Indian Journal of Experimental Biology
Vol. 62, December 2024, pp. 958-966
DOI: 10.56042/ijeb.v62i12.6850

Sini Decoction containing serum modulates the expression of angiogenesis-associated molecules in HUVECs by regulating the signalling pathway VEGF-Dll4/Notch1 and p38MAPK/ERK1/2/p-AKT

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Received 22 November 2023; revised 01 August 2024

Angiogenesis is crucial for tumour growth and metastasis. Vascular endothelial growth factor (VEGF) and Notch ligand Delta-like 4 (Dll4) play a critical role in vessel formation and angiogenesis. This study aimed to determine anti-angiogenesis effects of Sini Decoction serum on human umbilical vein endothelial cells (HUVECs). We prepared Sini Decoction serum in doses of 0.925, 1.85, 3.7 g/mL, and administered to HUVECs. HUVECs migration was evaluated using a wound healing assay. Formation of tube-like structures in HUVECs was observed using a tube-formation assay. Gene transcriptions of *Dll4*, *Notch1*, *VEGF receptor 2 (VEGFR-2)* and *VEGF* were examined using a PCR assay. Protein expressions of Janus kinase-2/signal transducer and activator of transcription 3 (*JAK2/STAT3)* molecules and signal molecules downstream of *VEGF/VEGFR* related to angiogenesis (including mitogen-activated protein kinase (p38MAPK), extracellular signal-regulated kinase-1/2 (ERK1/2), focal adhesion kinase (FAK) and phosphorylated protein kinase B (p-Akt)) were detected by western blot. Sini Decoction serum significantly inhibited cell migration and the formation of tube-shaped structures in HUVECs compared to Control group (P<0.05). Sini Decoction serum markedly decreased *Dll*4, *Notch1*, *VEGF*, and increased *VEGFR-2* gene transcriptions, compared to those of Control group (P<0.05). Sini Decoction serum significantly reduced expression of p-Akt, p38MAPK and ERK1/2, compared to Control group (P<0.05). Although a reduction in FAK and STAT3 expression was discovered in HUVECs administered by Sini Decoction serum, without statistical significance compared to Control group (P>0.05). In conclusion, Sini Decoction serum modulated the expression of angiogenesis-associated molecules in HUVECs by activating VEGF-Dll4/Notch1 and p38MAPK/ERK1/2/p-AKT signalling pathways.

Keywords: Traditional Chinese Medicine, Medical oncology, Treatment, Vascular endothelial cells

Angiogenesis, which exists in the tumour microenvironment, is the basis of tumour growth, invasion and metastasis[1,2]. Since the prevalence of lung cancer still ranks first and mortality was the leading cause of cancer-associated death[3], more and more scientists are paying attention to lung cancer for breakthroughs. Previous research[4] showed that vascular endothelial growth factor (VEGF) overexpresses in tumour tissues of patients with non-small cell lung cancer (NSCLC) and patients with small cell lung cancer (SCLC)[4]. Meanwhile, some studies[5,6] demonstrated that VEGF and its cognate receptor, VEGF receptor 2 (VEGFR-2), illustrating the essential relevance to angiogenesis and tumour metastasis. Therefore, scientists inferred that VEGF was a potential target in lung cancer. With in-depth research, more experimental evidence showed that the Notch signalling pathway was related to angiogenesis processes, while the Notch ligands Delta-like 4 (Dll4) plays a key role in tumour angiogenesis[7]. In fact, VEGF induced the expression of the Notch ligand Dll4, which further activated Notch in the adjacent cell by inhibiting the expression of VEGF receptors[8].

Traditional Chinese Medicine (TCM) has antitumour activity to some extent, albeit with an indecisive mechanism. In the theoretical system of Traditional Chinese Medicine, we review cancer as a pathogenic toxin that *Yin* prevails over *Yang*. To

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reinforce body immunity and then eliminate pathogenic toxins, we have applied *Wen-yang* therapy for cancers in recent years. The natural agents of medicinal plants have been used to treat cancers because of their bioactivities related to oxidative stress in other countries in the world[9-13]. As the representative prescription for *Wen-yang* therapy, Sini Decoction may be helpful for treating advanced lung cancer.

In our prophase studies, we found that Sini Decoction demonstrated an antitumour effect in Lewis lung cancer and inhibited the expression of serum VEGF[14]. Furthermore, Sini Decoction repressed the growth of the human lung adenocarcinoma cell line A549 (A549) by inducing cell apoptosis, which could be mediated by regulation of Bcl-2 family proteins[15]. In this study, we subsequently conducted a lot of research to explore whether the inhibitory effect of Sini Decoction is related to the Dll4/Notch signalling pathway and how Sini Decoction influences the Dll4/Notch and VEGF/VEGFR signalling pathways.

Materials and Methods

Animals

Twelve healthy male-specific pathogen-free Sprague-Dawley rats were purchased from Shanghai SLAC Laboratory Animal Limited Company (license No. SCXK 2019-0003). Rats aging from 4 weeks to 6 weeks and weighing 200 g to 250 g were used in this research. The rats were housed in cages under constant temperature and humidity with a 12 h-light/12 h-dark cycle and specific pathogen-free conditions.

Animal experiments have been approved by Animal Ethical Committee of Shuguang Hospital in Shanghai University of Traditional Chinese Medicine (Approval No. PZSHUTCM190308013). The animal experiments were also performed in accordance with the National Institutes of Health Guidelines for Care and Use of Laboratory Animals.

Preparation of Sini Decoction freeze-dried power

The Sini Decoction freeze-dried powder was first prepared. Sini Decoction contains *Fuzi* (*Aconitum carmichaelii*, produced in Sichuan province of China), *Ganjiang* (*Zingiberi officinale*, produced in Sichuan province of China) and *ZhiGancao* (*Glycyrrhiza uralensis*, produced in inner Mongolia of China) with a mixing ratio of 1.5:1:1. These herbs were purchased from Suzhou Shunhuitang Pharmaceutical Company (Suzhou, China). The preparation of Sini Decoction mainly includes two steps. Firstly, the mixture of herbs, including 225 g *Fuzi*, 150 g *Ganjiang*, 150 g *ZhiGancao*, were soaked in 1575 mL of water for 30 min. A total of 1050 mL of water was then added to the slag juice through heating reflux for 1 h. Second, herb residues were added 1575 mL of water and refluxed again for slag juice. The above two parts of the slag juice were combined, evaporated to dryness, and dissolved into liquid extract of volume less than 525 mL, namely about 1 g of crude drug/mL. The extraction was then weighed, cooled, overnight at -20°C and powdered by freeze drying. Finally, we weighed the powder and calculated the yield. The Sini Decoction freeze dried powder weighed 131 g and adjusted the contents of Sini Decoction at a concentration of 24.95 g/100 mL of 0.9% sodium chloride solution (0.2495 g/mL).

Preparation of Sini Decoction serums

We randomly divided 12 rats into four groups including one Control rats group and three experimental rats groups. Sini Decoctioncontaining serums were obtained as described in a previous study[16], with a few modifications. For rats in experimental groups, which were administered intragastricly with 0.2 mL of Sini Decoction serum (with a concentration of 0.2495 g/mL) twice daily for 5 days. For rats in the Control group, which were administered intragastricly with 0.2 mL 0.9% sodium chloride solution. After the last free administration with water for 2 h and fasted for 12 h, blood samples were collected from the abdominal artery by abdominal anaesthesia by intragastric administration of 0.3% pentobarbital at a dose of 1.5 mL/kg. The serum was then isolated by centrifuging and inactivated in a water bath at 56°C for 30 min. Finally, serum was filter sterilized using 0.22 μm pore diameter of membrane filter and packed/placed in a -80°C refrigerator in reserve. In our preliminary experiments, the biological activity (or chemical composition) of the Sini Decoction power and the Sini Decoction serum were analyzed and compared using HPLC analysis of the mass spectrometer as previously described[16]. The preliminary experiments showed that the main components, including cinnamic acid, ferulic acid, and puerarin, are the same in the Sini Decoction serum as the Sini Decoction power.

Cell culture and trial grouping

Human umbilical vein endothelial cells (HUVECs) were purchased from the Shanghai Institute of Biochemistry and Cell Biology of CAS (Shang,

China). HUVECs were cultured in M199 medium (Gibco BRL. Co. Ltd., Grand Island, New York, USA) containing 20% fetal bovine serum (FBS, Gibco BRL. Co. Ltd.) and supplemented with 1% gelatin (Sigma-Aldrich, St. Louis, Missouri, USA). The HUVECs were cultured at 37℃ with 5% CO_2 in air and passed every 2-3 days.

In this research, the HUVECs were divided into four groups, including a Control group and three experimental groups. For the experimental groups, HUVECs were administered Sini Decoction serum at a dose of 0.925 g/mL, 1.85 g/mL, 3.7 g/mL and assigned as low Sini Decoction serum group (L-SN), medium Sini Decoction serum group (M-SN), high Sini Decoction serum group (H-SN), respectively.

Wound healing assay

We used a marker pen to draw at least five lines with uniform space (0.5-1.0 cm) on the back of 6-well plates. Next, on the super-clean worktable, we collected and then inoculated HUVECs under logarithmic phase in the 6-well plates, making it covered with HUVECs in 24 h. The next day, we used 10 μL pipette tip vertically scratch along the lines on the front of 6-well plates, gently washing HUVECs three times with phosphate buffer saline (PBS, Beyotime Biotech., Shanghai, China) and adding serum-free medium. According to the settings of the model groups, we added the Sini Decoction serums, respectively into the mediums with the routine cultural method in the CO_2 incubator and took photos under the microscope after 48 h compared to the initial state. Then, the cell migration rate in HUVECs was calculated.

Tube formation assay

The liquid matrigel was mixed with serum-free extracellular matrix (ECM) in a ratio of 1:1. A total of 100 μL commixture was then added per well of the 48-well plates. We avoided bubbles during the process at room temperature and took 30 min for the solidification time. The content of the prepared single-cell HUVECs suspension made by serum-free ECM was 1×10^5/mL. The suspension, approximately 200 μL, was continued to inoculate in each well of 48-well plates as described above. Next, the HUVECs were cultured in an incubator at 37℃ and 5% CO_2. After the HUVECs adhered to the wall, the old culture medium of each well was replaced with 200 μL different culture medium containing various doses of Sini Decoction serum, keeping the culture of HUVECs. About 6-8 h later, we observed the formation of tube-like structures in HUVECs using an inverted microscope and captured the images with 3 visual fields in each well.

Western blot assay

The HUVECs were treated with RIPA buffer (Beyotime Biotech.), Phenyl-methane-sulfonyl fluoride (PMSF, Beyotime Biotech.), protease inhibitor, phosphatase inhibitor (100:1:2:2) and centrifuged under the condition of 1200 r/min at 4℃, after sufficiently splitting decomposition. The supernatant was assigned as the protein extract. After adding standard solutions of bovine serum albumin (BSA, Beyotime Biotech.), a total of 2 μL extracted protein samples were added to 96-well plates (Corning-Costar, Corning, NY, USA) and complemented with 18 μL PBS (Beyotime Biotech.). The protein concentration was measured using a bicinchoninic acid (BCA) detection kit (Merck & Co. Inc., Kenilworth, NJ, USA) as described by the manufacturer′s instructions. A total of 10 μL protein sample was loaded onto 10% separation gel (Beyotime Biotech.) and 5% stacking gel. Then sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime Biotech.) was performed and the separated proteins were electrotransferred onto polyvinylidene fluoride membrane (PVDF, Beyotime Biotech.) for 2 h under low temperature conditions with constant current (200 mA). The PVDF membrane was then soaked in 5% bovine serum albumin solution (Beyotime Biotech.) for blocking. The PVDF membrane was slowly shaken for 2 h at room temperature and then incubated with rabbit anti- Janus kinase-2 (JAK2, Cat. No. 3230), rabbit anti-signal transducer and activator of transcription 3 (STAT3, Cat. No. 12640), rabbit anti-mitogen-activated protein kinase (p38MAPK, Cat. No. 8690), rabbit anti-extracellular signal-regulated kinase-1/2 (ERK1/2, Cat. No. 4695), rabbit antifocal adhesion kinase (FAK, Cat. No. 13009), rabbit anti-phosphorylated protein kinase B (p-Akt, Cat. No. 4060) and rabbit anti-GAPDH (Cat. No. 5174) antibodies at 4℃ overnight. The PVDF membrane was incubated using goat anti-rabbit IgG labelled with horseradish peroxidase (HRP) (Cat. No. 7074) at 37℃ for 1 h. Both of the above primary and secondary antibodies were obtained from Cell Signalling Technology (Beverly, MA, USA). After the above incubation, the PVDF membrane was washed with Tris buffered saline Tween-20 (TBST,

Table 1 — Primers for the real-time PCR assay.

Genes	Forward sequence	Reverse sequence
VEGF	5'-AGGCCAGCACATAGGAGAGA-3'	5'-ACGCGAGTCTGTGTTTTTGC-3'
VEGF4-2	5'AACCATTGGTGAGACCATCGA-3'	5'-GGTTCCGGTTCCCGTCTT-3'
Dll4	5'-GCCCTTCAATTTCACCTGGC-3'	5'-CAATAACCAGTTCTGACCCACAG-3'
Notch1	5'-GCGACAACGCCTACCTCT-3'	5'-CTGCTGGCACAGTCATCC-3'
GAPDH	5'-GCATGGCCTTCCGTGTCCCC-3'	5'-GAGGGCAATGCCAGCCCCAG-3'

Beyotime Biotech.) for 10 min and 3 times. Western blot bands were soaked in electrochemiluminescence (ECL, Beyotime Biotech.) to develop images. Eventually, western blot images were analyzed with a UVP gel image scanning system (version: Labworks 4.6, Bio-Rad Laboratories, Hercules, CA, USA).

Real-time PCR assay

RNA was extracted from HUVECs with Trizol reagent (Beyotime Biotech.) and the concentration of which was measured using an enzyme-linked immunosorbent assay (ELISA). Next, by reverse transcription-polymerase chain reaction, complementary DNA (cDNA) was obtained from total RNA under the following conditions: 25 ℃ 10 min, 50℃ 30 min and 85℃ 5 min. An equal amount of cDNA was used to determine the gene transcriptions of *Dll4, Notch1, VEGF, VEGFR-2* under the following conditions of three stages: First: Initiation at 95℃ for 30 s, 95℃ for 10 s, extension at 60℃ for 30 s, with 40 cycles. Second: 95℃ for 15 s, annealing at 60℃ for 60 s. Finally, extension at 95℃ for 15 s. The real-time PCR assay was performed under an ABI 7300 real-time PCR equipment (ABI, Foster City, CA, USA). The premiers for the above PCR assay were listed in Table 1. The $2^{-\triangle\triangle Ct}$ method[17] was used to analyze the real-time PCR findings.

Statistical analysis

All data were represented as mean±standard deviation (SD) and analyzed using SPSS software 20.0 (IBM Corp., Armonk, NY, USA). Tukey's post hoc test validated one-way analysis of variance (ANOVA) was used to analyze significance among multiple groups. Student′s *t* test was used to analyze the difference between the two groups. The $P<0.05$ was considered the significant difference.

Results

Sini Decoction serum inhibited HUVECs migration

Cell migration was evaluated using a wound healing assay (Fig. 1A). The results showed that the HUVECs migration was markedly suppressed by the treatment of Sini Decoction serum at different dosages (L-SN, M-SN and H-SN group), compared to the Control group (Fig. 1B, $P<0.05$). Especially for administration with a high dose of Sini Decoction serum (H-SN group), in which the migration rate of HUVECs reached the lowest level compared to Control group (Fig. 1B, $P<0.001$). Meanwhile, the effects of Sini Decoction serum exhibited a dose-dependent manner.

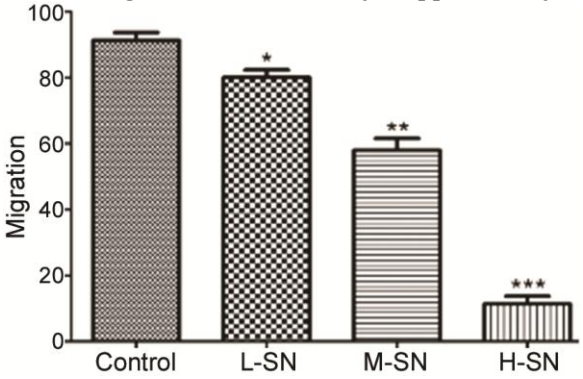

Fig. 1 — Evaluation of cell migration using a wound healing assay. (A) Images of the wound healing assay in each group at 48 h of culture. (B) Statistical analysis for HUVECs migration rate. [L-SN: HUVECs administered with 0.925 g/mL of Sini decoction serum. M-SN: HUVECs administered with 1.85 g/mL of Sini Decoction serum. H-SN: HUVECs administered with 3.7 g/mL of Sini Decoction serum. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ *vs.* Control group. Magnification, 200 ×]

Sini Decoction serum suppressed the formation of tube-like structures in VEGF-induced HUVECs

The tube-like structure could provide blood for angiogenesis, which was examined in this study (Fig. 2A). The results illustrated that the Sini Decoction serums (L-SN, M-SN and H-SN group) significantly suppressed tube-like structures compared to the Control group (Fig. 2B, $P<0.05$). Among the Sini Decoction serum doses (L-SN, M-SN and H-SN group), the high dosage (H-SN group) illustrated the lowest level of tube-like structures, compared to the Control group (Fig. 2B, $P<0.001$). Therefore, Sini Decoction serums demonstrated a notable anti-angiogenesis effect on HUVECs.

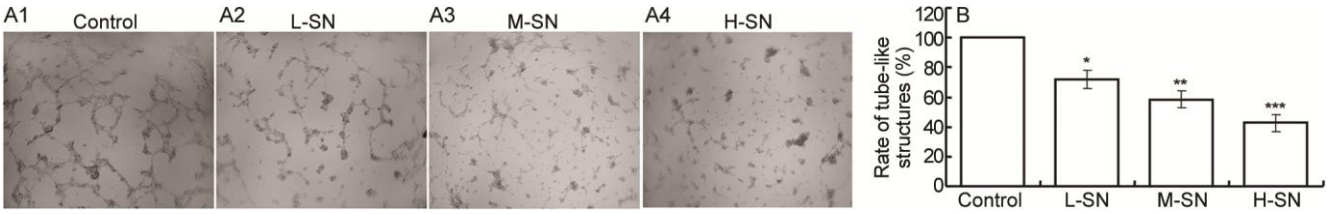

Fig. 2 — Formation of tube-like structures in VEGF-induced HUVECs. (A) Images of the formed tube-like structures in each group. (B) Statistical analysis for tube-like structures. [L-SN: HUVECs administered with 0.925 g/mL of Sini Decoction serum. M-SN: HUVECs administered with 1.85 g/mL of Sini Decoction serum. H-SN: HUVECs administered with 3.7 g/mL of Sini Decoction serum. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ *vs.* Control group. Magnification, 400 ×]

Sini Decoction serum attenuated transcription of tumour angiogenesis-associated genes

Due to the VEGF signalling pathway (key biomarker: VEGF and VEGFR-2) and the Dll4/Notch1 signalling pathway (key biomarker Dll4 and Notch1) are crucial to development of tumour angiogenesis, all of which were measured using a real-time PCR assay. Our findings indicated that Sini Decoction serums (L-SN, M-SN and H-SN group) significantly reduced gene transcription of *Dll4* (Fig. 3A), *Notch1* (Fig. 3B), *VEGF1* (Fig. 3D) and increased *VEGFR2* gene transcription (Fig. 3C) in HUVECs, compared to the Control group (Fig. 3, all $P<0.05$). Importantly, the effects of Sini Decoction serums on the above gene transcriptions were demonstrated in a dose-dependent manner, with a peak for HUVECs treated with Sini Decoction serum (3.7 g/mL, H-SN group) (Fig. 3). Therefore, 3.7 g/mL of Sini Decoction serum was applied in the following experiments.

Sini Decoction serum reduced JAK2 expression

In this study, the upstream signalling pathways, including the VEGF/VEGFR and the JAK2/STAT3 pathway, were examined using a western blot assay (Fig. 4A). Our findings showed that Sini Decoction serum remarkably decreased the expression of JAK2 in HUVECs when culturing for 12 h and 24 h (but not for 2 h and 6 h), compared with the Control group (Fig. 4B, $P<0.05$). However, there were no significant differences in STAT3 expression between the Control group and three groups of HUVECs administered by Sini Decoction serum (Fig. 4B, $P>0.05$). Therefore, Sini Decoction serum significantly modulated the JAK2/STAT3 signalling pathway.

Sini Decoction serum activated the p38 MAPK/ERK1/2 signalling pathway

VEGFR2 could promote tumour invasion and metastasis by activating downstream signal molecules, p38MAPK and ERK1/2,[18] therefore, both

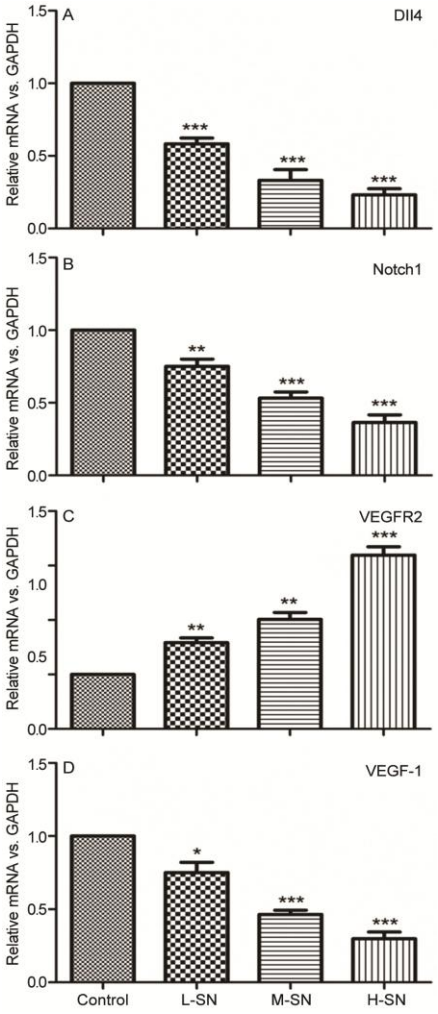

Fig. 3 — Effects of Sini Decoction serum treatment on the transcription of the *Dll4*, *Notch-1*, *VEGFR2*, *VEGF* gene in HUVECs, using a real-time PCR assay. (A) Effects of Sini Decoction serum on *Dll4* gene transcription. (B) Effects of Sini Decoction serum on *Notch-1* gene transcription. (C) Effects of Sini Decoction serum on *VEGFR2* gene transcription. (D) Effects of Sini Decoction serum on *VEGF* gene transcription. [L-SN: HUVECs administerted with 0.925 g/mL of Sini Decoction serum. M-SN: HUVECs administered with 1.85 g/mL of Sini Decoction serum. H-SN: HUVECs administered with 3.7 g/mL of Sini Decoction serum. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ *vs.* Control group]

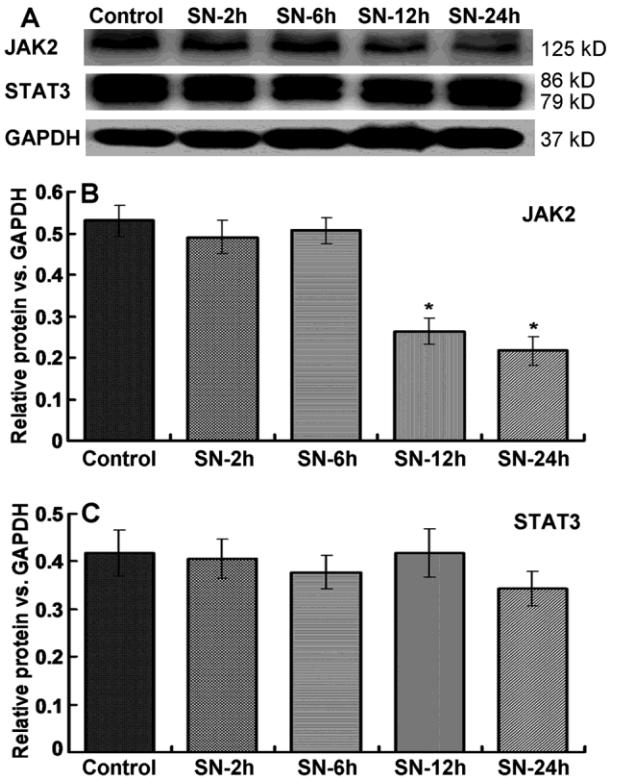

Fig. 4 — Measurement of JAK2 and STAT3 expression in HUVECs undergoing Sini Decoction serum treatments. (A) Western blot images for both JAK2 and STAT3 expression. (B) Statistical analysis for JAK2 expression in HUVECs. (C) Statistical analysis for STAT3 expression in HUVECs. [SN-2h, SN-6h, SN-12h and SN-24h represented the HUVECs administered with 3.7 g/mL of Sini Decoction serum for 2 h, 6 h, 12 h and 24 h, respectively. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ *vs.* Control group]

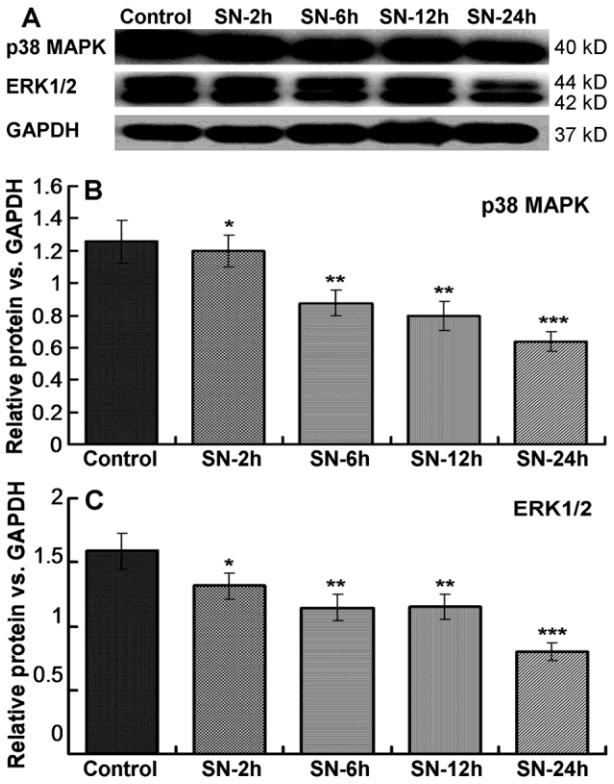

Fig. 5 — Effects of Sini Decoction serum administration on the expression of p38 MAPK and ERK1/2 molecules. (A) Western blot images for the expression of p38 MAPK and ERK1/2. (B) Western blot assay for p38 MAPK expression in HUVECs treated with Sini Decoction serum. (C) Statistical analysis for ERK1/2 expression in HUVECs treated with Sini Decoction serum. [SN-2h, SN-6h, SN-12h and SN-24h represented the HUVECs administered with 3.7 g/mL of Sini Decoction serum for 2 h, 6 h, 12 h and 24 h, respectively. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ *vs.* Control group]

were determined using a western blot assay (Fig. 5A). The present data showed that both the p38 MAPK molecule (Fig. 5B) and the ERK1/2 molecule (Fig. 5C) were markedly down-regulated in HUVECs at all the culture points (3.7 g/mL of Sini Decoction serum treated for 2 h, 6 h, 12 h and 24 h), compared to that of the Control group ($P<0.05$). Therefore, Sini Decoction serum remarkably activated the p38 MAPK/ERK1/2 signalling pathway.

Sini Decoction serum down-regulated the p-Akt molecule expression

Due to the existence of the FAK-VEGF-p-Akt axis, both FAK and p-Akt molecules were also examined using a western blot assay (Fig. 6A). Data showed that Sini Decoction serum did not demonstrate significant effects on FAK expression (Fig. 6B, $P>0.05$). However, Sini Decoction serum administration significantly decreased p-Akt expression (Fig. 6C) in HUVECs at 6 h, 12 h and 24 h culture points, compared to that of the Control group ($P<0.05$). Although the Sini Decoction serum had the strongest effect on the expression of p-Akt, no time-dependent manner was observed (Fig. 6).

Discussion

Cancer patients who fail chemotherapy or intolerance to chemotherapy, usually prefer the treatment of Traditional Chinese Medicine.[19] According to the theory of Chinese Medicine, patients with advanced lung cancer have weakened the resistance of the body to diseases.[20] In this case, Sini Decoction, as the representative prescription of *Wen-yang* therapy, would play a critical role in treating lung cancer. However, due to the complicated ingredients of traditional Chinese Medicine, such as the multiple anti-tumour effects, the mechanism for

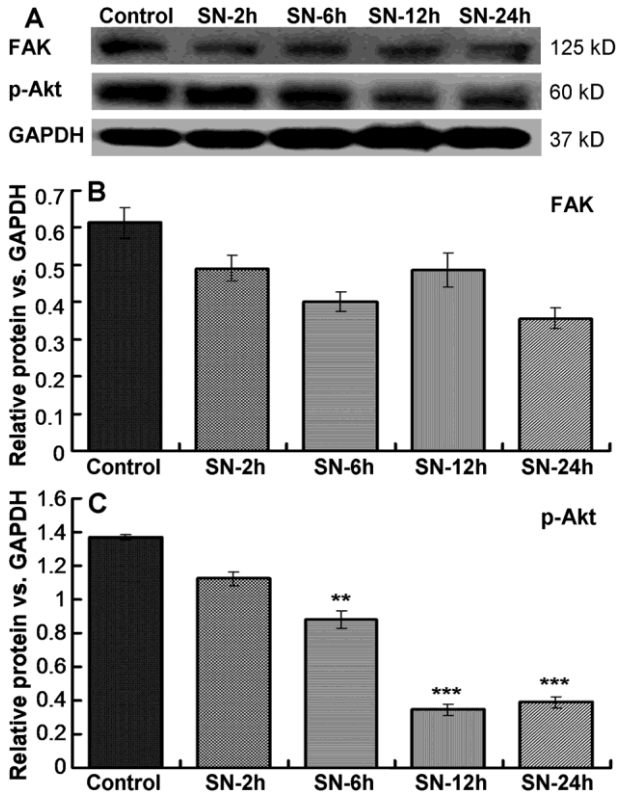

Fig. 6 — Evaluation of the expression of FAK and p-Akt molecules in HUVECs using a western blot assay. (A) Western blot images for both FAK and p-Akt expression. (B) Effects of Sini Decoction serum on FAK expression in HUVECs. (C) Effects of Sini Decoction serum on p-Akt expression in HUVECs. [SN-2h, SN-6h, SN-12h and SN-24h represented the HUVECs administered with 3.7 g/mL of Sini Decoction serum for 2 h, 6 h, 12 h and 24 h, respectively. $^{**}P<0.01$ and $^{***}P<0.001$ *vs.* Control group]

compound prescription has also been unknown until now.

According to the use of human umbilical vein endothelial cells (HUVECs) vascular endothelial cells experiments elsewhere, as well as the indefinite passage of cells, therefore, HUVECs were applied in this study. Due to the roles of HUVECs in lung cancer pathological processes, in this study, the migration of HUVECs and the formation of tube-shaped structures in HUVECs were evaluated. Our findings showed that Sini Decoction serum significantly inhibited HUVECs migration and obviously suppressed the tube-like structure formation in VEGF-induced HUVECs. These results are consistent with the effects of other Traditional Chinese Medicines on the proliferation and formation of HUVECs as reported by a previous study.[21]

Lung cancer always overexpresses the VEGF molecule, however, the Dll4 molecule plays more critical roles than the VEGF molecule in the tumour angiogenesis process in recent years[3,5]. Furthermore, there is a common connection between the Dll4/Notch signalling pathway and the VEGF/VEGFR signalling pathway. In stages of tumour angiogenesis, VEGF could activate VEGFR2 and simultaneously induce the expression of the Dll4 molecule. Interaction between Dll4 and its Notch1 receptor could promote the release of the Notch intracellular domain (NICD), which further inhibits the expression of the VEGFR2 molecule by regulating transcriptions of targeting genes of Notch signalling pathways[22,23]. VEGFR2 is related to the sprouting of new blood vessels, while Dll4 represses tumour angiogenesis[22,24]. In summary,

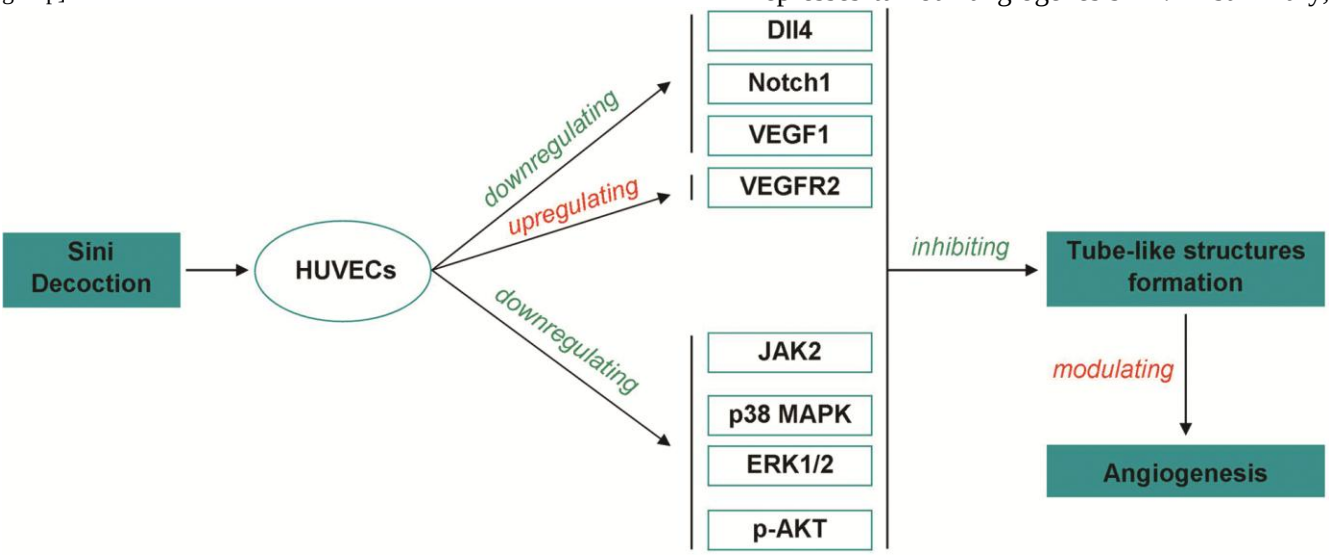

Fig. 7 — Sini Decoction modulates expression of angiogenesis-associated molecules in HUVECs *via* regulating VEGF-Dll4/Notch1 and p38MAPK/ERK1/2/p-AKT signalling pathway.

VEGF could induce Dll4 expression and is usually negatively regulated by Dll4/Notch signalling. Our results indicated that Sini Decoction serum attenuated the expression of genes associated with tumour angiogenesis, including *VEGF, VEGFR2, Dll4* and *Notch1,* in a dose-dependent manner. Therefore, Sini Decoction serum reduced HUVECs migration and suppressed tube-like structure formation in HUVECs by down-regulating VEGF expression and activating the Dll4/Notch1 signalling pathway.

The JAK2/STAT3 signalling pathway, downstream of the VEGF/VEGFR signalling pathway, involves VEGF expression and angiogenesis in the pathological process of lung cancer[25]. Furthermore, the VEGFR2 molecule could promote tumour cell invasion and metastasis by activating downstream signalling molecules, such as p38MAPK and ERK1/2[18]. Therefore, signalling molecules, including JAK2, STAT3, p38MAPK, and ERK1/2, were verified in HUVECs. Our results demonstrated that Sini Decoction serum significantly decreased the expression of JAK-2, p-AKT, p38MAPK, and ERK1/2, suggesting that Sini Decoction serum administration activates the signalling pathway of JAK2/p-Akt and p38 MAPK/ERK1/2. However, in this study, no effects of Sini Decoction serum on STAT3 and FAK expression were discovered. Therefore, we consider that Sini Decoction serum does not target STAT3, however, it targets the VEGF molecule. Additionally, Sini Decoction serum may exhibit the inhibitory effect on the expression of AKT, p38MAPK, and ERK1/2 by activating the other ways rather than modulating VEGFR2 expression.

Currently, more and more patients with advanced stage lung cancer are willing to accept Traditional Chinese Medicine (TCM) treatment, however, mechanisms that undergo TCM and associated compound prescriptions with significant clinical effects have not been fully clarified. Our study is precisely designed to explore whether Sini Decoction serum inhibits angiogenesis by activating the VEGF-Dll4/Notch signalling pathways, the above hypothesis being confirmed by our findings. However, in this study, it has not been evaluated whether Sini Decoction plays crucial roles in the effect of lung cancer angiogenesis (using lung cancer cell lines), which is a limitation of our study. Dll4 may show different effects on tumour angiogenesis in different histological types of lung cancer, while the biological effects of the Notch1 molecule in lung cancer are determined by oxygen concentration[26,27]. The lung cancer mainly includes squamous carcinoma, adenocarcinoma, and small cell lung cancer, however, the object of our study is limited and the experimental environment is also single. More samples are needed to be collected to verify the inhibitory effect of Sini Decoction on different types of lung cancer. Meanwhile, his study has not verified whether Sini Decoction generates changes in the molecular evaluation of lung cancer cells by activating or inhibiting specific molecular pathways, as well as in HUVECs. We have not determined the role of Sini Decoction in modulation of angiogenesis and identified molecular alterations of cytokines in other tumour cell lines, which will be clarified in a further study. Furthermore, the bioactive agents of medicinal plants could attenuate oxidative injury or oxidative stress in lung, kidney or other organs[28-32]. In the following studies, we would also investigate the effects of Sini Decoction on the oxidative stress in tumour cells or animal cancer model.

Conclusion

The present study based on the VEGF-Dll4/Notch signalling pathways was attempted to study the inhibitory effects of Sini Decoction serum on the expression of molecules with angiogenesis (Fig. 7). Meanwhile, this study was also expected to complement a close-blank domain in the mechanisms of Chinese herbal compound prescription in anti-tumour and provided the guideline for Sini Decoction in clinical application. Additionally, Sini Decoction serum also affected the expression of angiogenesis-associated molecules by activating the p38MAPK/ERK1/2/p-AKT signalling pathway. Follow-up experiments would have made further efforts to discover the effects of Sini Decoction on different types of lung cancers.

Funding statement

This study is supported by the Technological Project of Suzhou Health and Family Planning Commission: Diagnosis and treatment of advanced non-small cell lung cancer with TCM Comprehensive Treatment (LCZX201916) and Gusu Health Talents Program Training Project In Suzhou, China (No. GSWS2020081).

Acknowledgements

The authors are grateful to all of the participants in this work. Additionally, we take this opportunity to express our appreciation for the generous assistance that Dr. Qing Song and Dr. Binbin Cheng rendered to this article.

Conflict of interest

The authors have no conflict of interest to declare.

References

1 Folkman J, Role of angiogenesis in tumor growth and metastasis. *Semin. Oncol*, 29(2002) 15.

2 Li YQ, Li WL, Yu XH, Wu H, Wang YZ, Jin Y, Hao LL, Liu MM & Song XG, Mechanisms of Traditional Chinese Medicine Bushenantai granules in promoting angiogenesis at the maternal-fetal interface of recurrent spontaneous abortion mice. *J Tradit Chin Med*, 41(2021) 556.

3 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA & Jemal A, Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA. Cancer J Clin*, 68(2018) 394.

4 Alevizakos M, Kaltsas S & Syrigos KN, The VEGF pathway in lung cancer. *Cancer Chemoth. Pharm*, 72(2013) 1169.

5 Ellis LM & Hicklin DJ, VEGF-targeted therapy: mechanisms of antitumour activity. *Nat. Rev. Cancer*, 8(2008) 579.

6 Zhao YJ & Adjei A, Targeting angiogenesis in cancer therapy: moving beyond vascular endothelial growth factor. *Oncologist*, 20(2015) 660.

7 Liu Z, Fan F, Wang A, Zheng S & Lu Y, Dll4-Notch signaling in regulation of tumor angiogenesis. *J. Cancer Res. Clin. Oncol*, 140(2014) 525.

8 Benedito R, Rocha SF, Woeste M, Zamykal M, Radtke F, Casanovas O, Duarte A, Pytowski B & Adams RH, Notch-dependent VEGFR3 upregulation allows angiogenesis without VEGF–VEGFR-2 signalling. *Nature*, 484(2012) 110.

9 Salehi B, Selamoglu Z, S Mileski K, Pezzani R, Redaelli M, Cho WC, Kobarfard F, Rajabi S, Martorell M, Kumar P, Martins N, Subhra Santra T & Sharifi-Rad J. Liposomal Cytarabine as Cancer Therapy: From Chemistry to Medicine. *Biomolecules*, 9(2019) 773.

10 Kanwal N, Rasul A, Hussain G, Anwar H, Shah MA, Sarfraz I, Riaz A, Batool R, Shahbaz M, Hussain A & Selamoglu Z. Oleandrin: A bioactive phytochemical and potential cancer killer via multiple cellular signaling pathways. *Food Chem Toxicol*, 143(2020) 111570.

11 Rehman MFU, Akhter S, Batool AI, Selamoglu Z, Sevindik M, Eman R, Mustaqeem M, Akram MS, Kanwal F, Lu C & Aslam M. Effectiveness of Natural Antioxidants against SARS-CoV-2? Insights from the In-Silico World. *Antibiotics (Basel)*, 10(2021) 1011.

12 Abdulsattar Mosa A, Saleh Ali G & Selamoglu Z. Survey on the Oxidative Stress Status and the Role of Antioxidants in Acute Myeloid Leukemia Therapy. *J Pharm Care*, 11(2023) 165

13 Talas ZS, Bayraktar N, Ozdemir I, Gok Y & Yilmaz I. The effects of synthetic organoselenium compounds on nitric oxide in DMBA-induced rat liver. *J Environ Biol*, 30(2009) 591.

14 Gao JD, Chen JL & Li YJ, Influences of Sini Tang and different combinations of its medicinals on expression of serum vascular endothelial growth factor in mice with Lewis lung cancer. *J. Beijing Univ. Trad. Chin. Med*, 35(2012) 470.

15 Wang Q, Lu CJ & Gao JD, Sini Decoction Inhibits Lung Adenocarcinoma A549 Cell Growth by Promoting Cell Apoptosis. *Anal. Quant. Cytopathol. Histopathol*,41(2019) 93.

16 Chen W, Huang C, Yang C, Ge X, Huang W, Li X, Yang S & Liu S, Danggui Sini Decoction protected islet endothelial cell survival from hypoxic damage via PI3K/Akt/eNOS pathway. *Evid. Based Complement. Alternat. Med*, 2018(2018) 542103.

17 Livak KJ & Schmittgen TD, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 25(2001) 402.

18 Olsson AK, Dimberg A, Kreuger J & Claesson-Welsh L, VEGF receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol*, 7(2006) 359.

19 Wang N, Hou MS, Zhan Y, Shen XB & Xue HY, MALAT1 promotes cisplatin resistance in cervical cancer by activating the PI3K/AKT pathway. *Eur. Rev. Med. Pharmacol. Sci*, 22(2018) 7653.

20 Wan C, You S, Quan P, Song Y, Liu T, Lu J & Zheng P, Development and validation of the quality-of-life assessment system for lung cancer based on traditional Chinese Medicine. *Evid. Based Complement. Alternat. Med*, 2012 (2012) 945910.

21 Chiu JH, Chen FP, Tsai YF, Lin MT, Tseng LM & Shyr YM, Effects of Chinese medicinal herbs on expression of brain-derived neurotrophic factor (BDNF) and its interaction with human breast cancer MDA-MB-231 cells and endothelial HUVECs. BMC. *Complement. Altern. Med*, 17(2017) 401.

22 Kuhn C & Checa S, Computational Modeling to Quantify the Contributions of VEGFR1, VEGFR2, and Lateral Inhibition in Sprouting Angiogenesis. *Front. Physiol*, 10(2019) 88.

23 Tveriakhina L, Schuster-Gossler K, Jarrett SM, Andrawes MB, Rohrbach M, Blacklow SC & Gossler A, The ectodomains determine ligand function in vivo and selectivity of DLL1 and DLL4 toward NOTCH1 and NOTCH2 in vitro. *eLife*,7(2018) 40045.

24 Thomas JL, Baker K, Han J, Calvo C, Nurmi H, Eichmann AC & Alitalo K, Interactions between VEGFR and Notch signaling pathways in endothelial and neural cells. *Cell. Mol. Life. Sci*,70(2013) 1779.

25 Zhao M, Gao FH, Wang JY, Liu F, Yuan HH, Zhang WY & Jiang B, JAK2/STAT3 signaling pathway activation mediates tumor angiogenesis by upregulation of VEGF and bFGF in non-small-cell lung cancer. *Lung Cancer*,73(2011) 366.

26 Wael H, Yoshida R, Kudoh S, Hasegawa K, Niimori-Kita K & Ito T, Notch1 signaling controls cell proliferation, apoptosis and differentiation in lung carcinoma. *Lung Cancer*, 85(2014) 131.

27 Chen Y, De Marco MA, Graziani I, Gazdar AF, Strack PR, Miele L & Bocchetta M, Oxygen Concentration Determines the Biological Effects of NOTCH1 Signaling in Adenocarcinoma of the Lung. *Cancer Res*,67(2007) 7954.

28 Salehi B, Mnayer D & Özçelik B. Plants of the Genus Lavandula: From Farm to Pharmacy. *Nat Prod Commun*, 13(2018). doi:10.1177/1934578X1801301037.

29 Zara R, Rasul A, Sultana T, Jabeen F & Selamoglu Z. Identification of Macrolepiota procera extract as a novel G6PD inhibitor for the treatment of lung cancer. *Saudi J Biol Sci*, 29(2022) 3372.

30 Selamoglu Z, Özdemir İ, Çiftçi O & Çakır O. Propolis attenuates oxidative injury in brain and lung of nitric oxide synthaseinhibited rats. *J Pharm Care*, 1(2013) 45.

31 Talas ZS, Ozdemir I, Yilmaz I & Gok Y. Antioxidative effects of novel synthetic organoselenium compound in rat lung and kidney. *Ecotoxicol Environ Saf*, 72(2009) 916.

32 Habtemariam S, Daglia M, Sureda A, Selamoglu Z & Gulhan MF, Nabavi SM. Melatonin and Respiratory Diseases: A Review. *Curr Top Med Chem*, 17(2017) 467.