MiR-19a enhances cell proliferation, migration and invasion through improving lymphangiogenesis via targeting thrombospondin-1 in colorectal cancer

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MiR-19a enhances cell proliferation, migration and invasion through improving lymphangiogenesis via targeting thrombospondin-1 in colorectal cancer

Running title: MiR-19a promotes CRC progression by targeting THBS1

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Abstract

Colorectal cancer is a devastating disease with high mortality and morbidity and underlying mechanisms of miR-19a in CRC are poorly understood. In our study, dual-luciferase reporter assay was used to evaluate the binding of miR-19a with THBS1. Cell viability, migration and invasion were assessed by MTT, wound healing and transwell assays, respectively. Tube-formation assay of HLECs was used to evaluate lymphangiogenesis ability and tumor xenograft assay was used to measure tumor growth. Results showed that miR-19a was up-regulated and THBS1 was down-regulated in CRC tissues and cells. MiR-19a inhibitor suppressed survival, migration and invasion and inhibited the MMP-9 and VEGFC. Further mechanistic study identified that THBS1 was a direct target of miR-19a. THBS1 silencing attenuated the above effects induced by miR-19a inhibitor. Furthermore, miR-19a inhibitor suppressed migration and tube-formation abilities of HLECs via targeting THBS1-MMP-9/VEGFC signaling. And the inhibition of miR-19a also suppressed tumor growth and lymphatic tube formation in vivo. In conclusion, miR-19a inhibition suppressed the viability, migration and invasion of CRC cells and suppressed migration and tube formation abilities of HLECs, as well as inhibited tumor growth and lymphatic tube formation in vivo via targeting THBS1.

Keywords: Colorectal cancer, miR-19a, migration, invasion, lymphangiogenesis, THBS1, VEGFC
Introduction

Colorectal cancer (CRC), is a devastating disease with high mortality and morbidity (Lieberman 2015). More than 1.2 million new cases have been annually diagnosed with CRC (Valle 2016). Among all cancers, CRC affects about 10% male patients and about 9.2% female patients (Siegel et al. 2017). Currently, surgery and radiation therapy with adjuvant chemotherapy are the main strategies for treatment of CRC (Wolf et al. 2018). However, despite the development of surgical and adjuvant therapeutic techniques, the prognosis of CRC patients is still poor due to the tumor metastasis. In CRC patients, around 60% patients may eventually develop metastasis, which is also the primary cause for the high mortality (Li et al. 2018). CRC develops metastatic growth by spreading to other organs and tissues such as lung, bone marrow, blood and draining lymph nodes (LNs). In recent years, the metastasis of CRC to LNs, which provides an escape route for the tumor cells to enter the circulation, has attracted attentions of many researchers (Gu et al. 2018). Therefore, unmasking the mechanisms of proliferation, invasion, migration and lymphangiogenesis of CRC cells will provide deeper insights for pathogenesis and treatment of CRC.

MicroRNAs (miRNAs), which are small endogenous RNAs, play roles in many biological processes and diseases such as cell proliferation, apoptosis, tumorigenesis and cancer metastasis (Pereira et al. 2013). Among numerous miRNAs, miR-19a is recognized as an oncogene in various tumors, such as colorectal cancer, prostate cancer and myeloma (Hao et al. 2015; Liu et al. 2017; Lu et al. 2015). Previous reports also suggested that miR-19a was elevated in CRC (Zhang et al. 2012). However, the deeper mechanisms for roles of miR-19a are still unclear.

Thrombospondin-1 (THBS1) is an extracellular matrix (ECM) protein with 450 kDa. Studies have shown THBS1 affects many cellular activities, including cell proliferation, apoptosis, invasion
and migration (Sargiannidou et al. 2001). Importantly, the inhibitory effects of THBS1 on angiogenesis were also documented (Taraboletti et al. 2015). THBS1 was served as a potential endogenous blocker of capillary morphogenesis in vivo (Lawler 2002). It was reported that THBS1 could suppress angiogenesis of vascular endothelial cells via regulating MMP-9 activity (Qian et al. 1997). Additionally, the absence of THBS1 resulted in the up-regulation of vascular endothelial growth factor (VEGF) and VEGFR2 (Chu et al. 2013). Studies also showed THBS1 could inhibit inflammatory lymphangiogenesis in mice model of inflammation-induced corneal neovascularization (Claus et al. 2011). However, no study illuminated the regulation effects of THBS1 on lymphangiogenesis of CRC cells. Recently, the bioinformatics analysis predicted that THBS1 was a potential target of miR-19a (Sundaram et al. 2011). Furthermore, up to now, the roles of THBS1 in the miR-19a-induced promotion of cell proliferation and metastasis of CRC are unknown.

In this paper, we demonstrated the up-regulation of miR-19a in CRC tissues as well as cell lines. We showed for the first time that elevated level of miR-19a could enhance cell proliferation, migration, invasion and tumor growth through improving lymphangiogenesis ability by directly targeting THBS1. Our findings might provide evidences for the molecular mechanisms underlying CRC progression and might also provide new potential targets for CRC treatment.
Materials and Methods

Patients and tissues

Totally 30 pairs of CRC tissues and matched adjacent normal tissues were collected from the patients diagnosed with CRC. The procedure of human sample collection was approved by the Ethics Committee of Affiliated Hospital of ZunYi Medical College (ZunYi, GuiZhou, China). Written informed consent was obtained from all patients. The tissue samples were immediately frozen and stored at -80 °C till further assays. Clinical characteristics of all patients were collected and shown in Table 1.

Cell culture

Normal human colon mucosal epithelial cell line NCM460 and human CRC cell lines Caco-2, HCT15, HT29 and SW480 were all purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (Sigma-Aldrich, St. Louis, MO, USA) along with penicillin (100 µg/mL) and streptomycin (100 µg/mL) at 37 °C in a humidified atmosphere of 5% CO₂.

Cell transfection

Briefly, the cells at log-phase were seeded in 6-well plates at 5×10⁵ cells/well. After 12 h incubation in serum free medium, the cells were transfected with miR-19a inhibitor or miR-19a NC (negative control), shTHBS1 or shNC and pcDNA3.1-THBS1 or pcDNA3.1-NC (Shanghai GenePharma Co., Ltd., Shanghai, China) using lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. After 24 or 48 h of transfection, cells were used
for subsequent experiments. Untreated cells were used as blank control.

**Dual-luciferase reporter assay**

The wild type (WT) or mutant (MUT) 3'-UTR of THBS1 was cloned into the psiCHECK2 vectors (Promega Corporation, Madison, WI, USA). Phusion Site-Directed Mutagenesis Kit (Thermo Fisher) was used to construct the mutant-type 3'-UTR of THBS1. The SW480 cells in 24-well plates were transfected with psiCHECK2 vectors containing the wild type or mutant 3'-UTR of THBS1, in the presence of miR-19a inhibitor or not. Luciferase assays were performed 48 h after transfection using a Bright-Glo™ Luciferase Assay System (Promega, USA).

**MTT assay**

Cell viability was evaluated after 12 h, 24 h, 36 h, 48 h and 72 h after transfection using MTT assay. Briefly, the cells seeded in 96-well plates at density of 3×10⁴/mL were cultured at 37 °C and 5% CO₂. Then 10 µL MTT solution (5 mg/mL, Sigma, St. Louis, MO, USA) was added. The cells were subsequently cultured for another 4 h at 37 °C and 5% CO₂. After removing MTT, 50 µL DMSO (Sigma, St. Louis, MO, USA) was added and cells were further incubated at 37 °C for 10 minutes. Then the optical density (OD) was measured at 490 nm by a plate reader (SpectraMax® M5e, Molecular Devices). All the OD data of treated groups were corrected by subtracting the culture medium-only OD.

**Wound healing assay**

SW480 cells or human lymphatic endothelial cells (HLECs) (5×10⁴/well) after indicated
treatments were seeded in 24-well plates and incubated at 37 °C. Briefly, cells were gently scratched using a 200 µL pipette tip. After 24 h incubation, the non-adherent cells were removed and pictures were photographed to record the wound width under a bright-field microscope (Zeiss, Germany). Distances were analyzed by ImageJ software (Rasband, NIH, US). The migration distances of cells was measured by the following formula: \( \frac{(W_{0h} - W_{24h})}{W_{0h}} \times 100\% \).

**Transwell assay**

Briefly, cells were seeded in the upper compartment of transwell chambers (Corning, USA) that were pre-coated with Matrigel. As a chemoattractant, the bottom compartments were added with DMEM containing 20% FBS. After incubation for 24 h at 37°C, the non-invading cells were erased with a cotton swab and the invaded cells on the lower surface were fixed with 4% paraformaldehyde and stained with Giemsa. The images were photographed in random fields under a light microscope (Zeiss, Germany).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA according to the manufacturer’s instructions. Then the first strand cDNA kit (Sigma, Munich, Germany) was used to convert RNA to cDNA. PCR reactions were performed using a SYBR Green PCR kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) on an ABI 7300 Thermocycler (Applied Biosystems, Foster City, CA, USA). GAPDH and U6snRNA were used as internal controls for mRNA and miRNA, respectively. The \( 2^{-\Delta\Delta Cq} \) method was used to calculate relative expression levels. The specific primers used were listed as follows:
miR-19a forward: 5’-TTTTTACTAATTTTGTGTACTTTTATTGTGATGATGT-3’; reverse: 5’-CACTTTTAGTGCTACAGAAAGCTGTCA-3’;

THBS1 forward: 5’-TTGTCTTTTGGAACCACACCA-3’; reverse: 5’-CTGGACAGCTCATCACAGG-3’;

MMP-9 forward: 5’-ACGACATAGACGGCATCCA-3’; reverse: 5’-GCTGTGTTCTGTTGCTCATCCA-3’;

VEGFC forward: 5’-CAGTTACGGTCTGTGTCATCCAGTGA-3’; reverse: 5’-GGACACACATGGAGGTTTAA-3’;

GAPDH forward: 5’-GGGCTGCTTTTAACTCTGGTAAT-3’; reverse: 5’-ATGGGTGGAATCATATTGGAAC-3’;

U6snRNA forward: 5’-GCTTCGGCAGCACATATACTAAAAT-3’; reverse: 5’-CGCTTCACGAATTTGCGTCAT-3’.

Tube-formation assay of human lymphatic endothelial cells (HLECs)

For HLECs tube formation assay, HLECs were treated with conditioned medium of SW480 cells transfected with miR-19a NC, miR-19a inhibitor, miR-19a inhibitor and shNC, miR-19a inhibitor and shTHBS1, respectively. The HLECs tube formation assay was performed as previously (Kong et al. 2007). In brief, 100 μL of cell suspension of HLECs was added into 24-well plates with growth factor-induced Matrigel. After incubation for 4-6 h at 37 °C and 5% CO₂, cells were visualized a light microscope (Zeiss, Germany). The images of the capillary network were photographed and the number of junctions was counted using the Image J software (Rasband, NIH, USA).
Xenograft tumor model

For tumor xenograft assay, 18 male BALB/C nude mice (4~6 weeks) were purchased from SJA Laboratory Animal company (Hunan, China). All animals were kept in a light-controlled room under a 12 h/12 h light/dark cycle and controlled temperature (23-25°C), and had free access to food and water. In particular, any effort was put to avoid unnecessary pain of the animals. The whole study was approved by the Institutional Animal Care Committee at Affiliated Hospital of ZunYi Medical College. The SW480 cells stably transfected with miR-19a inhibitor or miR-19a NC were implanted by subcutaneous injection in the dorsal side of the hind limb. The tumor volumes were measured every 5 days until 30 days after injection. Tumor weights were also evaluated.

Immunohistochemistry assay

For LYVE-1 (a marker for lymphatic endothelial cells) staining, tumor tissues were immediately collected after resection. All tissues were fixed with 10% formalin, embedded by paraffin and sectioned. Tissue sections were then de-paraffinized in xylene and rehydrated in a series of diluted alcohol. Samples were then immersed with 3% H₂O₂ and incubated with the primary antibody of LYVE-1 (Abcam, ab33682, 1/100) at 4°C overnight. Tissues were then incubated with corresponding second antibody at 37°C for 30 min and stained with diaminobenizidine (DAB). Samples were observed using an optical microscope (Zeiss, Germany).

Western blot analysis

The concentration of the extracted proteins was measured with a BCA assay kit (EMD Millipore,
Samples were then separated with 10% SDS-PAGE and transferred to PVDF membranes. Subsequently, the membranes were blocked in 5% nonfat milk and immunoblotted overnight at 4 °C with the primary antibodies. Then the membranes were incubated with secondary antibodies for 1 h at room temperature. All antibodies used in this study were purchased from Abcam (Cambridge, MA, USA) as listed below: primary antibodies against THBS1 (ab231115, 1/5000), MMP-9 (ab73734, 1/2000), VEGFC (ab9546, 1/2000), LYVE-1 (ab33682, 1/1000) and GAPDH (ab8245, 1/500), secondary antibody HRP-labeled goat anti-rabbit IgG (ab7090, 1/500). Bands were developed using RapidStep™ ECL Reagent (EMD Millipore) and the band intensity was measured for calculating relative protein expression. Quantification of visualized bands was performed by densitometry using Image J software (Rasband, NIH, US).

**Statistical analysis**

All experiments were performed at least for three times in triplicate, with one representative experiment shown. Data were expressed as mean ±SD. Statistical analysis was performed using Student’s \( t \) test (two tailed) between two groups or one-way analysis of variance (ANOVA) followed by Tukey post hoc test for multiple comparison by SPSS software version 20.0. The correlation between miR-19a expression and clinicopathological characteristics of CRC patients was assessed by the Chi-squared test. Differences were considered statistically significant at \( p < 0.05 \).
Results

Expression of miR-19a, THBS1, MMP-9 and VEGFC in colorectal cancer tissues and colorectal cancer cell lines

The expression of miR-19a, THBS1, MMP-9 and VEGFC in CRC and normal tissues was detected with RT-qPCR assay. Results revealed that level of miR-19a, and mRNA levels of MMP-9 and VEGFC were all significantly higher in tumor tissues, while expression of THBS1 was significantly lower than that in normal tissues (Figure 1A-D). Furthermore, the expression of miR-19a was also much higher in tumors with lymph nodes metastasis than that without metastasis (Figure 1E). Consistently, level of miR-19a, as well as levels of MMP-9 and VEGFC were also shown to be significantly higher in CRC cell lines including Caco-2, HCT15, HT29 and SW480 compared to normal human colon mucosal epithelial cells NCM460, whereas the protein and mRNA levels of THBS1 were remarkably lower in CRC cell lines (Figure 1F-I). As shown in Table 1, high miR-19a expression was associated with the advanced clinical stage (p <0.05) and lymphatic metastasis (p <0.05) in CRC patients. These results showed that miR-19a and THBS1 might have crucial effects on tumorigenesis of CRC. Since the most significant expression alteration of miR-19a, THBS1, MMP-9 and VEGFC was observed in SW480 cells, SW480 cells were selected for the next experiments.

miR-19a directly targeted on THBS1

Bioinformatic prediction by software (targetscan) identified that THBS1 was predicted to be targeted by miR-19a. As shown in Figure 2A, miR-19a inhibitor caused the rapid decrease of miR-
19a level, and inhibition of miR-19a resulted in around 2 folds induction of THBS1 mRNA level. Similarly, the protein level was also up-regulated upon miR-19a inhibitor treatment (Figure 2B and C). Luciferase reporter assay showed that miR-19a inhibitor caused dramatical increase in luciferase activity of wild type of 3′-UTR of THBS1. When mutations were introduced into the potential 3′-UTR of miR-19a binding sites, the up-regulation of reporter activities induced by miR-19a inhibitor was abolished (Figure 2D and E). These results suggested THBS1 was a direct target of miR-19a in CRC cells.

**miR-19a regulated the proliferation, migration and invasion of CRC cells by targeting THBS1**

To investigate roles of miR-19a on CRC progression and whether THBS1 mediated the oncogenic effects of miR-19a on CRC cells, cells were transfected with miR-19a inhibitor in the presence or absence of shTHBS1. As shown in Figure 3A-D, when miR-19a inhibitor was applied, the expression of miR-19a was remarkably inhibited and the expression of THBS1 was significantly enhanced. However, silencing of THBS1 significantly reversed the induction of mRNA and protein levels of THBS1 by miR-19a inhibitor. Interestingly, silencing of THBS1 also increased the expression of miR-19a compared to the cells transfected with only miR-19a inhibitor (Figure 3A). Thus we further investigated whether overexpression of THBS1 would alter expression of miR-19a and found that overexpression of THBS1 could dramatically suppress the expression of miR-19a, suggesting THBS1 could also negatively regulate miR-19a expression (Figure 3B). We discussed this phenomenon in Discussion section. Meanwhile, transfection of miR-19a inhibitor significantly inhibited the cell viability and led to the decrease of migration and invasion abilities compared to the
miR-19a NC treated group. However, silencing of THBS1 antagonized the effects of miR-19a inhibitor on the cell viability, migration and invasion, which demonstrated that the above abilities were enhanced again (Figure 3E-I). These results indicated miR-19a regulated cell proliferation, migration and invasion of CRC cells through regulation of THBS1.

miR-19a affected HLECs migration and tube formation via targeting THBS1-MMP-9/VEGFC signaling

We further speculated that miR-19a might affect HLECs migration and tube formation via targeting THBS1-MMP-9/VEGFC signaling. As shown in Figure 4A, miR-19a inhibitor induced the decrease in mRNA levels of MMP-9 and VEGFC, whereas THBS1 silencing abolished the effects induced by miR-19a inhibitor and resulted in the enhancement of the mRNA levels of MMP-9 and VEGFC. Similarly, miR-19a inhibitor also induced the decrease in protein levels of MMP-9 and VEGFC, whereas THBS1 silencing also abolished such effects (Figure 4B and C). Furthermore, wound healing assay showed that the conditioned medium from SW480 cells with miR-19a inhibitor treated group resulted in the decreased cell migration of HLECs, whereas inhibition of THBS1 significantly reversed such effects and caused the induction of migration ability of HLECs (Figure 4D and E). Moreover, conditioned medium from SW480 cells with miR-19a inhibition robustly inhibited the tube formation of HLECs, which was also abolished by shTHBS1 (Figure 4F and G). All these results suggested miR-19a inhibitor could restrain migration ability and tube formation ability of HLECs via targeting THBS1-MMP-9/VEGFC signaling.
Inhibition of miR-19a suppressed tumor growth and lymphangiogenesis in vivo

At last, we used tumor xenograft assay to investigate effects of miR-19a inhibition on tumor growth and lymphangiogenesis. As shown in Figure 5A-C, when treated with cells transfected with miR-19a inhibitor, the tumor growth was significantly inhibited compared to both the miR-19a NC treated or the blank control group. Expressions of miR-19a, as well as MMP-9, VEGFC and LYVE-1 were all significantly down-regulated in tumor tissues after miR-19a inhibition treatment, while expression of THBS1 was remarkably enhanced when miR-19a was suppressed (Figure 5D-F). LYVE-1 staining also showed inhibition of miR-19a significantly reduced the lymphatic tube formation (Figure 5E). All these results indicated that the inhibition of miR-19a could suppress tumor growth and lymphatic tube formation in vivo.

Discussion

Due to the high mortality and morbidity of CRC, it is imperative to explore more effective treatments to improve survival of CRC patients. In the present study, we revealed that miR-19a was elevated in colorectal cancer tissues and cells, and we for the first time showed inhibition of miR-19a dramatically inhibited cell viability, migration and invasion of CRC cells, as well as suppressed migration and tube formation abilities of HLECs, and also attenuated tumor growth and lymphatic tube formation in vivo, which was mediated by directly targeting THBS1.

The abnormal expression of miR-19a was reported in various cancers. The level of miR-19a was significantly elevated in glioma tissues and cell lines (Shao et al. 2014). MiRNA microarray analysis showed that miR-19a was over-expressed in gastric cancers (Nair et al. 2012). Fu et al., found that
miR-19a was over-expressed in prostate cancer tissues (Fu et al. 2018). It was also demonstrated miR-19a level was increased in ovarian cancer and could enhance the survival of ovarian cancer cells (Wang et al. 2018). Additionally, miR-19a promoted gastric cancer progression by directly targeting PITX1 (Qiao et al. 2018). In the current study, we also found miR-19a was highly expressed in CRC tissues and cells, which was consistent with other researches. We also found high miR-19a expression was associated with the advanced clinical stage and lymphatic metastasis in CRC patients. However, few studies focused on the involvement of THBS1 in CRC and no study demonstrated the relationship between THBS1 and miR-19a in CRC development. In the present research, we demonstrated for the first time the expression of THBS1 was reduced in both CRC tissues and cell lines, and miR-19a could directly target THBS1 and affect the proliferation and metastasis of CRC cells.

Roles of miR-19a on proliferation and metastasis of cancer cells were reported in many studies. Wang et al. showed miR-19a promoted development of colitis-associated colorectal cancer through regulation NF-κB signaling (Wang et al. 2016). It was also shown in lung cancer, both miR-19a and miR-19b could enhance cell proliferation and migration (Gu et al. 2017). Feng et al. also demonstrated miR-19a could induce cell proliferation, migration and invasion by targeting PMEPA1 in prostate cancer cells (Feng et al. 2016). In the present study, we found the inhibition of miR-19a significantly decreased the cell proliferation, invasion and migration of CRC cells, while silencing of THBS1 reserved these effects. Moreover, inhibition of miR-19a could also inhibit tumor growth and lymphatic tube formation in vivo. It was considered miR-19a could suppress the expression of THBS1 and THBS1 might be a target for miR-19a in neuroblastoma cells. In our research, we firstly demonstrated that miR-19a directly targeted THBS1 in CRC and we are the first to confirm the results by using dual-luciferase reporter assay. Besides, we also demonstrated that the overexpression of
THBS1 could dramatically suppress the expression of miR-19a, and negatively regulate miR-19a expression. This result might need more studies to reveal the mechanisms in the future.

THBS1 is a tumor-specific ECM protein to modulate angiogenesis. So far, the functions of THBS1 in regulation of angiogenesis were controversial. Up-regulation of THBS1 was reported to be related with the promotion of angiogenic features in the gastric cancer (Zhang et al. 2003). It was also reported THBS1 could up-regulate levels of MMP-2, MMP-9 and other metalloproteinases (John et al. 2009). Pal et al. showed THBS1 promoted migration of cancer cells and enhanced MMPs levels through the integrin signaling, and thus promoted invasion of oral squamous cell carcinoma (Pal et al. 2016). On the other hand, THBS1 was reported to be negatively correlated with the degree of invasion of the primary tumor to other adjacent organs and with microvessel count (MVC) (Zhang et al. 2003). The ratios of VEGF/THBS1, VEGFC/THBS1, and Ang-2/THBS1 significantly correlated with the higher MVC (Tanaka et al. 2002). However, up to now, few studies focused on effects of THBS1 on lymphangiogenesis of CRC cells and no study demonstrated relationship between THBS1 and miR-19a in CRC. In line with the findings of THBS1 as an anti-angiogenic factor, we demonstrated miR-19a inhibitor could induce the decrease of cell migration of HLECs, the expression of MMP-9 and VEGFC, as well as the tube formation ability of HLECs by directly inducing the expression of THBS1. But, THBS1 silencing abolished all the effects induced by miR-19a inhibitor, indicating miR-19a inhibitor could restrain migration and tube formation abilities of HLECs via targeting THBS1-MMP-9/VEGFC signaling.

In summary, we demonstrated miR-19a was elevated in colorectal cancer tissues and cells. Inhibition of miR-19a could inhibit the cell viability, migration and invasion of CRC cells as well as
suppress cell migration and tube formation of HLECs, and attenuate tumor growth and lymphatic tube formation \textit{in vivo}, which was mediated by targeting THBS1. These results might give deeper insights for development of CRC and might provide novel targets for CRC treatment.
Acknowledgements

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Conflict

The authors declare that there are no conflicts of interest.
References


Figure Legends

**Figure 1.** Expression of miR-19a, THBS1, MMP-9 and VEGFC in CRC tissues and CRC cell lines.
(A-D) RT-qPCR analyses of miR-19a (A), MMP-9 (B), THBS1 (C), and VEGFC (D) expression in CRC tumor tissues and paired normal tissues, respectively. (E) RT-qPCR analyses of expression of miR-19a in CRC tumor tissues with or without LNs metastasis. (F and G) Western blotting analyses of THBS1, MMP-9 and VEGFC expression in CRC cells. (H and I) RT-qPCR analyses of miR-19a, THBS1, MMP-9 and VEGFC expression in CRC cells. Data are expressed as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001.

**Figure 2.** MiR-19a directly targeted on THBS1. (A) Expression of miR-19a and THBS1 in SW480 cells transfected with miR-19a inhibitor or NC by RT-qPCR. (B and C) Expression of THBS1 in SW480 cells transfected with miR-19a inhibitor or NC by Western blotting. (D) Predictive binding sites for miR-19a and THBS1. (E) Luciferase activity in THBS1-WT and THBS1-WUT transfected with miR-19a inhibitor or NC. Data are expressed as the mean ± SD. *P<0.05.

**Figure 3.** MiR-19a regulated the proliferation, migration and invasion of CRC cells by targeting THBS1. (A) Expression of miR-19a and THBS1 in SW480 cells transfected with miR-19a NC, miR-19a inhibitor, miR-19a inhibitor and shNC, miR-19a inhibitor and shTHBS1 by RT-qPCR. (B) Expression of miR-19a in cells after indicated treatments by RT-qPCR. (C and D) Expression of THBS1 in SW480 cells transfected with miR-19a NC, miR-19a inhibitor, miR-19a inhibitor and shNC, miR-19a inhibitor and shTHBS1 by Western blotting. (E) Cell viability of SW480 cells transfected with miR-19a NC, miR-19a inhibitor, miR-19a inhibitor and shNC, miR-19a inhibitor and shTHBS1 by MTT assay. (F and G) Cell migration of SW480 cells transfected with miR-19a
NC, miR-19a inhibitor, miR-19a inhibitor and shNC, miR-19a inhibitor and shTHBS1 by wound healing assay. (H and I) Cell invasion of SW480 cells transfected with miR-19a NC, miR-19a inhibitor, miR-19a inhibitor and shNC, miR-19a inhibitor and shTHBS1 by transwell assay. Data are expressed as the mean ± SD. *P<0.05 and **P<0.01. 

Figure 4. MiR-19a affected migration and tube formation of HLECs via targeting THBS1-MMP-9/VEGFC signaling. (A) Expression of MMP-9 and VEGFC in SW480 cells transfected with miR-19a NC, miR-19a inhibitor, miR-19a inhibitor and shNC, miR-19a inhibitor and shTHBS1 by RT-qPCR. (B and C) Expression of MMP-9 and VEGFC in SW480 cells transfected with miR-19a NC, miR-19a inhibitor, miR-19a inhibitor and shNC, miR-19a inhibitor and shTHBS1 by Western blotting. (D and E) Cell migration of HLECs treated with conditioned medium of SW480 cells transfected with miR-19a NC, miR-19a inhibitor, miR-19a inhibitor and shNC, miR-19a inhibitor and shTHBS1 respectively by wound healing assay. (F and G) HLECs tube formation treated with conditioned medium of SW480 cells transfected with miR-19a NC, miR-19a inhibitor, miR-19a inhibitor and shNC, miR-19a inhibitor and shTHBS1 by tube-formation assay. Data are expressed as the mean±SD. *P<0.05 and **P<0.01.

Figure 5. Inhibition of miR-19a suppressed tumor growth and lymphangiogenesis in vivo. (A) Tumor growth of mice injected with cells stably transfected with miR-19a inhibitor or miR-19a NC by tumor xenograft assay. (B and C) Tumor volume and weight curves of mice injected with cells transfected with miR-19a inhibitor or miR-19a NC. (D) Expression of miR-19a in tumor tissues of mice by qRT-PCR. (E and F) Expression of THBS1, MMP-9, VEGFC, LYVE-1 in tumor tissues of mice by
western blotting. (G) LYVE-1 staining by immunohistochemistry assay. Data are expressed as the mean±SD. *$P<0.05$ and **$P<0.01$. 
Figure 1

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Figure 2

168x84mm (300 x 300 DPI)
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Figure 5
Table 1 Associations between miR-19a expression and clinicopathologic characteristics of colorectal cancer patients

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