Triptolide Inhibited Liver Cancer Growth Based Through SPTLC2-S1P Signal Pathway

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Triptolide Inhibited Liver Cancer Growth Based through SPTLC2-S1P Signal Pathway

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Abstract

Background: Hepatocellular carcinoma is a cancer that has a high incidence in men, and its incidence is increasing year by year. Studies show that angiogenesis plays an important role in the formation of tumors, not only providing nutrients to tumor cells, but also closely related to tumor growth and metastasis. So, how to find new anti-vascular and anti-tumor targets for the pathogenesis of liver cancer is a key issue that needs to be resolved.

Methods: After treating HUVEC and HepG2 cells with different concentrations of TP, the relationship between TP's anti-vascular and anti-tumor activities and sphingolipids was investigated respectively. Then, the three-dimensional co-culture model was used to explore the correlation between HUVEC and HepG2 cells, and to find the relationship between it and sphingolipids.

Results: TP can inhibit the tube formation process of HUVEC cells. Through PCR Array, PCR and Western Blot experiments, it is found that it may achieve this effect by down-regulating SPTLC2. TP can also inhibit the proliferation, migration and invasion of HepG2 cells through the same mechanism. In the three-dimensional co-culture model of HUVEC and HepG2 cells, it was found for the first time that HUVEC can promote the biological process of HepG2 cells. It was found through ELISA and Western Blot experiments that it may be achieved through the S1P/S1PR5 pathway, and TP was found in the dosing experiment. It can significantly inhibit the induction of HUVEC on HepG2 cells.

Conclusions: These data confirm that the level of SPTLC2 may be related to the anti-vascular and anti-tumor effects of TP. The data also showed that there is a correlation between the viability of HepG2 cells and HUVEC cells, which may be related to the expression of S1P/S1PR5. Ultimately, these data may help discover new anti-tumor targets.

Keywords: Triptolide, liver cancer, SPTLC2, S1P, S1PR5

Introduction

Hepatocellular carcinoma is a cancer that has a high incidence in men, and its incidence is
increasing year by year [1]. The pathogenesis of liver cancer is a very complicated process composed of a series of related links mediated by various risk factors such as chronic viral hepatitis, alcohol abuse, non-alcoholic steatohepatitis, and type 2 diabetes [2]. Studies show that angiogenesis plays an important role in the formation of tumors, not only providing nutrients to tumor cells, but also closely related to tumor growth and metastasis [3]. In addition, these new vascular tissues can provide blood and nutrients for the continued growth of tumors, and tumor tissues can further promote the regeneration of blood vessels through a variety of ways, thereby forming a vicious circle, so modern medicine believes that cutting off the vascular tissues of tumors can "Starved to death" tumor. So, how to find new anti-vascular and anti-tumor targets for the pathogenesis of liver cancer is a key issue that needs to be resolved. Only by cutting off the nutritional supply of tumor tissue and inhibiting the further growth of tumors can liver cancer be fundamentally overcome.

In recent years, with the further study, it is found that in addition to being the basic component of cell membrane, sphingolipids also participate in a variety of signal transduction pathways and play an important role in the occurrence and development of various diseases. The metabolism of sphingolipids is a key pathway in cancer biology, and its metabolites ceramide, sphingosine and sphingosine-1-phosphate (S1P) together regulate tumor cell death, proliferation and drug resistance, as well as host angiogenesis and inflammation [4]. Ceramide is produced by the hydrolysis of sphingolipids, which can also de novo synthesis of ceramide by the precursor dihydroceramide, which is converted to ceramide by dihydroceramide desaturase to induce tumor cell apoptosis [5]. Ceramide is hydrolyzed by a ceramidase to produce sphingosine, which is phosphorylated by sphingosine kinases (SK1 and SK2) to produce S1P. S1P is dephosphorylated by S1P phosphatase 1 and 2 and is degraded by S1P lyase, which cleaves S1P to produce phosphoethanolamine and hexadecenal. In addition to intracellular targets, S1P also binds to and activates the G protein-coupled receptor family, S1P receptor 1-5 (S1PR1-5), which regulates the biological activity of cells. Studies in many cancer cell lines indicate that S1P induces proliferation and inhibits ceramide-induced apoptosis. Ceramide is a key factor in sphingolipid metabolism, and Serine palmitoyltransferase (SPT) is a key enzyme for de novo synthesis of ceramide. SPT in mammals is a heterodimer composed of two subunits, namely SPTLC1 and SPTLC2, and SPTLC3 is the third subunit found in 2009 [6]. SPT is a class of substances with pre-inflammatory and pre-apoptotic properties that can be involved in subsequent responses by activating multiple protein kinases and phosphatases downstream of the inflammatory response, or by generating S1P as a second messenger [7].

Triptolide(TP) is one of the main active ingredients extracted from the roots, stems and leaves of Tripterygium wilfordii. It is a small molecule compound with anti-tumor, anti-angiogenesis, anti-inflammatory and pro-apoptotic effects [8]. TP has anti-liver cancer [9,10], ovarian cancer [11-14], lung cancer [15-17], gastric cancer [18,19], breast cancer [20,21] effects.

Based on the above facts, this experiment explores the effects of TP on liver cancer from the three perspectives of anti-vascularity, tumor suppression and tumor microenvironment, and finds the connection between TP’s anti-liver cancer effect and sphingolipid. In this study, we first studied the effect of TP on human umbilical vein endothelial cells (HUVEC) and its possible mechanism, explored the effect of TP on angiogenesis, and looked for the effect of TP on angiogenesis New target. Secondly, we studied the effect of TP on liver cancer cell HepG2, and further explored the new target of TP against liver cancer. As we all know, as one of the members
of the tumor microenvironment, vascular endothelial cells not only form vascular nutrition tumor
tissue, but also penetrate the entire tumor tissue. So does endothelial cell itself have a certain
effect on tumor cells? Then, we further explored the interaction between HUVEC and HepG2 cells
in the three-dimensional co-cultivation mode and its possible mechanism, and studied the
influence of TP on the co-cultivation system.

Materials and Methods

Cell grouping and transfection

Human umbilical vein endothelial cells (HUVEC) were purchased from the North Branch of the
Institute of Biotechnology, and the liver cancer HepG2 cells were purchased from the Chinese
Academy of Sciences and cultured in 10% (v/v) fetal bovine serum and 1% (v/v)
Penicillin-streptomycin in DMEM. Cultures were placed at 37°C in a humidity incubator with 5%
CO₂. When the cells are in good condition and are as long as about 80%, they are used in
subsequent experiments. Cultured cells were divided into siR-SPTLC2 group, siR-NC group,
SPTLC2 group, SPTLC2-NC group and blank group and transfected with siR- SPTLC2, siR-NC,
SPTLC2, SPTLC2-NC, and no treatment was done, respectively. In addition, a medium
concentration of celestrol was added to each group.

Cell proliferation assay

Cell viability was determined by the CCK-8 assay. Briefly, HUVEC and HepG2 cells were
adjusted to a density of 4 x 10^4 cells/ml, plated into 96-well plates overnight, 100 μl per well, and
treated with different concentrations of celestrol. The HUVEC group was treated with 12.5 nM, 25
nM, 50 nM TP, DMSO as the negative control, and endostatin as the positive control (8 mg/L),
and the time was set at 24, 48, and 72 hours. The HepG2 group was treated with 1 μM, 2 μM, and
4 μM of TP, and DMSO was used as a negative control, and the time was set at 24 and 48 hours.
The HUVEC and HepG2 co-culture groups were set to 1 to 4 days, 2 x 10^3 HepG2 cells (200
μl/well) were added to the upper chamber of the 24-well co-culture chamber, and 5 x 10^3 HUVEC
cells were added to the lower chamber (500 μl/well). After the cells were treated for a specific
time, the medium in the 96-well plate was aspirated, and 100 μl of CCK-8-containing medium
(CCK-8 reagent: medium = 1:10) was added. Transferred the co-culture group upper chamber to a
new 24-well plate containing 500 μl of the above CCK-8 medium. After 1 to 4 hours, measure the
absorbance at 450 nm with a microplate reader

Cell migration and invasion assay

The migration ability of the treated HUVEC cells and HepG2 cells was determined using a
24-well two-compartment transwell assay. The TP treatment of each group of HUVEC cells and
HepG2 were resuspended in serum-free DMEM. 200 μl of the cell suspension was added to the
upper chamber of the transwell, and 500 μl of the complete medium was added to the lower
chamber. In the co-culture group, the density of HepG2 cells was adjusted to 5x10^4/200 μl, and
the density of the lower chamber HUVEC was adjusted to 2x10^4/500 μl. After 24 hours, the upper
chamber was taken out and washed with PBS three times, 4% tissue cell fixative was fixed for 1
hour, washed again with PBS for 3 times, 0.1% crystal violet stained for 30 minutes, PBS was
washed 3 times, gently wipe the cells inside the chamber with a cotton swab, and finally
photographed with a microscope. After the end of the photographing, each group of chambers was
decolorized for 5 minutes in 500 μl of a 10% (v/v) acetic acid solution, and the absorbance was measured at 550 nm.

The cell invasion assay was similar to the cell migration assay except that the transwell membrane was pretreated with Matrigel and the HepG2 cell density was adjusted to 3 x 10^5 cells/200 μl.

**Cell adhesion assay**

The pre-cooled Matrigel was placed in a pre-cooled 96-well plate at 50 μl per well and allowed to air dry. 2% BSA 100 μl was added to each well and blocked for 1 h. 3x10^4 HUVEC cells were seeded in a 96-well plate with 100 μL of cell suspension per well. After 1 hour, the 96-well plate was taken out, washed twice with PBS, 100 ul of the above CCK-8 mixed solution was added, and incubation was continued for 1 to 4 hours in a cell culture incubator, and finally the absorbance at 450 nm was analyzed by a microplate reader.

**Cell tube formation assay**

Matrigel, 96-well plates and tips were placed in a 4 °C refrigerator overnight. On the next day, remove the Matrigel on the ice box, add 60 ul of Matrigel to each well of the 96-well plate to evenly spread the wells, do not have air bubbles, let stand at 4 ° C for 5 minutes, then place in the cell incubator for 1 hour. HUVEC cells were digested with 0.25% trypsin to prepare a cell suspension, counted on a cell counting plate, adjusted to a cell density of 8x10^4/100 μl, and added to a 96-well plate plated with Matrigel, 100 μl of cell suspension per well, take pictures after 4-8h.

**PCR-array and RT-PCR assay**

Total RNA from cells was prepared using TRNzol Universal (TIANGEN, Beijing, China) according to the manufacturer’s protocol. Absorbance was measured at 260 and 280nm to assess the quantity and purity of RNA. The cDNA was prepared from total RNA (1 μg) with a reverse transcriptase (RT) Primer Mix using the PrimeScript RT reagent Kit with gDNA Eraser (TIANGEN, Beijing, China) according to the manufacturer’s instructions. Subsequent PCR amplification was carried out using a Bio-Rad CFX Manager 3.1 system (Bio-Rad, Hercules, CA) under the following conditions: 40 cycles at 95°C for 15s and at 60°C for 60s. Amplified products were monitored by measuring the increase of the dye intensity of the SYBR Green that binds to double-strand DNA amplified by PCR. GAPDH was used as an internal control. SPTLC2 forward primer (5'-3'): CAGATTGCTTGAGGCCAGGAGTTC; SPTLC2 reversed primer (5'-3'): AGTGGTGTGATCTTGCGTCATTGC.

**Western blotting**

After the HUVEC and HepG2 cells are treated with the drug or transfected under the above conditions, the protein is extracted for subsequent experiments. The cells were subsequently lysed with RIPA buffer(Solarbio, China), according to the manufacturer’s recommendations. Equivalent amounts of protein were separated by 10% SDS-PAGE and transferred to a PVDF membrane. The membrane blocked in 5% nonfat milk in TBST for 1h at room temperature and then incubated with primary antibodies at 4 °C overnight. The immunoblots were then incubated with a secondary antibody at room temperature. Finally, the antigen-antibody complex on the membrane was visualized using ECL plus and X-ray film.
Enzyme linked immunosorbent assay (ELISA)
The HUVEC and Hep G2 cells co-culture for 1 to 4 days, 2 × 10⁵ HepG2 cells (2 ml/well) were
added to the upper chamber of the 6-well co-culture chamber, and 1 × 10⁵ HUVEC cells (1
ml/well) were added to the lower chamber. After the cells were treated for a specific time, the
supernatants were collected. It was detected by enzyme-linked immunosorbant assay according to
the manufacturer's protocol.

Statistical analysis
All data were represented by mean±SD. GraphPad Prism 5.0 software was applied to statistical
analysis, and significance between groups was ascertained by one-way ANOVA compared with
least significant difference. When the P-value was less 0.05, the analysis was accepted as
statistical difference.

Results
TP inhibits HUVEC cell proliferation, migration, adhesion and tube formation
Firstly, we assessed the effects of TP on HUVEC cells proliferation. Figure 1A presented that TP
treatment remarkably inhibited the proliferation of HUVEC cells in a dose and time dependent
manner. The proliferation of HUVEC cells were both dramatically decreased after treatment with
8 mg/L endostatin and 8 mg/L endostatin + TP 12.5 nM. Considering the results, we decided to
use 24h for subsequent experiments. Figure 1B presented that TP treatment significantly inhibited
the migration of HUVEC cells in a dose-dependent manner. The migration of HUVEC cells were
both dramatically decreased after treatment with 8 mg/L endostatin and 8 mg/L endostatin + TP
12.5 nM. The adhesion and tube formation of HUVEC cells after TP treatment were also analyzed
in our research. The results of Figure 1C and 1D showed that TP treatment remarkably inhibited
HUVEC cells adhesion and tube formation in a dose-dependent manner. The adhesion and tube
formation of HUVEC cells were both dramatically decreased after treatment with 8 mg/L
endostatin and 8 mg/L endostatin + TP 12.5 nM.

TP down-regulates the expression of SPTLC2 in HUVEC cells
Firstly, we treated HUVEC cells with 25 nM TP and extracted RNA for PCR-array assay for
screening for altered genes. As presented in Figure 2A, SPTLC2 changes the most, which implied
that SPTLC2 might be involved in the effects of TP on HUVEC cells proliferation, migration,
adhesion and tube formation. As presented in Figure 2B and 2C, we further verify the expression
of SPTLC2 by RT-PCR and Western blotting.

SPTLC2 participates in the effects of TP on HUVEC cell proliferation, migration, adhesion
and tube formation
To verify the roles of SPTLC2 in HUVEC cells proliferation, migration, adhesion and tube
formation, SPTLC2 and siR-SPTLC2 were transfected into HUVEC cells, respectively. Figure 3A
showed that siR-SPTLC2 transfection down-regulated the expression of SPTLC2, while SPTLC2
transfection up-regulated the expression of SPTLC2 in HUVEC cells. Figure 3B presented that
HUVEC cells proliferation was notably inhibited by SPTLC2 suppression and markedly enhanced
by SPTLC2 overexpression. Figure 3C presented that HUVEC cells migration was notably
inhibited by SPTLC2 suppression and markedly enhanced by SPTLC2 overexpression. The results
of Figure 3D and 3E showed that SPTLC2 suppression remarkably inhibited HUVEC cells adhesion and tube formation, SPTLC2 overexpression enhanced HUVEC cells adhesion and tube formation. Finally, we explored the mechanism. As shown in Figure 3F, SPTLC2 can affect the expression of S1P. The down-regulation of SPTLC2 can inhibit the expression of S1P, and the up-regulation of SPTLC2 can promote the expression of S1P. Based on this, we believe that SPTLC2 is likely to regulate various biological processes of cells by regulating the expression of S1P.

**TP inhibits HepG2 cell proliferation, migration and invasion**
Firstly, we assessed the effects of TP on HepG2 cells proliferation. Figure 4A presented that TP treatment remarkably inhibited the proliferation of HepG2 cells in a dose and time dependent manner. Considering the results, we decided to use 24h for subsequent experiments. Figure 4B and 4C presented that TP treatment significantly inhibited the migration and invasion of HepG2 cells in a dose-dependent manner.

**TP down-regulates the expression of SPTLC2 in HepG2 cells**
As presented in Figure 4D and 4E, TP treatment dramatically down-regulated the expression of SPTLC2 in HepG2 cells in a concentration-dependent manner.

**SPTLC2 participates in the effects of TP on HepG2 cell proliferation, migration and invasion**
To verify the roles of SPTLC2 in HepG2 cells proliferation, migration and invasion, SPTLC2 and siR-SPTLC2 were transfected into HepG2 cells, respectively. Figure 5A showed that siR-SPTLC2 transfection down-regulated the expression of SPTLC2, while SPTLC2 transfection up-regulated the expression of SPTLC2 in HepG2 cells. Figure 5B presented that HepG2 cells proliferation was notably inhibited by SPTLC2 suppression and markedly enhanced by SPTLC2 overexpression. The results of Figure 5C and 5D showed that SPTLC2 suppression remarkably inhibited HepG2 cells migration and invasion, SPTLC2 overexpression enhanced HepG2 cells migration and invasion. Finally, we explored the mechanism. As shown in Figure 5E, SPTLC2 can affect the expression of S1P. The down-regulation of SPTLC2 can inhibit the expression of S1P, and the up-regulation of SPTLC2 can promote the expression of S1P. Based on this, we believe that SPTLC2 is likely to regulate various biological processes of cells by regulating the expression of S1P.

**HUVEC cells may induce proliferation, migration and invasion of HepG2 cells through the S1P-S1PR₃ pathway.**
In order to verify the effect of HUVEC cells on proliferation, migration and invasion of HepG2 cells, HUVEC cells and HepG2 cells were co-cultured to further detect the proliferation, migration and invasion of HepG2 cells. As shown in Figure 6A, HUVEC cells promoted the proliferation of HepG2 cells in a time-dependent manner. Figure 6B and 6C presented that HUVEC cells significantly promote migration and invasion of HepG2 cells. To further understand its mechanism of action, we further examined changes in S1P in medium and changes in S1PR₃ expression in HepG2 cells. As shown in Figure 6D, the level of S1P increased as the co-culture time increased. As shown in Figure 6E, S1PR1 and S1PR2 of HepG2 cells also increased with increasing
co-culture time. However, S1PR3 has the opposite trend.

**TP inhibits the proliferation, migration and invasion of HepG2 cells induced by HUVEC cells.**

HUVEC cells were treated with different concentrations of TP and then co-cultured with HepG2 cells. As shown in figure 7A, it was found that the proliferation of HepG2 cells decreased with the increase of treatment concentration. As shown in Figure 7B and 7C, the induction of migration and invasion of HepG2 cells by HUVEC cells treated with TP was also attenuated.
Fig. 1 TP inhibits HUVEC cell proliferation, migration, adhesion and tube formation. (A) The viability of HUVEC cells after 0, 12.5, 25, 50 nM TP and DMSO, endostatin (8 mg/L) treatment for 24, 48 and 72 h were measured using cell counting kit-8 (CCK-8) assay, (x±s, n=4). (B) Transmembrane migration of cells in each group (×100) and absorbance of cells passing through the compartment in each group. (C) Effect of TP on the adhesion of HUVEC cells. (D) Effect of TP on the angiogenesis of HUVEC cells(×100). * P<0.05, ** P<0.01 versus Control group.
Fig. 2 TP down-regulates the expression of SPTLC2 in HUVEC cells. (A) HUVEC cells were treated with the optimal concentration of TP (25 nM), and the changed genes were screened by PCR ARRAY assay. The above results are all absolute values of the △Ct value. (B) SPTLC2 mRNA expression in HUVEC cells was tested by RT-PCR assay. (C) SPTLC2 protein expression in HUVEC cells was checked by Western blot assay. * P<0.05, ** P<0.01 versus Control group.
Fig. 3 SPTLC2 participates in the effects of TP on HUVEC cell proliferation, migration, adhesion and tube formation. (A) SPTLC2 protein expression in HUVEC cells was checked by Western blot assay. (B) Effect of SPTLC2 on the proliferation of HUVEC cells. (C) Transmembrane migration of cells in each group (×100) and absorbance of cells passing through the compartment in each group. (D) Effect of SPTLC2 on the adhesion of HUVEC cells. (E) Effect of SPTLC2 on the angiogenesis of HUVEC cells (×100). (F) Effect of DEGS1 on S1P expression. * P<0.05, ** P<0.01 versus Blank group.
Control             DMSO            TP-1 μM
                   TP-2 μM            TP-4 μM

C

Control         DMSO   TP-1 μM
TP-2 μM      TP-4 μM

D

Control         DMSO   TP-1 μM
TP-2 μM      TP-4 μM

E

SPTLC2

β-actin

Control         DMSO   TP-1 μM
TP-2 μM      TP-4 μM
**Fig. 4** TP inhibits HepG2 cell proliferation, migration and invasion and down-regulates the expression of SPTLC2 in HepG2 cells. (A) HepG2 cells were treated with 0 μM, 1 μM, 2 μM, 4 μM of TP and DMSO for 24h and 48h, finally cell viability was measured by CCK-8 assay, (x±s, n=4). (B) Transmembrane migration of cells in each group (×100) and absorbance of cells passing through the compartment in each group. (C) Invasion of cells in each group (×100) and absorbance of cells passing through the compartment in each group. (D) SPTLC2 mRNA expression in HepG2 cells was tested by RT-PCR assay. (E) SPTLC2 protein expression in HepG2 cells was checked by Western blot assay. * P<0.05, ** P<0.01 versus Control group.
C

Blank  siR-NC  siR-SPTLC2  SPTLC2-NC  SPTLC2

TP-2 μM  siR-NC+ TP  siR-SPTLC2 + TP  SPTLC2-NC+ TP  SPTLC2+ TP

D

Blank  siR-NC  siR-SPTLC2  SPTLC2-NC  SPTLC2

TP-2 μM  siR-NC+ TP  siR-SPTLC2 + TP  SPTLC2-NC+ TP  SPTLC2+ TP
Fig. 5 SPTLC2 participates in the effects of TP on HepG2 cell proliferation, migration and invasion. (A) SPTLC2 protein expression in HepG2 cells was checked by Western blot assay. (B) Effect of SPTLC2 on the proliferation of HepG2 cells. (C) Transmembrane migration of cells in each group (×100) and absorbance of cells passing through the compartment in each group. (D) Invasion of cells in each group (×100) and absorbance of cells passing through the compartment in each group. (E) Effect of DEGS1 on S1P expression. * P<0.05, ** P<0.01 versus Blank group.
Fig. 6 HUVEC cells may induce proliferation, migration and invasion of HepG 2 cells through the S1P-S1PR3 pathway. (A) HUVEC cells can promote the proliferation of HepG 2 cells, and their proliferative effects increase as co-culture time increases. (B) HUVEC cells can promote the migration of HepG 2 cells. (C) HUVEC cells can promote the invasion of HepG 2 cells. (D) The content of S1P in the culture system was determined by an ELISA assay. (E) S1PR3 protein expression in HepG 2 cells was checked by Western blot assay. * P<0.05, ** P<0.01 versus Non-co-culture group.
Fig. 7 TP inhibits the proliferation, migration and invasion of HepG 2 cells induced by HUVEC cells. (A) HUVEC cells were treated with different concentrations of TP and then co-cultured with HepG 2 cells. It was found that the proliferation of HepG 2 cells decreased with the increase of treatment concentration. (B) HUVEC cells were treated with different concentrations of TP and then co-cultured with HepG 2 cells. It was found that the migration ability of HepG 2 cells decreased with the increase of treatment concentration. (C) HUVEC cells were treated with different concentrations of TP and then co-cultured with HepG 2 cells. It was found that the invasive ability of HepG 2 cells decreased with the increase of treatment concentration. * P<0.05, ** P<0.01 versus Control group.

Discussion
As is known to all, angiogenesis plays an important role in tumor growth and metastasis. In
addition to transferring tumor cells, the newly formed vascular tissue can also provide a continuous supply of nutrients to the tumor tissue[22]. Therefore, modern medicine believes that cutting off the tumor vascular tissue can "starve" the tumor. However, as a member of the tumor microenvironment, vascular endothelial cells not only form vascular vegetative tumor tissues, but also run through the entire tumor tissue, so do endothelial cells themselves have some influence on tumor cells? Angiogenesis is associated with many tumors, especially solid tumors such as liver cancer and breast cancer. TP is one of the anti-tumor drugs that has attracted much attention in recent years, which can not only inhibit tumor angiogenesis, but also inhibit the biological processes of various tumors. In recent years, with further studies, it has been found that nerve sphingolipid is not only a basic component of cell membrane, but also involved in a variety of signal transduction pathways and plays an important role in the occurrence and development of various diseases, especially tumors[4]. With this in mind, we hypothesized whether there was some connection between TP's anti-tumor effects and sphingolipid. Based on the above facts, we designed there experiments to explore: Firstly, we explored the mechanism between TP and HUVEC, and explored the connection between TP's inhibition of angiogenesis and nerve sphingolipid; Then, we further explore the connection between the antitumor effects of TP and nerve sphingolipids. Finally, we continued to study the effect of HUVEC on tumor cells and its possible mechanism.

Firstly, we verify TP anti-angiogenesis effect in vitro, in order to guarantee the reliability of the data we choose the endostatin as a positive drug, within our experiment data showed endostatin can significantly inhibit the whole process of HUVEC cells into tube (proliferation, migration, adhesion, and angiogenesis), TP the inhibitory effect of HUVEC cells into tube ability compared with the positive control is a bit weak, but still have statistical significance (P < 0.01). So, is there an inevitable connection between TP's antivascular effect and sphingolipid? We screened the changes of 48 nerve sphingolipin-related genes by PCR Array experiment. According to the experimental results, we found that compared with the control group, the change of SPTLC2 in the TP group was larger (P<0.01), which is likely to be a new target of TP's anti-angiogenesis. And SPTLC2 is nerve sheath one of the key enzyme of lipid metabolic pathway, the new synthesis of ceramide in SPTLC2 generated under the action of ceramide, and ceramide is closely related to cell apoptosis, it can promote cell apoptosis, ceramide further under the action of ceramide enzyme hydrolysis to produce sphingosine, sphingosine kinase S1P further its phosphorylation, whereas S1P can affect the tumor microenvironment, thus promotes the transfer of tumor and growth[23]. Based on this, we further detected the expression of SPTLC2 by Western Blot and RT-PCR, and the results showed that the expression of SPTLC2 decreased significantly with the increase of TP concentration (P<0.01), which greatly attracted our interest. The transfection model of SPTLC2 was further constructed and the results were detected by Western Blot. After the successful establishment of the model, we further tested the tube-forming ability of the transfected SPTLC2 cells. The results showed that the tubulogenesis of HUVEC cells was significantly improved after SPTLC2 was elevated. However, after knocking down SPTLC2, its pipe forming ability decreased significantly (P<0.01). Based on the above results, we believe that SPTLC2 is likely to be a new target of TP's antivascular effect, and TP is likely to play the antivascular effect by down-regulating SPTLC2. We then further explored the association between SPTLC2 and TP anti-tumor, and our experimental data showed that TP significantly inhibited the proliferation, migration, and invasion of HepG2 cells (P<0.01). Since angiogenesis is closely related to the
occurrence and development of tumor, and TP is known to have both anti-vascular and anti-tumor
effects, is it possible for TP to play both anti-vascular and anti-tumor effects through the same
mechanism? According to this conjecture, we by Western Blot and RT-PCR experiments further
testing SPTLC2 expression in HepG2 cells, the results showed with the increase of concentration
of TP its expression decreased significantly (P<0.01), it could also be the result hints SPTLC2 TP
antitumor effect of new targets, so as in HUVEC cells we also build the model of transfection
HepG2 cells. The data showed that when SPTLC2 was elevated, the proliferation, migration and
invasion of HepG2 cells were significantly increased. However, after knocking down SPTLC2, it
decreased significantly (P<0.01). Based on the above experimental results, we believe that
SPTLC2 is likely to be a new target of TP's anti-tumor effect, and TP is likely to play an
anti-tumor effect by down-regulating SPTLC2. Finally, we constructed the transfection model of
HUVEC and HepG2 cells, and after the model was successfully constructed, we further detected
the change of S1P expression. The data showed that the expression of S1P in the two cells
decreased after SPTLC2 was knocked down. After up-regulating SPTLC2, the expression of S1P
in both cells increased. Based on the above results, we believe that TP may have an anti-vascular
and anti-tumor effect by down-regulating SPTLC2 and eventually affecting the expression of S1P.

Subsequently, we further explored the effects of endothelial cells as a member of the tumor
microenvironment on tumor cells. We found that HUVEC cells promote the biological processes
(proliferation, migration, and invasion) of liver cancer cell line HepG2 cells by using a
three-dimensional co-culture model, and we found for the first time that endothelial cells promote
the biological behavior of breast cancer cells. So, what mechanism is it implemented by? Based on
the three-dimensional co-culture model, we speculate that HUVEC cells may secrete a substance,
which is likely to be water-soluble and can diffuse freely in the medium, thus acting on HepG2
cells and binding to its corresponding receptors on the cell membrane, thus promoting its
development. So what is this substance and its receptor? As described in our introduction, S1P, the
metabolite of spongolipid, is a water-soluble substance, which can play a variety of roles in the
development of various tumors by combining with S1PRs. So, is it through this pathway that
HUVEC cells promote HepG2 cells? With these questions in mind, we further detected the content
of S1P in the co-culture system, and the data showed that the content of S1P increased
significantly with the extension of the co-culture time (P<0.01). Does the corresponding S1PR3 on
HepG2 cells also change? The changes of S1PR1-3 were further detected by Western Blot. The
results showed that S1PR1 and S1PR2 were significantly up-regulated with the extension of
coculture time, while S1PR3 was significantly down-regulated with the extension of co-culture
time (P<0.01). Based on the above experimental results, we believe that the promotion effect of
HUVEC cells on liver cancer cells is probably realized through the S1P-S1PRs pathway. So can
HUVEC cells promote liver cancer cells that can be inhibited by TP? With this problem in mind,
we further pretreated HUVEC cells with different concentrations of TP, co-cultured them with
HepG2 cells after a certain period of time, and then detected the proliferation, migration and
invasion ability of HepG2 cells after a certain period of time. Experimental data showed that with
the increase of TP treatment concentration, the ability of HUVEC cells to induce HepG2 cells
decreased significantly. Based on this result, we concluded that TP could inhibit the induction of
HUVEC cells to tumor cells in addition to its significant anti-vascular and anti-tumor effects.
Study strengths and limitations
This study explored the effects of TP on liver cancer from the perspectives of anti-vascular, anti-cancer and tumor microenvironment, and discovered the connection between the anti-liver cancer effect of TP and sphingolipids. New targets have been discovered for anti-vascular and anti-tumor research, which will further promote the study of tumorigenesis mechanisms. This study has several limitations. The regulatory effect of SPTLC2 in other tumors needs further experimental verification, and the relationship between TP’s anti-tumor effect and SPTLC2 in vivo also needs further experimental research.

Conclusions
These data confirm that the level of SPTLC2 may be related to the anti-vascular and anti-tumor effects of TP. The data also showed that there is a correlation between the viability of HepG2 cells and HUVEC cells, which may be related to the expression of S1P/S1PRs. Ultimately, these data may help discover new anti-tumor targets.

Abbreviations
HUVEC: Human umbilical vein endothelial cells; TP: Triptolide; PCR: polymerase chain reaction; S1P: sphingosine-1-phosphate; S1PRs: sphingosine-1-phosphate receptors; SPTLC2: Serine palmitoyltransferase 2; siRNA: small interfering RNA; OD: optical density; ELISA: enzyme-linked immuno sorbent assay

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Authors’ contributions
LL-J and QY-T conceived and designed the experiments; LL-J and MF-Z performed the experiments; LL-J and QY-T analyzed the data; LL-J and QY-T wrote the paper. The author(s) read and approved the final manuscript.

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Availability of data and materials
The data in this article is dependable. The original data is available from the corresponding authors.

Ethics approval and consent to participate
This manuscript does not contain any studies with human participants or animals performed by any of the authors.

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Figure 1

TP inhibits HUVEC cell proliferation, migration, adhesion and tube formation. (A) The viability of HUVEC cells after 0, 12.5, 25, 50 nM TP and DMSO, endostatin (8 mg/L) treatment for 24, 48 and 72 h were measured using cell counting kit-8 (CCK-8) assay, (x±s, n=4). (B) Transmembrane migration of cells in
TP down-regulates the expression of SPTLC2 in HUVEC cells. (A) HUVEC cells were treated with the optimal concentration of TP (25 nM), and the changed genes were screened by PCR ARRAY assay. The above results are all absolute values of the ΔCt value. (B) SPTLC2 mRNA expression in HUVEC cells was tested by RT-PCR assay. (C) SPTLC2 protein expression in HUVEC cells was checked by Western blot assay. * P<0.05, ** P<0.01 versus Control group.
Figure 3

SPTLC2 participates in the effects of TP on HUVEC cell proliferation, migration, adhesion and tube formation. (A) SPTLC2 protein expression in HUVEC cells was checked by Western blot assay. (B) Effect of SPTLC2 on the proliferation of HUVEC cells. (C) Transmembrane migration of cells in each group (×100) and absorbance of cells passing through the compartment in each group. (D) Effect of SPTLC2 on
TP inhibits HepG2 cell proliferation, migration and invasion and down-regulates the expression of SPTLC2 in HepG2 cells. (A) HepG2 cells were treated with 0 μM, 1 μM, 2 μM, 4 μM of TP and DMSO for 24h and 48h, finally cell viability was measured by CCK-8 assay, (x±s, n=4). (B) Transmembrane migration of cells in each group (×100) and absorbance of cells passing through the compartment in each group. (C) Invasion of cells in each group (×100) and absorbance of cells passing through the compartment in each group. (D) SPTLC2 mRNA expression in HepG2 cells was tested by RT-PCR assay. (E) SPTLC2 protein expression in HepG2 cells was checked by Western blot assay. * P<0.05, ** P<0.01 versus Control group.
Figure 5

SPTLC2 participates in the effects of TP on HepG2 cell proliferation, migration and invasion. (A) SPTLC2 protein expression in HepG2 cells was checked by Western blot assay. (B) Effect of SPTLC2 on the proliferation of HepG2 cells. (C) Transmembrane migration of cells in each group (×100) and absorbance of cells passing through the compartment in each group. (D) Invasion of cells in each group (×100) and absorbance of cells passing through the compartment in each group. (E) Effect of DEGS1 on S1P expression. * P<0.05, ** P<0.01 versus Blank group.
HUVEC cells may induce proliferation, migration and invasion of HepG 2 cells through the S1P-S1PRS pathway. (A) HUVEC cells can promote the proliferation of HepG 2 cells, and their proliferative effects increase as co-culture time increases. (B) HUVEC cells can promote the migration of HepG 2 cells. (C) HUVEC cells can promote the invasion of HepG 2 cells. (D) The content of S1P in the culture system was...
determined by an ELISA assay. (E) S1PRS protein expression in HepG 2 cells was checked by Western blot assay. * P<0.05, ** P<0.01 versus 15 Non-co-culture group.

Figure 7

TP inhibits the proliferation, migration and invasion of HepG 2 cells induced by HUVEC cells. (A) HUVEC cells were treated with different concentrations of TP and then co-cultured with HepG 2 cells. It was found that the proliferation of HepG 2 cells decreased with the increase of treatment concentration. (B) HUVEC cells were treated with different concentrations of TP and then co-cultured with HepG 2 cells. It was found that the migration ability of HepG 2 cells decreased with the increase of treatment concentration. (C) HUVEC cells were treated with different concentrations of TP and then co-cultured with HepG 2 cells. It was found that the invasive ability of HepG 2 cells decreased with the increase of treatment concentration. * P<0.05, ** P<0.01 versus Control group.