Tropical Journal of Pharmaceutical Research December 2022; 21 (12): 2475-2581
ISSN: 1596-5996 (print); 1596-9827 (electronic)
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Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v21i12.11

Original Research Article

Sini San inhibits breast cancer cell migration and angiogenesis via the HIF-1/VEGF pathway

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Sent for review: 8 August 2022 *Revised accepted: 29 November 2022*

Abstract

Purpose: *To investigate the effects of Sini San (SNS) on breast cancer (BC), and the mechanism of action.*
Methods: *MDA-MB-231 and SK-BR-3 cells were used as breast cancer cell models. Cell viability, migration, and invasion were determined by CCK-8, Transwell and wound healing assays, respectively. SNS mechanism of action and its anti-cancer effect were investigated by network pharmacological analysis, and further verified by Immunoblot.*
Results: *Sini San inhibited the proliferation of the breast cancer (BC) cells., and also suppressed the migration as well as the invasion of BC cells, and also restrained the angiogenesis of BC cells. In performing the network pharmacological analysis of Sini Powder in the treatment of BC, 337 drug-disease targets were obtained. PPI network was established through String, and GO and KEGG enrichment analysis was performed on the target sites. KEGG analysis showed that genes were enriched in HIF-1 and VEGF pathways.*
Conclusion: *Sini San suppressed cell migration as well as angiogenesis via the HIF-1 /VEGF pathway.*

Keywords: *Breast cancer, Sini San (Sini San), Angiogenesis, Cell migration, MDA-MB-231, SK-BR-3 cells, HIF-1/VEGF pathway*

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INTRODUCTION

Breast cancer (BC) is the most common cancer in women, and its incidence is rapidly increasing worldwide, with about 1.7 million new cases each year [1]. However, for women who have been diagnosed, survival has improved, but median survival is still very low. Most metastatic or recurrent breast tumors are incurable, and dramatically improving the survival rate remains the main aspiration of researchers [2]. Angiogenesis is the basic process of tumor malignant progression, and it plays an important role in early tumor metastasis [3]. BC carcinogenesis is a complex biological process,

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and the mechanism of its occurrence is still unclear, requiring further exploration.

Under the condition of lack of western medicine for tumor microenvironment, Traditional Chinese medicine is playing an increasingly important role in tumor prevention and treatment by adopting a multi-target and holistic regulation approach based on a holistic concept and syndrome differentiation [4]. Sini SAN (SNS) is a classical Chinese medicine formula which originated from the treatise on Febrile Diseases in Han Dynasty [5]. It contains four plant medicines: Bupleurum, Paeonia lactiflora, Immaturus aurantii and licorice [6]. Sini SAN can be used as a candidate drug for hepatocellular carcinoma[7]. More and more studies have shown that paeoniflorin in Paeoniflorin, Saponin A in Bupleurum, naringin in Immaturus aurantii and glycyrrhetinic acid in glycyrrhiza glycyrrhiza have anti-breast cancer effects [8]. However, the possible effects of SNS on BC and the possible mechanism are still unclear and need further study.

VEGF is a glycoprotein that plays a variety of roles in tumor angiogenesis, stimulates the formation of new blood vessels and lymphatic vessels, and increases vascular permeability [9]. VEGF primarily stimulates endothelial cells through its receptor VEGFR-1 (FLT-1) or VEGFR-2 (FLK-1)[10]. The interaction between VEGF and VEGFR-2 activates tyrosine amino acid residues contained in the intracytoplasmic tail of the receptor [11]. VEGF is also a downstream target of hypoxia-inducible factor-1 α (HIF-1α)[8]. Several drugs played the anti-cancer role by targeting VEGF and HIF-1.

In this study, Sini SAN was shown to inhibit breast cancer cell migration and angiogenesis through network pharmacological molecular docking. Sini SAN may regulate the HIF-1 /VEGF pathway to play an anticancer role in BC treatment.

EXPERIMENTAL

Cell culture

MDA-MB-231 and MCF-7 breast cancer cell line cells were accessed from the ATCC (Manassas, VA, USA) and kept in DMEM medium supplied with FBS and antibiotics. The cells were maintained in a sterile culturing hood supplied with 5 % CO_2.

SNS formula preparation

The SNS formula was prepared following the clinical prescriptions containing 10 g Bupleurum Chinese DC. (Radix Bupleuri), 10 g Paeonia lactiflora Pall. (Radix Paeoniae Alba), 10 g Citrus × aurantium L. (Fructus Aurantii Immaturus), 10 g Glycyrrhiza uralensis Fisch. (Radix Glycyrrhizae), which were soaked for 1 h and refluxed at 95 °C in distilled water for 1 h. SNS was given at the concentration of 200 μg /mL, 400 μg /mL, and 800 μg /mL respectively.

CCK-8 assay

Breast cancer cells were trypsinized and inoculated into 96-well plates. After overnight incubation, the cells were added with CCK-8 solutions. Absorbance of each well was determined using the ELx 800 Universal microplate reader (BioTek, Inc.) at 490 nm wavelength.

Colony formation assay

Adherent cells were plated into 6-well plates and maintained for 2 weeks. Then cell colonies were fixed with methanol for 30 min and then stained with 1.5 % crystal violet at room temperature for 10 min. cell colonies were counted manually.

Transwell assay

The cells ($2x10^5$ cells) were inoculated into the upper chamber (8.0 μm membrane pores) in DMEM containing 20 % matrigel. Then the cells in the upper chambers were induced to migrate to the bottom chambers possessing complete medium with 10 % FBS. Cells in the top chamber were taken out by cotton swabs, and the remaining cells were fixed in 4 % paraformaldehyde and stained using 0.2 % crystal violet. The cells that migrated or invaded the bottom surface of the filter were counted.

Tube formation assay

The plate was coated with 50 μL of growth factor reduced Corning Matrigel matrix (Corning Lifesciences, USA) and incubated at 37 °C for 45 min to solidify the Matrigel. Tubular structures mimicking angiogenesis were examined using inverted phase contrast microscope.

Database building of compound targets and disease targets

In order to search the potential targets of the Bupleurum (Bupleurum SAPonin A, eugenol, stigmasterol, Coumarin, linolenic acid), Paeoniflorin (paeoniflorin, β -sitosterol), Immaturus aurantii (naringin, naringin), Glycyrrhizin (Glycyrrhetinic acid, glycyrrhizin, formononetin), the ingredients targets were

obtained through the Swiss target prediction website (http://www.swisstargetprediction.ch/), and the targets were obtained from the genecard website (https://www.genecards.org/). Using R language and breast cancer in the ingredients target intersection, Map Wayne was plotted. PPI network was then established for the targets through the String website (https://string-db.org/), and GO and KEGG enrichment analysis was conducted for the targets through R language.

Immunoblot assay

The entire protein content was extracted using RIPA buffer (Beyotime) and protein concentration was measured with BCA kit. The proteins were then resolved by SDS-PAGE, and transferred to PVDF membranes. Then the membranes were blocked with 5 % BSA in TBST. Subsequently, the membranes were incubated with primary antibodies targeting HIF-1a (1:1000, Abcam), VEGFA (1:1000, Abcam), and beta-actin (1:20000, Abcam) and secondary antibodies for 2 h, and then visualized with ECL kit.

Statistical analysis

This was performed using GraphPad 7.0. Data were presented as mean ± SEM. Statistical comparison was achieved by Student's t-test, and p <0.05 considered significantly different.

RESULTS

SNS inhibits breast cancer cell proliferation

The role of SNS in breast cancer cell proliferation was analyzed using CCK-8 assay. As shown in Figure 1 A, SNS treatment led to reduced cell proliferation in a dose dependent manner, and SNS induced reduced colony formation both in MDA-MB-231 and MCF-7 cells (Figure 1 B and C). Thus, SNS significantly suppressed cell proliferation in breast cancer cells.

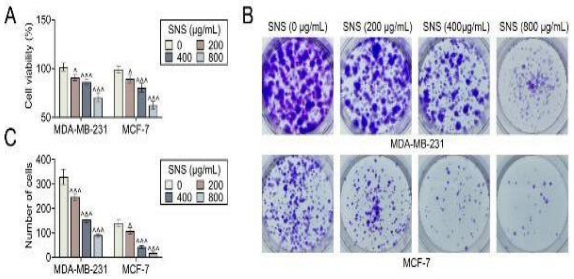

Figure 1: SNS inhibits breast cancer cell proliferation. A: The cell viability in MDA-MB-231 and MCF-7 cells were determined by CCK-8 assay; B, C: The colony formation in MDA-MB-231 and MCF-7 cells were detected. ^P < 0.05, ^^p < 0.01, ^^^p < 0.001

SNS alleviates cell migration and invasion in breast cancer

The cell migration and invasion were determined by transwell and wound healing assay. SNS addition led to reduced migration ability in breast cancer cells (Figure 2 A and B). Moreover, SNS induced impaired wound healing both in MDA-MB-231 and MCF-7 cells (Figure 2 C and D). These results suggest that SNS is associated with impaired cell migration and invasion in breast cancer.

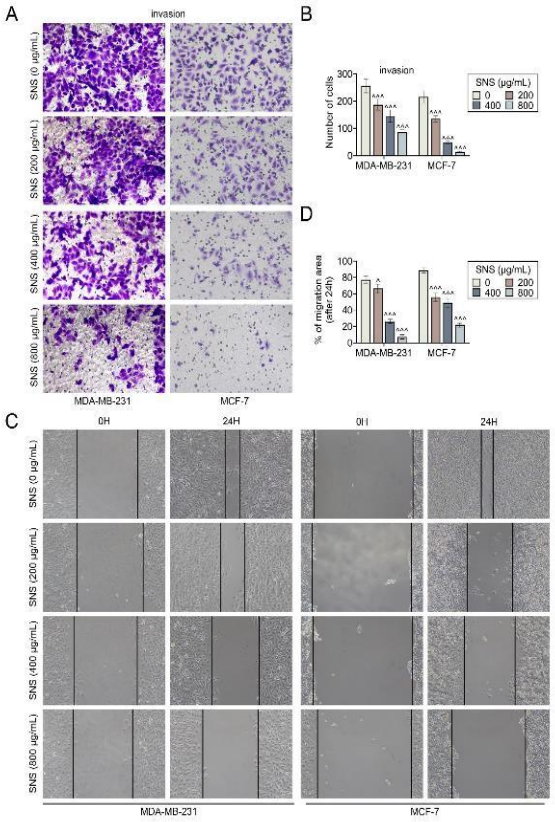

Figure 2: SNS alleviates cell migration and invasion in breast cancer. A, B: The cell migration in MDA-MB-231 and MCF-7 cells were determined by transwell assay; B, C: The cell invasion in MDA-MB-231 and MCF-7 cells were determined by wound healing. ^P < 0.05, ^^p < 0.01, ^^^p < 0.001

SNS Inhibits tube formation

In order to study the effect of SNS on angiogenesis *in vitro*, matrigel tube formation assay was performed. As shown in Figure 3, the tube-like structures in SNS treated cells decreased significantly at the doses of 200, 400 and 800 μM of SNS, and the branch point numbers were reduced in SNS treated cells (Figure 3).

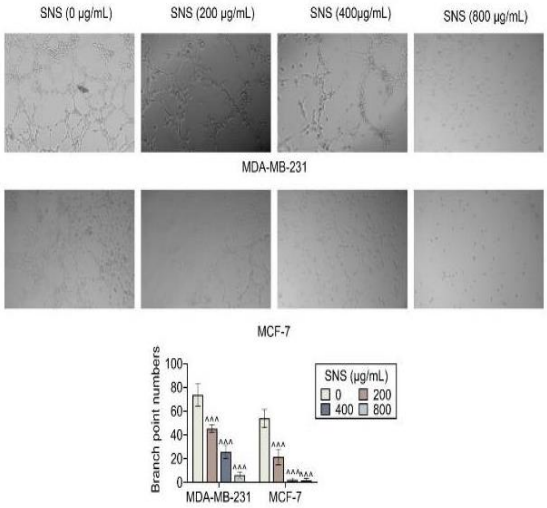

Figure 3: SNS inhibits tube formation. The angiogenesis in MDA-MB-231 and MCF-7 cells were determined by tube formation assay. ^P < 0.05, ^^p < 0.01, ^^^p < 0.001

Potential target of SNS in alleviating cell migration and invasion in breast cancer

As revealed by compound-target network (Figure 4 and Figure 5), there were Bupleurum (bupleurum saponin A, eugenol, marosterol, curmarin, linolenic acid), Paeoniflorin (paeoniflorin, β -sitosterol), Poncirus aurantii (naringin, naringin), and glycyrrhizin (glycyrrhetinic acid, glycyrrhizin, formononetin) in the SNS. Swiss Target Prediction website (http://www.swisstargetprediction.ch/) was used to obtain 370 ingredients targets. Through the genecard website (https://www.genecards.org/), 15,298 breast cancer targets were obtained. Using R language and breast cancer in the ingredients target intersection, map Wayne, 337 targets were identified (Figure 4 A). PPI network was established for the 337 targets through the String website (https://string-db.org/) (Figure 4 B and C), and GO and KEGG enrichment analysis was conducted for the targets through R language (Figure 4 D - G). KEGG analysis revealed that the gene was enriched in HIF-1 and VEGF pathways.

SNS regulates HIF-1 /VEGF pathway to play an anticancer role in breast cancer

After the bioinformatic analysis of the SNS anticancer function in breast cancer, the HIF-1/VEGF pathway was analyzed. As shown in Figure 5, SNS cells showed suppressed levels of HIF-1α and VEGFA. In all, SNS regulated the HIF-1 /VEGF pathway which plays an anticancer role in breast cancer.

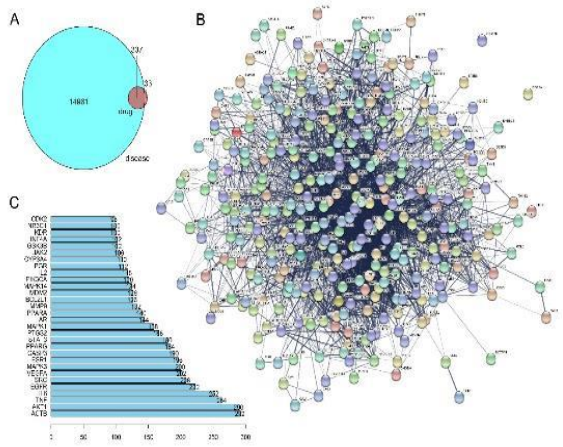

Figure 4: Potential target of SNS in alleviating cell migration and invasion in breast cancer. A, B, C: PPI network of SNS in breast cancer. ^P < 0.05, ^^p < 0.01, ^^^p<0.001

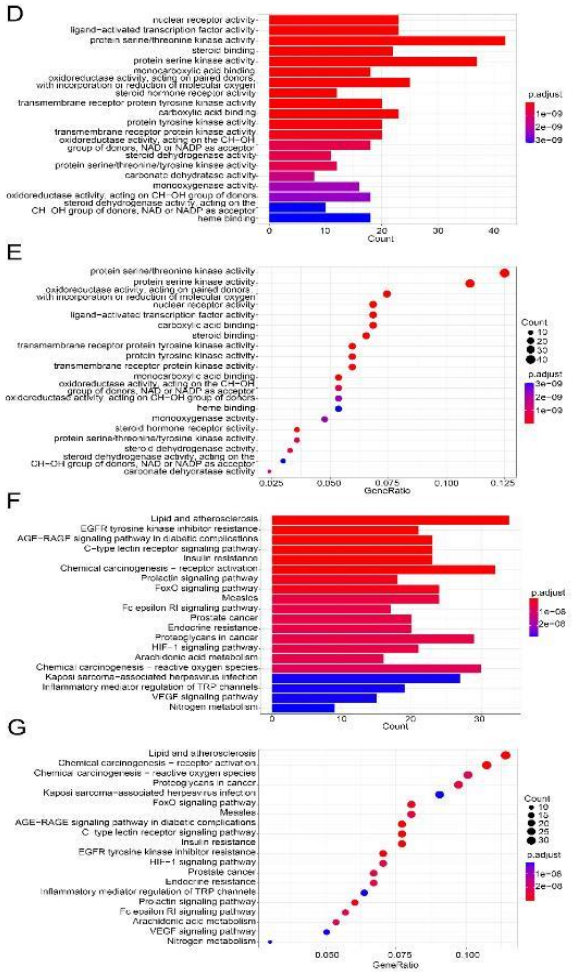

Figure 5: Potential target of SNS in alleviating cell migration and invasion in breast cancer. D, E, F, G: GO and KEGG enrichment analysis of targets both in SNS in breast cancer. ^P < 0.05, ^^p < 0.01, ^^^p<0.001

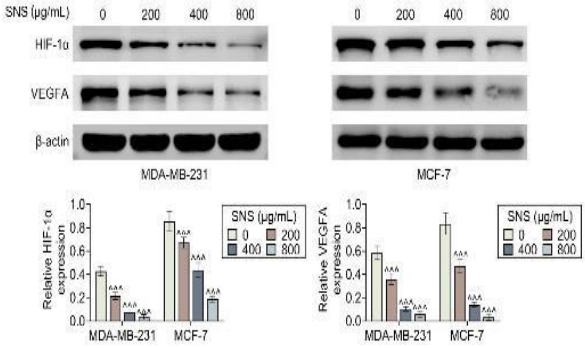

Figure 5: SNS regulates HIF-1 /VEGF pathway to play an anticancer role in breast cancer. A: The protein level of HIF-1 /VEGF pathway in in MDA-MB-231 and MCF-7 cells. ^$P < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$

DISCUSSION

Breast cancer is an out-of-control proliferation of mammary gland epithelial cells due to a variety of carcinogenic factors [12,13]. The early stage of the disease is often manifested as breast lumps, nipple discharge, axillary lymph node enlargement and other symptoms, and the late stage can be manifested as the distant metastasis of cancer cells, multiple organ lesions and the direct threat to the patient's life [14,15]. According to the latest data from the International Agency for Research on Cancer (IARC) in 2018, breast cancer is the most common cancer among women worldwide, accounting for 24.2 % of all cancers, with 52.9 percent occurring in developing countries.

In China, the incidence of breast cancer is increasing every year, as more than 300,000 women are diagnosed with breast cancer every year [16]. The rise in breast cancer is particularly pronounced in eastern coastal areas and large cities with developed economies. To combat this disease, new and more effective drugs are still needed. In this study, Sini SAN has been shown to be capable of inhibiting breast cancer cell migration and angiogenesis, and through network pharmacological molecular docking. Sini SAN could serve as a drug for BC.

Traditional Chinese medicine (TCM) prescriptions are characterized by multiple components and targets [10]. Traditional experimental methods have not been successful in characterizing the underlying complex pharmacological mechanisms. In addition, TCM is not used globally due to the current lack of understanding of specific activities and mechanisms. In order to ensure effective clinical application, it is necessary to clarify the scientific basis and mechanism of the beneficial effects of TCM [11], and web-based pharmacology analysis can help illuminate complex pathways. In this study, network pharmacological analysis combined with experimental validation was used to study the role and mechanism of SNS in breast cancer.

Sini San can be used as a candidate drug in the treatment of various tumors [6,17]. More and more studies have shown that paeoniflorin in Paeoniflorin, Saponin A in Bupleurum, naringin in Immaturus aurantii and glycyrrhetinic acid in glycyrrhiza glycyrrhiza have anti-breast cancer effects [10,18]. In this study, the anti-BC effect of Sini SAN was confirmed.

A total of 370 targets of the active ingredients in sini SAN single drug were screened, and 337 drug-disease targets were obtained by intersection with the breast cancer targets in Genecard (15298 targets) and the drawing of a Venn diagram. A PPI network was established through String, and GO and KEGG enrichment analysis was performed on the target sites. KEGG analysis showed that the genes were enriched in HIF-1 and VEGF pathways. Therefore, the possible mechanisms underlying Sini SAN suppressing BC was revealed.

Sini SAN could also regulate the HIF-1/VEGF pathway to play an anticancer role in BC treatment. The interaction between VEGFR-2 and VEGFR-2 activates the tyrosine amino acid residues contained in the intracytoplasmic tail of the receptor, which triggers different signaling casements in endothelial cells [4,7]. HIF-1/VEGF pathway plays an important role in the progression and metastasis of a variety of tumors, especially breast cancer. So it may serve as a potential therapeutic target for breast cancer. Several drugs also intervene in this pathway to treat BC.

CONCLUSION

Sini SAN inhibits breast cancer cell migration and angiogenesis. Furthermore, network pharmacological molecular docking indicates that Sini SAN may regulate HIF-1 /VEGF pathway to play an anticancer role in BC treatment. Sini SAN is, therefore, a promising drug for BC.

DECLARATIONS

Acknowledgements

None provided.

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Tingting Zhang and Chen Lu designed the study and carried them out; Mingming Lv and Shengwang Du supervised the data collection, analyzed the data, and interpreted the data; and Xinjun Wu and Changqin Wang prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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