detected using western blotting.

**Cell immunofluorescence assay**

First, we placed sterile glass coverslips into 24 well plates. Next, 2 × 10⁴ KYSE140 and KYSE450 cells were seeded into the wells. After attachment to the surface of the coverslips, cells were cultured with 0, 5, 10 or 20 μM circsiliol for 24 h. After washing in PBS, the cells were fixed using 4% paraformaldehyde for 30 min. Following another 3 times washes in PBS, special primary antibody were added into the cells at 4°C for 15 h and then incubated in the second fluorescent antibodies with diisopropylamine (DIPA) at room temperature for 2 h. After washed by PBS, the coverslips were transferred to glass slides with an anti-fluorescence quenching agent. The results were analyzed using Image-Pro Plus v6.0.

**Native gel electrophoresis**

The Native gel electrophoresis assay was conducted based on the Gel System protocol of the NativePAGE™ Novex® Bis-Tris. Cells were incubated for 30 min on ice in 1× sample buffer (50 mM Bis-Tris, 0.1% n-dodecyl-β-D-maltoside, 6 N HCl, 50 mM NaCl, 0.001% Ponceau S, 10% Glycerol, pH 7.2). After 30 min 20,000 g centrifugation, the supernatant was collected, and the concentration was measured using BCA kit. NativePAGE™ gel (4–16%) electrophoresis was performed at 200 V constant voltages. After running for 30 min the Cathode Buffer-Dark Blue was changed with Light Blue and electrophoresis was resumed for another 80 min. The gel was then transferred in 1× NuPAGE® Transfer Buffer at 100 mA constant for 1 h and the membrane was fixed with 20 mL of 8% acetic acid for 15 min and immunodetection was directly performed.

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**Table 1** The site mutant primers of TYK2

| Name | Forward primer (5’ to 3’) | Revers primer (5’ to 3’) |
|------|--------------------------|------------------------|
| 1A   | CTTGCACTGGTCGCTCGAGT     | GCCGTATTCCATGACCACTGCA |
| 1A   | GGTCCAGATTCGTCGATAAT    | GCCGTATTCCATGACCACTGCA |
| 2A   | GGTCCAGATTCGTCGATAAT    | GCCGTATTCCATGACCACTGCA |

1A (Val 981 to Ala); 1A (Pro 982 to Ala); 2A (double mutant to Ala)
Nuclear and cytoplasmic protein extraction
We utilized the Nuclear and Cytoplasmic Protein Extraction kit to analyze the STAT3 nuclear translocation. After harvesting ESCC cells treated with cirsiliol for 24 h, we extracted nuclear proteins according to the instructions of manufacturer. The STAT3 protein levels in nuclear and cytoplasmic extracts were subsequently quantified using the BCA kit and detected via immunodetection.

Western blotting
Cells were suspended in cell lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, and 1 mM phenylmethylsulphonyl fluoride, pH 8.0) and incubated on ice for 30 min. After centrifugation (12,000 g, 10 min), the supernatant was collected as whole cell extracts. The concentration of the extracts was measured using a BCA kit. Via gel electrophoresis and transfer, the extracts were transferred to polyvinylidene fluoride membranes. After blocking with 5% non-fat milk in 1× PBS, the membranes were incubated with TYK2, p-STAT3, STAT3, c-myc, Bcl-2, Mcl-1 or GAPDH antibodies. These proteins on the membrane were detected using a chemiluminescence reagent.

CDX mouse model
KYSE450 cells were utilized to prepare the CDX mouse model. First, the cells were infected with the mock, shTYK2#2, and shTYK2#3 packaged virus. After selection by puromycin, cells were expanded in culture. After acquiring sufficient numbers of cells, 1 × 10^7 KYSE450 cells were harvested and seeded in the right flank of each mouse. After 3 weeks, the volume of the tumors was measured every 2 days. The tumor weight was measured when the tumor volume reached 1 cm^3.

PDX mouse model
Six weeks old female severe combined immunodeficient (SCID) mice were bought from Vital River Labs (Beijing, China) and kept in a 12/12 h light/dark cycle condition (SCID) mice were bought from Vital River Labs (Beijing, China) and kept in a 12/12 h light/dark cycle condition. PDX tumor tissues were removed when the tumor volume reached 1 cm^3, and then the tumors were extracted and euthanized.

Immunohistochemistry (IHC) analysis
Tumor tissues embedded in paraffin were used for IHC staining. After deparaffinization, antigen unmasking, and blocking by 5% goat serum for 40 min at room temperature, the slices were incubated with antibodies against Ki-67, p-STAT3 (Tyr705), and TYK2 at 4 °C for 15 h, and then incubated with the secondary antibody followed by DAB (3, 3′-diaminobenzidine) staining. After counter-staining and dehydration, the slices were mounted on glass coverslips with neutral resin. The slices were photographed (100 × magnification) and analyzed using the Aperio ImageScope software program.

Statistical analysis
In this study, GraphPad Prism8.0 was used to conduct all statistical analysis and quantitative results were showed as mean ± SD. The unpaired Student’s t-test or one-way analysis of variance (ANOVA) was used to compare significant differences. *p < 0.05, **p < 0.01, and ***p < 0.001 were used to show significance for each experiment.

Results
TYK2 is highly expressed in ESCC and negatively associated with patient survival
The protein levels of TYK2 were evaluated by IHC staining in ESCC tissue array (Fig. 1a). The results showed that TYK2 protein levels were higher in tumors than in adjacent tissue (Fig. 1b). TYK2 protein levels were significantly increased in stage 3 and stage 4 compared with adjacent tissues (Fig. 1c). A lower survival rate was also observed in TYK2 protein level highly ESCC patients (Fig. 1d). Then we utilized UALCAN to assess the gene information in the TCGA database [24]. In the UALCAN data base, bioinformatics analysis showed that the TYK2 mRNA level was significantly up-regulated both in EAC and ESCC (Fig. 1f). Furthermore, TYK2 mRNA was also upregulated in other 22 kinds of cancers (Fig. 1e). Similarly, TYK2 mRNA level in UALCAN data base was significantly up-regulated in all ESCC tumors stages (Fig. 1g). The correlation between TYK2 protein level and ESCC tissue array clinic pathologic characteristics are shown in Table 2.

Knocking down of TYK2 suppressed the growth of ESCC
To evaluate the role of TYK2 in ESCC, we checked the protein level of TYK2 in immortalized esophagus cell SHEE and ESCC cell lines. The results showed that TYK2 protein levels in ESCC Cell lines were higher than immortalized esophagus cell (Fig. 2a, up panel). Then highly expressed TYK2 KYSE140 and KYSE450 cell lines were selected for further knockdown assays. Results showed that shTYK2#2 and shTYK2#3 decreased the
Fig. 1 (See legend on next page.)
protein levels of TYK2 notably in both cell lines (Fig. 2a, down panel). After knocking down of TYK2, the growth of KYSE140 and KYSE450 were suppressed (Fig. 2b). Similarly, the anchor independent cell growth was also attenuated in the shTYK2#2 and shTYK2#3 groups (Fig. 2c). Furthermore, the tumor growth of Cell-derived xenograft (CDX) mice model was reduced after TYK2 knockdown (Fig. 2d & e and Supplementary 1a) and the average of tumor weight in the TYK2 knockdown group was lower than the control group in the CDX mice model (Fig. 2f). In addition, after TYK2 knockdown, the cell cycle was arrested at the G2/M phase in both KYSE140 and KYSE450 cell lines (Supplementary 1b). Similarly, cell migration was also decreased following TYK2 knockdown in KYSE140 and KYSE450 (Supplementary 1c).

Then, we overexpressed TYK2 in KYSE30, KYSE410 and KYSE510 cell lines. After transfected with pcDNA3.1-TYK2–3 × Flag or vehicle plasmid for 36 h, cells were seeded for cell proliferation and anchorage-independent cell growth assay. Results showed that the cell proliferation and cell colony formation were increased (Fig. 3a and b). For the related mechanism study, we found that the STAT3 phosphorylation was inhibited after TYK2 knockdown in KYSE140 and KYSE450 cell lines (Fig. 3c). In summary, these data indicated that TYK2 played an oncogenic role in ESCC growth.

**TYK2 is a target of cirsiliol**

Since TYK2 had a positive effect on ESCC proliferation, we attempted to identify an inhibitor of TYK2; hence we used an *in-silico* docking assay to screen compounds from natural compound library. Our results showed that cirsiliol could bind to TYK2. The chemical structure of cirsiliol and cirsiliol-TYK2 docking model are shown in Fig. 4a. The results predicted that cirsiliol achieves binding at PRO982 and VAL981 in the ATP binding pocket (Fig. 4b). Furthermore, pull-down assay showed that Sepharose 4B-coupled-cirsiliol could bind directly to endogenous and recombinant TYK2 protein ex vitro (Fig. 4c). Similarly, SPR assay also showed that the binding affinity between cirsiliol and TYK2 protein increased over time in a concentration dependent manner (Fig. 4d). The determined equilibrium dissociation constant (KD) of cirsiliol was approximately 0.8 μM (Fig. 4e).

We then evaluated whether PRO982 and VAL981 docking sites were important for the binding ability of cirsiliol. After single and double mutation, the amino sites, the cell
lysate were harvested for pull down assay, which showed that the binding ability of cirsiliol was decreased after mutating the docking site to Alanine (Fig. 4f). In addition, kinase activity assay was utilized to detect the inhibitory effects of cirsiliol on TYK2 activity. Results showed that cirsiliol significantly suppressed the activity of TYK2 at concentrations of 10 μM and 20 μM (Fig. 4g & h).

Cirsiliol suppresses ESCC growth
Cell proliferation and anchorage-independent cell growth assays were performed to confirm the anti-cancer effects of cirsiliol. Results showed that cirsiliol significantly suppressed the activity of TYK2 at concentrations of 10 μM and 20 μM (Fig. 4g & h).

Cirsiliol inhibitory effects on ESCC partly depend on TYK2
Our data indicated that cirsiliol could effectively inhibit the proliferation and colony formation of ESCC cells. However, it remains unknown whether the inhibitory
The effect of cirsiliol is dependent on expression of TYK2. Thus, we treated TYK2 knockdown ESCC cells with cirsiliol. After treatment with cirsiliol for 72 h, cell viability was assessed in KYSE140 and KYSE450 cell lines in mock, shTYK2#2 and shTYK2#3 groups to evaluate their sensitivity to cirsiliol. Results indicated that the cell viability of KYSE140 with cirsiliol treatment in the mock group was 13.3, 55.5% lower than those of shTYK2#2 and shTYK2#3 groups, respectively (Fig. 5d upper panel). Similarly, compared with the mock group of KYSE450, the cell viability of shTYK2#2 and shTYK2#3 was increased by 82.5 and 20.2% after cirsiliol treatment, respectively (Fig. 5d lower panel). Moreover, the inhibitory effect of cirsiliol on clone formation was also weakened after TYK2 knockdown (Fig. 5e & f). Then, we rescued the knockdown cells with vehicle, wild type TYK2, and double mutant TYK2. After transfected for 36 h, cell proliferation assay and western blot were conducted. Results showed that after transfected with wild type TYK2, cells sensitivity to cirsiliol was increased when compared with vehicle (Fig. 5g), however, no significant changes were observed in cell proliferation ability and sensitivity in the mutant group. Western blot results showed that after rescuing TYK2 in knockdown cells, wild type and mutant type TYK2 both overexpressed in knockdown cell lines (Fig. 5h).

Cirsiliol inhibits TYK2 downstream signaling

Our previous data indicates that cirsiliol can inhibit the activity of TYK2 in vivo; hence, we investigated the downstream signaling molecule of TYK2. We performed immunofluorescence assays to detect the variation of STAT3 (Tyr705) phosphorylation and total STAT3. Results showed that STAT3 (Tyr705) phosphorylation was inhibited after cirsiliol treatment while the total STAT3 signal did not change (Fig. 6a & b). In addition, we utilized native gel electrophoresis to investigate the change in STAT3 dimerization. Results showed that STAT3...
dimerization was decreased in both KYSE140 and KYSE450 cell lines after cirsiliol treatment (Fig. 6c). The cytoplasmic and nuclear proteins extracting assay was performed to evaluate the STAT3 localization change after cirsiliol treatment. Results showed that fewer STAT3 was present in the cell nucleus than the control group after cirsiliol treatment in both cell lines (Fig. 6d). Furthermore, western blotting was conducted to detect the signaling pathway associated with TYK2. The results showed that cirsiliol inhibited the phosphorylation of STAT3 in both KYSE140 and KYSE450 cells. The expression levels of STAT3-targeting gene, including Mcl-1, c-myc, and cyclin D1, were decreased in a dose-dependent pattern after cirsiliol treatment (Fig. 6e). Thus, the above data supported that cirsiliol inhibited ESCC by blocking the TYK2/STAT3 pathway.

Fig. 4 Cirsiliol binds with TYK2 and inhibits the kinase activity. a Computational docking model between cirsiliol and TYK2. b The detailed binding site of cirsiliol on TYK2. c Upper panel: the binding ability of cirsiliol on endogenic TYK2 in vitro, obtained via pull down assay. Down panel: the binding ability of cirsiliol to recombinant TYK2 protein. d The change of affinity response intensity with the passage of time. -60 to 0 s was set as the time before inject cirsiliol solution; 0–120 s was set as contact time between cirsiliol and TYK2; 120–180 s was set as dissociation time. e The variation of response intensity with the increase of cirsiliol concentration. f Upper panel: cirsiliol binding ability with mutant TYK2. Down panel: the protein level of TYK2 in 293 T cell line. 1A (V981A), 1’A (P982A), 2A (double mutant), WT: wild type. g Kinase assay performed with cirsiliol and TYK2. h p-STAT3 inhibition analyzed by ImageJ in three independent assays (n = 3, *p < 0.05, **p < 0.01). Student's unpaired t-test in (h). KD, dissociation constant
Fig. 5 (See legend on next page.)
Cirsiliol inhibits ESCC patient-derived xenograft (PDX) growth

In order to assess the anti-tumor activity of cirsiliol in vivo, LEG73 and LEG104 cases with high level of TYK2 were selected from the ESCC PDX specimen repository to develop the PDX mouse model (Fig. 7a & b). After administration of cirsiliol via oral gavage, the tumors volumes of high cirsiliol concentration treatment group in LEG73 case were suppressed when compared with the vehicle group (Fig. 7c). Similarly, the tumor volumes of both cirsiliol treatment groups in LEG104 case were also reduced (Fig. 7c). After sacrificing the mice and harvesting tumors, the tumor pictures in both cases were shown in Fig. 7c. The average tumor weights of high cirsiliol concentration group in both cases were obviously lower than vehicle group (Fig. 7d). After analyzing the tumor weights in each group, the average tumor growth inhibition rate of the high concentration treatment group in the two cases were 50.21 and 45.12% compared with the vehicle group, respectively (Fig. 7d). Furthermore, the tumors were stained with Ki-67 and p-STAT3 specific antibody for mechanism study after tissue slice preparation. The representative IHC staining images are shown in Fig. 6e. IHC analysis revealed that cirsiliol inhibited the phosphorylation of STAT3 and reduced Ki-67 protein level significantly after cirsiliol treatment (50 mg/kg) in LEG73 and LEG104 cases (Fig. 7e). Mice’s body weights were not significantly changed between each group after cirsiliol treatment (Supplementary 1e). The acute toxicity test results showed that cirsiliol had no effects on the mice body weight and white blood cell when comparing with vehicle group at the concentration of 50 mg/kg (Supplementary 1f).

**Discussion**

Esophageal squamous cell carcinoma is an aggressive cancer worldwide. Due to lack of effective therapeutic targets and related drugs, the five-year survival rate of ESCC is still less than 20%. Thus, finding effective therapeutic targets and inhibitors for ESCC is highly interested.

Previous studies showed that TYK2 is overexpressed in several cancer cells and be supposed to a potential therapeutic target [25, 26]. In Malignant peripheral nerve sheath tumors (MPNST), TYK2 induces cell proliferation and promotes MPNST progression through inhibiting cell death [27]. In prostate cancer, TYK2 influences the invasiveness of prostate cancer cells [28]. While, in osteosarcoma cell lines, TYK2 is essential for cancer cell survival [29]. Furthermore, TYK2 inhibition could also block the invasiveness of breast cancer cells [30]. Herein, we investigated the role of TYK2 in ESCC and found that the mRNA and protein levels of TYK2 were expressed highly in the most tumor tissues (Fig. 1b-e). The tissue array results showed that TYK2 protein levels were positively correlated with the tumor clinical stage while it was negatively correlated with patient survival rate in ESCC (Fig. 1c-d). However, due to the limited clinical samples, we could not validate the relationship between TYK2 and patient survival rate using the TCGA data base. Also, since TYK2 protein level differs in various adjacent tissues, potential side-effects for later target therapy may be a challenge. Despite this limitation, our findings illustrate the clinical significance of our research. Combined with TYK2 knockdown and overexpression assays in ESCC cell lines, results confirmed the positive role of TYK2 in ESCC proliferation.

Previous reports showed that cirsiliol induced radio sensitization in non-small cell lung cancer cell lines and inhibited interleukin (IL)-6-induced STAT3 activation [22, 31], and that cirsiliol could restrain the colony formation and migration of melanoma cells [32]. However, the underlying mechanisms and related targets have not been elucidated. In the present study, we utilized computational docking model to exhibit that cirsiliol could bind to TYK2 (Fig. 4). In addition, we also confirmed this by using pulldown assay and SPR assay (Fig. 4). Furthermore, utilizing kinase assay, we verified that cirsiliol inhibited the kinase activity of TYK2 in vitro rather than detecting the signal only in cell lines. These data show that cirsiliol is an inhibitor of TYK2.

PDX models have high stability and resemblance to human tumors. Thus, they are the preferred translational tools in preclinical studies and widely applied
Fig. 6  Cirsiliol inhibits ESCC through TYK2-STAT3 signaling pathways. a Immunofluorescence staining of KYSE140 and KYSE450 cells were treated for 24 h, and then stained for p-STAT3 (Tyr705) and STAT3 (100x magnification). b The analysis of p-STAT3 fluorescence intensity in KYSE140 and KYSE450 cells. Analyzed by student’s unpaired t-test (*p < 0.05, **p < 0.01). c The change of STAT3 dimer formation after treated by cirsiliol in KYSE140 and KYSE450 cells. d The Nucleus localization variation of STAT3 after treated with 20 μM cirsiliol in KYSE140 and KYSE450 cells. e The effects of cirsiliol on the TYK2-related signal pathway in KYSE140 and KYSE450 cells. IOD, Integrated Optical Density.
in drug discovery [33]. To evaluate the inhibitory effects of cirsiliol on ESCC, two cases of ESCC PDX models which expressed TYK2 at high levels were selected for our in vivo study. Results showed that cirsiliol decreased both tumor volume and tumor weight obviously (Fig. 7c-d). IHC staining of Ki67 and p-STAT3 showed that cirsiliol significantly suppressed tumor cell proliferation in the PDX mice model. In this study, we reported TYK2 directly regulated the phosphorylation of STAT3 in ESCC which was confirmed by TYK2 knockdown, overexpression, and kinase assays (Figs. 3c, d, and 4g, respectively). Taken together, these findings indicated that cirsiliol inhibited ESCC tumor growth in vivo by targeting TYK2. Previous
studies have shown that STAT3 activation subsequently increased c-myc, Bcl-2, and Mcl-1 transcription, thereby inducing cancer cell proliferation and survival [34, 35]. Therefore, we assessed the p-STAT3 and c-myc, Bcl-2, and Mcl-1 protein levels in KYSE140 and KYSE450 cells after cirsiliol treatment. Data showed that the STAT3 phosphorylation was inhibited and the protein levels of its downstream pathway were decreased after cirsiliol treatment. In summary, cirsiliol inhibited the activity of TYK2, which in turn decreased the phosphorylation and dimerization of STAT3 which inhibited its effects on gene transcription (Fig. 7f).

Conclusion
Our results demonstrated that TYK2 is a promising therapeutic target against ESCC. Cirsiliol could bind to TYK2 and inhibit its kinase activity. Cirsiliol also inhibited ESCC growth in vitro and in vivo by blocking TYK2/STAT3 signaling pathway. Our results suggest that the proper application of cirsiliol may be a beneficial chemo-preventive strategy for ESCC patients with high TYK2 levels.

Abbreviations
ESCC: Esophageal squamous cell carcinoma; PDX: Patient-derived xenograft; SPR: Surface plasmon resonance; STAT3: Signal transducer and activator of transcription 3; TPM: Transcription per million; TYK2: Tyrosine kinase 2

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13046-021-01903-z.

Additional file 1: Supplementary 1. (a) The TYK2 protein levels in each group of CDX mice tissues. (b) After knockdown TYK2, the cell cycle state in KYSE140 and KYSE450. (c) The variation of cell migration ability after TYK2 knockdown in KYSE140 and KYSE450. (d) The toxicity of cirsiliol on SHEE cell line. (e) The average body weight of mice in each group of LEG73 and LEG104 after treated by cirsiliol (n = 9 for LEG73, n = 8 for LEG104). (f) After continuous gavage administration for 2 weeks, the toxicity on mice body weight and white blood cell (WBC) were checked. Left panel: the average body weight of mice in each group after continuous treatment for 2 weeks for acute toxicity test (n = 3); right panel: the number of WBC after treated by cirsiliol. The mouse hematology was analyzed by PROKAN PE-6800. ANOVA was used for analysis in (b, c, d, e and f); no significant difference compared with control group was observed in (d, e and f).

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Authors’ contributions
XC Jia performed the overall experiments and wrote the original draft. CT Huang, YM Hu, XL Li, and WN Nie conducted the data analysis. Q Wu and FF Liu established PDX mice model. HY Chen performed computational docking. ZG Dong and KD Liu Supervised and design the Project. The author(s) read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article [and its supplementary information files].

Declarations
Ethics approval and consent to participate
All animal experiments and clinical samples were performed following the Declaration of Helsinki and approved by the Zhengzhou University Ethics Committee (Zhengzhou, Henan, China) and Consent as obtained for all cancer tissues utilized in the study from the cancer patients.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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