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Research Article

Keywords: Triptolide, liver cancer, SPTLC2, S1P, S1PRS

Posted Date: April 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-466452/v1>

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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/S1PRS 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/S1PRS. Ultimately, these data may help discover new anti-tumor targets.

Keywords: Triptolide, liver cancer, SPTLC2, S1P, S1PRS

Introduction

Hepatocellular carcinoma is a cancer that has a high incidence in men, and its incidence is

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

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

34 Triptolide(TP) is one of the main active ingredients extracted from the roots, stems and leaves
35 of *Tripterygium wilfordii*. It is a small molecule compound with anti-tumor, anti-angiogenesis,
36 anti-inflammatory and pro-apoptotic effects [8]. TP has anti-liver cancer [9,10], ovarian cancer
37 [11-14], lung cancer [15-17], gastric cancer [18,19], breast cancer [20,21] effects.

38 Based on the above facts, this experiment explores the effects of TP on liver cancer from the
39 three perspectives of anti-vascularity, tumor suppression and tumor microenvironment, and finds
40 the connection between TP's anti-liver cancer effect and sphingolipid. In this study, we first
41 studied the effect of TP on human umbilical vein endothelial cells (HUVEC) and its possible
42 mechanism, explored the effect of TP on angiogenesis, and looked for the effect of TP on
43 angiogenesis New target. Secondly, we studied the effect of TP on liver cancer cell HepG2, and
44 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 4×10^4 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, 2×10^3 HepG2 cells (200 µl/well) were added to the upper chamber of the 24-well co-culture chamber, and 5×10^3 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 $5 \times 10^4/200$ µl, and the density of the lower chamber HUVEC was adjusted to $2 \times 10^4/500$ 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

1 decolorized for 5 minutes in 500 μ l of a 10% (v/v) acetic acid solution, and the absorbance was
2 measured at 550 nm.

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

6 7 **Cell adhesion assay**

8 The pre-cooled Matrigel was placed in a pre-cooled 96-well plate at 50 μ l per well and allowed to
9 air dry. 2% BSA 100 μ l was added to each well and blocked for 1 h. 3×10^4 HUVEC cells were
10 seeded in a 96-well plate with 100 μ L of cell suspension per well. After 1 hour, the 96-well plate
11 was taken out, washed twice with PBS, 100 μ l of the above CCK-8 mixed solution was added, and
12 incubation was continued for 1 to 4 hours in a cell culture incubator, and finally the absorbance at
13 450 nm was analyzed by a microplate reader.

14 15 **Cell tube formation assay**

16 Matrigel, 96-well plates and tips were placed in a 4 °C refrigerator overnight. On the next day,
17 remove the Matrigel on the ice box, add 60 μ l of Matrigel to each well of the 96-well plate to
18 evenly spread the wells, do not have air bubbles, let stand at 4 °C for 5 minutes, then place in the
19 cell incubator for 1 hour. HUVEC cells were digested with 0.25% trypsin to prepare a cell
20 suspension, counted on a cell counting plate, adjusted to a cell density of 8×10^4 /100 μ l, and added
21 to a 96-well plate plated with Matrigel, 100 μ l of cell suspension per well, take pictures after 4-8h.

22 23 **PCR-array and RT-PCR assay**

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

35 36 **Western blotting**

37 After the HUVEC and HepG2 cells are treated with the drug or transfected under the above
38 conditions, the protein is extracted for subsequent experiments. The cells were subsequently lysed
39 with RIPA buffer(Solarbio, China), according to the manufacturer's recommendations. Equivalent
40 amounts of protein were separated by 10% SDS-PAGE and transferred to a PVDF membrane. The
41 membrane blocked in 5% nonfat milk in TBST for 1h at room temperature and then incubated
42 with primary antibodies at 4 °C overnight. The immunoblots were then incubated with a secondary
43 antibody at room temperature. Finally, the antigen-antibody complex on the membrane was
44 visualized using ECL plus and X-ray film.

Enzyme linked immunoSorbent assay (ELISA)

The HUVEC and HepG2 cells co-culture for 1 to 4 days, 2×10^5 HepG2 cells (2 ml/well) were added to the upper chamber of the 6-well co-culture chamber, and 1×10^5 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 $\pm$ 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-S1PR_s 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 S1PR_s 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

1 co-culture time. However, S1PR3 has the opposite trend.

2

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

5 HUVEC cells were treated with different concentrations of TP and then co-cultured with HepG2
6 cells. As shown in figure 7A, it was found that the proliferation of HepG2 cells decreased with the
7 increase of treatment concentration. As shown in Figure 7B and 7C, the induction of migration
8 and invasion of HepG2 cells by HUVEC cells treated with TP was also attenuated.

9

10 A

11

12 B

13

14

15

16 C

17

1 D

4

5 **Fig. 1** TP inhibits HUVEC cell proliferation, migration, adhesion and tube formation. (A) The

6 viability of HUVEC cells after 0, 12.5, 25, 50 nM TP and DMSO, endostatin (8 mg/L) treatment

7 for 24, 48 and 72 h were measured using cell counting kit-8 (CCK-8) assay, ($\bar{x} \pm s$, $n=4$). (B)

8 Transmembrane migration of cells in each group ($\times 100$) and absorbance of cells passing through

9 the compartment in each group. (C) Effect of TP on the adhesion of HUVEC cells. (D) Effect of

10 TP on the angiogenesis of HUVEC cells($\times 100$). * $P < 0.05$, ** $P < 0.01$ versus Control group.

11

12 A

B

13 C

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 ΔC_t 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.

B

1 C

7

8 D

9

10 E

F

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 ($\times 100$) 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 ($\times 100$). (F) Effect of DEGS1 on S1P expression. * $P < 0.05$, ** $P < 0.01$ versus Blank group.

A

B

Control

DMSO

TP-1 μM

TP-2 μM

TP-4 μM

C

Control DMSO TP-1 μM TP-2 μM TP-4 μM

D

E

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

A

B

1 C

6

7 D

13

14

1 E

2

3 **Fig. 5** SPTLC2 participates in the effects of TP on HepG2 cell proliferation, migration and
 4 invasion. (A) SPTLC2 protein expression in HepG 2 cells was checked by Western blot assay. (B)
 5 Effect of SPTLC2 on the proliferation of HepG 2 cells. (C) Transmembrane migration of cells in
 6 each group (×100) and absorbance of cells passing through the compartment in each group. (D)
 7 Invasion of cells in each group (×100) and absorbance of cells passing through the compartment in
 8 each group. (E) Effect of DEGS1 on S1P expression. * P<0.05, ** P<0.01 versus Blank group.

9

10 A

11

12 B

13

14 Non-co-culture Co-culture

15

C

Non-co-culture Co-culture

1 D

2

3 E

8

9

10 **Fig. 6** HUVEC cells may induce proliferation, migration and invasion of HepG 2 cells through the
11 S1P-S1PR_s pathway. (A) HUVEC cells can promote the proliferation of HepG 2 cells, and their
12 proliferative effects increase as co-culture time increases. (B) HUVEC cells can promote the
13 migration of HepG 2 cells. (C) HUVEC cells can promote the invasion of HepG 2 cells. (D) The
14 content of S1P in the culture system was determined by an ELISA assay. (E) S1PR_s protein
15 expression in HepG 2 cells was checked by Western blot assay. * P<0.05, ** P<0.01 versus
16 Non-co-culture group.

17

18 A

19

1 B

3 Control TP-12.5 nM TP-25 nM TP-50 nM

6 C

Control TP-12.5 nM TP-25 nM TP-50 nM

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

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

19 Firstly, we verify TP anti-angiogenesis effect in vitro, in order to guarantee the reliability of the
20 data we choose the endostatin as a positive drug, within our experiment data showed endostatin
21 can significantly inhibit the whole process of HUVEC cells into tube (proliferation, migration,
22 adhesion, and angiogenesis), TP the inhibitory effect of HUVEC cells into tube ability compared
23 with the positive control is a bit weak, but still have statistical significance ($P < 0.01$). So, is there
24 an inevitable connection between TP's antivasular effect and sphingolipid? We screened the
25 changes of 48 nerve sphingolipin-related genes by PCR Array experiment. According to the
26 experimental results, we found that compared with the control group, the change of SPTLC2 in
27 the TP group was larger ($P < 0.01$), which is likely to be a new target of TP's anti-angiogenesis. And
28 SPTLC2 is nerve sheath one of the key enzyme of lipid metabolic pathway, the new synthesis of
29 ceramide in SPTLC2 generated under the action of ceramide, and ceramide is closely related to
30 cell apoptosis, it can promote cell apoptosis, ceramide further under the action of ceramide
31 enzyme hydrolysis to produce sphingosine, sphingosine kinase S1P further its phosphorylation,
32 whereas S1P can affect the tumor microenvironment, thus promotes the transfer of tumor and
33 growth[23]. Based on this, we further detected the expression of SPTLC2 by Western Blot and
34 RT-PCR, and the results showed that the expression of SPTLC2 decreased significantly with the
35 increase of TP concentration ($P < 0.01$), which greatly attracted our interest. The transfection model
36 of SPTLC2 was further constructed and the results were detected by Western Blot. After the
37 successful establishment of the model, we further tested the tube-forming ability of the transfected
38 SPTLC2 cells. The results showed that the tubulogenesis of HUVEC cells was significantly
39 improved after SPTLC2 was elevated. However, after knocking down SPTLC2, its pipe forming
40 ability decreased significantly ($P < 0.01$). Based on the above results, we believe that SPTLC2 is
41 likely to be a new target of TP's antivasular effect, and TP is likely to play the antivasular effect
42 by down-regulating SPTLC2. We then further explored the association between SPTLC2 and TP
43 anti-tumor, and our experimental data showed that TP significantly inhibited the proliferation,
44 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 sphingolipid, is a water-soluble substance, which can play a variety of roles in the development of various tumors by combining with S1PR_s. 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 S1PR_s 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 co-culture 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-S1PR_s 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

Acknowledgements

Thanks to the scientific research team of the Hepatobiliary and Pancreatic Surgery Laboratory of Guilin Medical College Affiliated Hospital. Special thanks to Dr. Xiao Juan for his guidance on this paper.

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.

Funding

This study was supported by the National Natural Science Foundation of China (Grants Nos. 81360665, 81760747) and also partially supported by the Guangxi Municipal Natural Science Foundation Grants 2015GXNSFAA139114 and 2018GX NSFAA050147. The present study was also supported in part by the Lijiang Scholar Award in Guilin, Hundred Talents Program “the Introduction of Overseas High-Level Talents in Colleges and Universities in Guangxi”, and the High Level of Innovation Team and Outstanding Scholars Program in Colleges and Universities in Guangxi.

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.

Consent for publication

Authors provide formal consent to publish work.

Competing interests

The authors declare no conflict of interest

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Figures

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, ($\bar{x} \pm s$, $n=4$). (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.

Figure 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 ΔC_t 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 ($\times 100$) 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($\times 100$). (F) Effect of DEGS1 on S1P expression. * $P < 0.05$, ** $P < 0.01$ versus Blank group.

Figure 4

TP inhibits HepG2 cell proliferation, migration and invasion and down-regulates the expression of SPTLC2 in HepG 2 cells. (A) HepG 2 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, ($x\pm s$, $n=4$). (B) Transmembrane migration of cells in each group ($\times 100$) and absorbance of cells passing through the compartment in each group. (C) Invasion of cells in each group ($\times 100$) and absorbance of cells passing through the compartment in each group. (D) SPTLC2 mRNA expression in HepG 2 cells was tested by RT-PCR assay. (E) SPTLC2 protein expression in HepG 2 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 HepG 2 cells was checked by Western blot assay. (B) Effect of SPTLC2 on the proliferation of HepG 2 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.

A

B

C

D

E

Figure 6

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.