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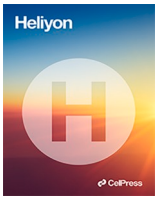

Research article

Dextran sulfate inhibits proliferation and metastasis of human gastric cancer cells via miR-34c-5p

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ABSTRACT

Background: Gastric cancer (GC) is a malignant tumor with a high global mortality rate that is currently difficult to treat. Dextran sulfate (DS), a safe anti-tumor agent, can effectively inhibit the malignant biological behavior of gastric cancer; however, its mechanism of action is not fully understood. Therefore, this study aimed at elucidate the potential mechanisms of action.

Methods: In this study we used DS to intervene in lentivirus-transfected gastric cancer cells to observe the effect of DS on miR-34c-5p. RT-qPCR, CCK-8, clone formation assay, wound healing assay, transwell assay and western blot were used to examine whether DS affects the proliferation and metastasis of gastric cancer cells via miR-34c-5p. The results were validated using in vivo experiments.

Results: Our data confirmed that DS up-regulated miR-34c-5p expression in human gastric cancer cells. Moreover, DS intervention enhanced the inhibitory effect of miR-34c-5p over-expression on the proliferation, invasion, and migration of gastric cancer cells, and partially reversed the promotive effect of miR-34c-5p on the proliferation, invasion, and migration of gastric cancer cells. In addition, DS could affect the activation of the MAP2K1/ERK signaling pathway through the up-regulation of miR-34c-5p, thereby inhibiting the malignant biological behavior of gastric cancer. Finally, it was demonstrated that DS could also inhibit the expression of MAP2K1 in vivo, which in turn inhibits the activation of the ERK signaling pathway to exert anti-cancer effects.

Conclusion: DS may inhibit the proliferation and metastasis of gastric cancer cells by regulating miR-34c-5p, which may be a new option for clinical treatment.

1. Introduction

Gastric cancer (GC) is one of the most common malignant tumors in Asia [1]. Owing to its low early diagnosis rate and susceptibility to abdominal metastasis, GC has a high mortality rate and low 5-year survival rate [2,3]. To diagnose gastric cancer early and

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improve its prognosis, there is an urgent need to find effective anti-tumor drugs and diagnostic targets for the clinical treatment of gastric cancer.

Dextran sulfate (DS) has received much attention as a new anti-tumor drug with high safety, low toxicity and long-lasting effects, and its use in the clinical treatment of gastric cancer may play a significant role. Previous studies have shown that dextran sulfate can play an anti-cancer role in gastric cancer by down-regulating the expression of Nrf2, HO-1, HIF-1α and ITGβ1 [4–6], but the specific regulatory mechanism of DS in gastric cancer needs to be further explored.

MicroRNAs (miRNAs) are a group of single-stranded small molecule RNAs that can regulate the post-transcriptional levels of target mRNAs by complementary binding to their 3′-untranslated regions (UTRs) and participate in regulating the biological behavior of many tumors, possessing great potential as clinical therapeutic targets [7,8]. For example, APBB2, SRPK1 and TRIAP1 were highly expressed in gastric adenocarcinoma, hepatocellular carcinoma cslls and osteosarcoma cells. miR-30a-3p, miR-155 and miR-539 can inhibit the proliferation and metastasis of gastric adenocarcinoma, hepatocellular carcinoma cells and osteosarcoma cells by targeting APBB2, SRPK1 and TRIAP1 [9–11]. In recent years, an increasing number of researchers have focused on the relationship between anti-tumor drugs and miRNAs, and have tried to prove that the molecular mechanisms of anti-tumor drug action in tumors are achieved by affecting the expression of miRNA. As a result, several molecules and drugs with targeting effects have been discovered and used in clinical trials [12]. For example, Sheng et al. found that curcumin could inhibit the malignant progression of prostate cancer by targeting miR-9 to inhibit the malignant progression of prostate cancer [13]; metformin can down-regulate miR-21-5p to inhibit breast cancer cell proliferation and metastasis [14]; and sevoflurane, a volatile anesthetic, can inhibit the malignant progression of laryngeal squamous cell carcinoma in vitro via the miR-26a/FOXO1 axis [15]. Our previous study found that miR-34c-5p inhibits the malignant biological behavior of gastric cancer, and miR-34c-5p was directly target mitogen-activated protein kinase kinase 1 (MAP2K1, MEK1) to exert inhibitory effects [16]. DS could similarly inhibit gastric cancer progression, whereas MAP2K1, a key factor in the MEK/ERK signaling pathway, directly phosphorylates ERK and activates the pathway. Therefore, in this study, we sought to demonstrate that the anti-gastric cancer effect of DS was associated with miR-34c-5p and further explore whether the operational mechanism involved is related to the activation of the ERK signaling pathway, in order to provide a theoretical basis for the clinical treatment of gastric cancer.

2. Materials and methods

2.1. Cell culture

Normal gastric mucosal epithelial cells GES-1 and human gastric cancer cells MKN-45 were purchased from Shanghai Zhongqiao Xinzhou Biotechnology Co Ltd, human gastric cancer cells HGC-27 were purchased from Wuhan Prosperity Life Science Co Ltd, human gastric cancer cells BGC-823 were purchased from Beijing Golden Amethyst Bio-medicine Science and Technology Co Ltd, human gastric cancer cells MKN-28 were kindly given to us by Affiliated Hospital of North China University of Traditional Chinese Medicine, and human gastric cancer cells AGS and SGC-7901 were kindly given to us by East China Normal University. All cells were cultured in RPMI-1640 culture medium (Biological Industries) and 37 °C, 5 % CO_2 incubator, where RPMI-1640 culture medium contained 10 % fetal bovine serum (Biological Industries) and 1 % penicillin mixture.

2.2. Cellular lentivirus transfection

The following products were bought from GenePharma: miR-34c-5p mimic, mimic negative control (NC), miR-34c-5p inhibitor and inhibitors negative control (NC). Small interfering RNA (siRNA) of MAP2K1, MAP2K1 over-expression plasmid, and empty plasmid (Vector) were purchased from Sangon Biotech. INVI DNA RNA transfection reagent was applied for transfection.

2.3. Preparations of DS

DS is an anti-tumor drug, and previous studies by the group have shown that 0.3 % concentration of DS solution could exert the maximum anti-tumor effect. Therefore, DS (Sigma) was dissolved in phosphate-buffered saline (PBS) for cell culture and then sterilized using a 22-μm filter, to obtain a final concentration of 0.3 % [4].

2.4. Quantitative real-time polymerase chain Reaction(RT-qPCR)

MiRNA was extracted from tissues and cells using the E.Z.N.A.® miRNA Kit, and total RNA was extracted from HGC-27 and MKN-28 cells after transfection with lentivirus according to the TRIzol® reagent instructions, followed by reverse transcription of RNA into cDNA using the PrimeScript™ RT reagent kit (Takara), and amplification was performed using the TB Green® Premix Ex Taq™ II kit (Takara). Amplification was performed, U6 and GAPDH were used as internal parameters and the $2^{-\triangle\triangle ct}$ method was used to quantify the relative expression levels of the genes. The primer sequences used were as follows:

Gene	Primer sequences(5'–3')
miR-34c-5p-F	GCCAGGCAGTGTAGTTAGCT
miR-34c-5p-R	ATCCAGTGCAGGGTCCGAGG

(continued on next page)

(*continued*)

MAP2K1–F	ATGTTTGGGTGCCAGGTGGA
MAP2K1-R	GGTCGGCTGTCCATTCCGTA
U6–F	CGCTTCGGCAGCACATATAC
U6-R	TTCACGAATTTGCGTGTCAT
GAPDH-F	GACTCATGACCACAGTCCATGC
GAPDH-R	AGAGGCAGGGATGAT GTTCTG

2.5. Western blot

Total protein from tissues or cells were extracted using the Whole Protein Extraction Kit (KeyGEN BioTECH), and proteins were quantified using the BCA Protein Content Assay Kit (KeyGEN BioTECH). Equal amounts of proteins were separated by 10 % SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, USA) for closure. The membranes were incubated with primary antibodies overnight at 4 °C. Primary as follows: E-cadherin (1:300, Santa Cruz Biotechnology), N-cadherin (1:1000, Bioss), Vimentin (1:1000, Bioss), MAP2K1 (1:1000, Affinty), ERK (1:1000, Cell Signaling Technology), P-ERK (1:2000, Cell Signaling Technology), PCNA (1:1000, Affinty), GAPDH (1:1000, Proteintech), and tubulin (1:1000, CWBIO). Images were quantified using the Amersham Imager 600 Gel Imaging Analysis System. All experiments were performed in triplicate.

2.6. Cell proliferation assay

Cell proliferation capacity was assessed using the Cell Counting Kit8 (APExBIO). Cells were seeded at 5000 per well in 96-well culture plates. After the cells were attached to the wall in each group, the medium was changed, where the DS intervention group was replaced with a solution containing 0.3 % DS, after which the cell viability was determined at 0 h, 24 h, 48 h and 72 h. For the colony formation experiments, 1×10^3 cells per well were inoculated into 6-well culture plates at 37 °C and 5 % CO_2. After the cells were attached to the wall, different culture media were followed to each group according to the subgroups, such as the DS group, the miRNA-34c-5p-mimics + DS group, and the miRNA-34c-5p-inhibitor + DS group needed to undergo a 0.3 % DS solvent change. After 14 days, cells were fixed with 4 % paraformaldehyde and stained with 0.1 % crystal violet. Colony formation was assessed by counting the number of stained colonies. All experiments were performed in triplicate.

2.7. Cell migration and invasion assays

A wound healing assay was performed to detect the migration ability of cells. Cells were inoculated into 6-well plates at a ratio of 5×10^5 cells/well, discarded the old medium when each group of cells was full grown, put a straight ruler under the 6-well plate, and used a 200 μl pipette tip to draw a vertical line vertically along the straight ruler. Photographs were taken under a microscope at 0 h and 24 h to record the distance of the scratch. The ability of gastric cancer cells to migrate and invade was measured using transwell chambers (8 μm pore size membrane). Cells were resuspended in serum-free medium, adjusted to 5×10^5 cells/ml, inoculated into the upper chamber of the transwell, RPMI-1640 medium containing 10 % FBS or solution containing 0.3 % DS was added to the lower chamber, and incubated at 37 °C and 5 % CO_2 to determine cell migration ability, and matrix gel Matrigel (BD Biosciences, USA) was spread in the upper chamber of the small chamber to measure cell invasion ability. After incubation for 24 h, cells in the upper chamber were gently wiped using sterile cotton swabs, fixed with 4 % paraformaldehyde for 20 min, stained with 0.1 % crystal violet for 15 min, and photographed under a microscope to observe the number of cells penetrating the membrane. All experiments were performed in triplicate.

2.8. In vivo tumorigenesis

Twelve BALB/c male nude mice aged 5–6 weeks and weighing 17–19g were selected as experimental subjects, and 12 nude mice were randomly divided into the control group and the DS group (a group of 6 mice). HGC-27 cells were resuspended in saline, and cells number were adjusted to 1×10^7 cells/ml. The groups and numbers were marked on the body of the nude mice with a black marker, and cell suspension was injected into the peritoneal cavity of the nude mice with a 1 ml syringe. On the 2nd postoperative day, 1 ml of saline was given to each nude mouse in the control group, and 1 ml of DS solution per nude mouse in the experimental group. After 14 days, the mice were executed. The tumors were weighed. The greater omentum and liver tissues were also collected and total RNA and tissue proteins were extracted for experimental analysis. The tumors were removed and their length and width were measured manually using a caliper, and the tumor volume in mm^3 was calculated by the formula: $\text{Volume}=(\text{width})^2 \times \text{length}/2$ [11].

2.9. Statistical analysis

The experimental data were statistically analyzed and plotted using SPSS 23.0 and Graphpad Prism 8.0. The test results of each group are expressed as mean ± standard deviation ($\bar{x}\pm SD$), and a two-sample *t*-test was used to compare the means between two groups, one-way ANOVA was used to compare the means of multiple groups, and the LSD test was used to compare the means of multiple groups. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. DS upregulates mir-34c-5p expression in gastric cancer cells

Six gastric cancer cell lines (MKN-28, AGS, MKN-45, BGC-823, SGC-7901, and HGC-27) and normal gastric mucosal cells (GES-1) were selected to detect the relative expression of miR-34c-5p. The results showed that miR-34c-5p mRNA expression in gastric cancer cell lines was lower than that in normal gastric mucosal cells (Fig. 1A). Changes in miR-34c-5p mRNA expression was detected by RT-qPCR after intervention with 0.3 % DS solution in gastric cancer cells, and the results showed that the expression level of miR-34c-5p in gastric cancer cells was significantly increased after DS intervention (Fig. 1B), suggesting that DS may exert its anti-cancer effect by up-regulating the expression of miR-34c-5p.

3.2. DS inhibits proliferation, invasion and migration of gastric cancer cells by regulating mir-34c-5p

To verify that DS inhibits the malignant biological behavior of gastric cancer by regulating miR-34c-5p expression, we first transfected the miR-34c-5p mimic and mimic negative control into HGC-27 cells and the miR-34c-5p inhibitor and inhibitor negative control into MKN-28 cells. Transfection was observed under an inverted fluorescence microscope and the results showed that the cells in the mimics-NC, miR-34c-5p-mimics, inhibitor-NC and miR-34c-5p-inhibitor groups all expressed green fluorescent protein with >90 % transfection efficiency and successful transfection (Fig. 2A).

Next, the transfected cells were treated with 0.3 % DS solution, and the proliferation, invasion and migration ability of gastric cancer cells were detected by CCK-8, clone formation, wound healing, and transwell assays. The results showed that DS intervention could enhance the inhibitory effect of miR-34c-5p over-expression on the proliferation, invasion and migration of gastric cancer cells. In contrast, inhibition of miR-34c-5p promoted the proliferation, invasion and migration of gastric cancer cells, and after DS intervention, the cell proliferation, invasion and migration abilities of DS + miR-34c-5p-inhibitor group were significantly attenuated, suggesting that DS treatment reverses the promotional effect on gastric cancer cells after knockdown of miR-34c-5p (Fig. 2B–G). Expression of proliferation, invasion and migration related factors was detected by western blot assay. The results showed that the expression levels of PCNA, Vimentin and N-cadherin in the DS + miR-34c-5p-mimics group were significantly lower, while the expression levels of E-cadherin were significantly higher than those in the miR-34c-5p-mimics group. Compared with the miR-34c-5p-inhibitor group, the expression levels of PCNA, Vimentin and N-cadherin in the DS + miR-34c-5p-inhibitor group were significantly lower and the expression level of E-cadherin was significantly higher(Fig. 2H–I). The above results indicated that the tumor suppressive effect of DS was significantly enhanced when miR-34c-5p was over-expressed, and conversely, the tumor suppressive effect of DS was attenuated when miR-34c-5p was inhibited.

3.3. DS downregulates MAP2K1 expression and inhibits activation of ERK signaling pathway in gastric cancer

Since our previous study showed that miR-34c-5p directly targets MAP2K1. MAP2K1 can directly phosphorylates ERK and constitutes the MEK/ERK signaling pathway involved in tumor progression. To verify the effect of DS on MAP2K1, RT-qPCR revealed that DS significantly down-regulated the expression of MAP2K1 mRNA in gastric cancer cells (Fig. 3A). The results of the western blot assay demonstrated that DS intervention significantly down-regulated the protein expression of MAP2K1 and P-ERK, which means that it inhibits the activation of the ERK signaling pathway (Fig. 3B).

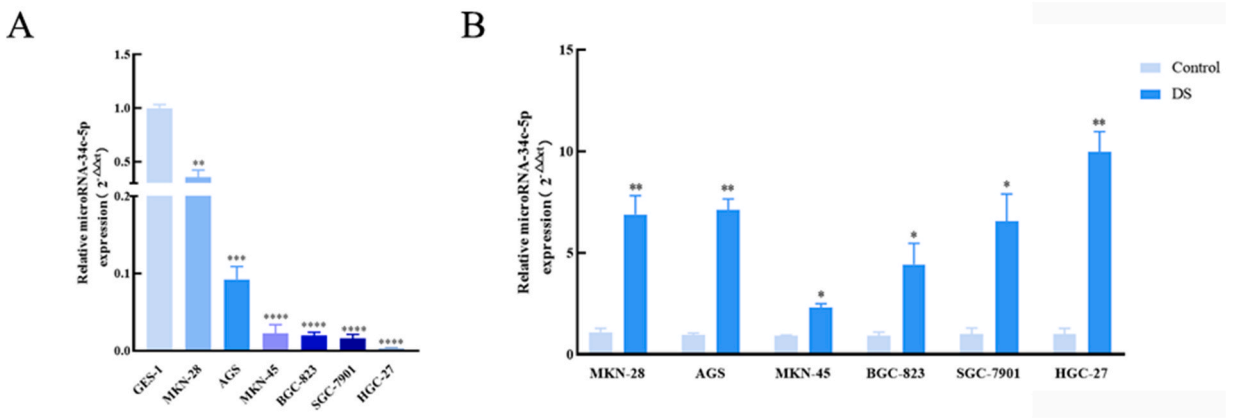


Fig. 1. DS up-regulates miR-34c-5p expression in gastric cancer cells. (A) Expression of miR-34c-5p in normal gastric mucosa epithelial cell line (GES-1) and six gastric cancer cell lines (MKN-28, AGS, MKN-45, BGC-823, SGC-7901, HGC-27). (B) RT-qPCR to detect changes in miR-34c-5p expression in gastric cancer cells after DS intervention.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

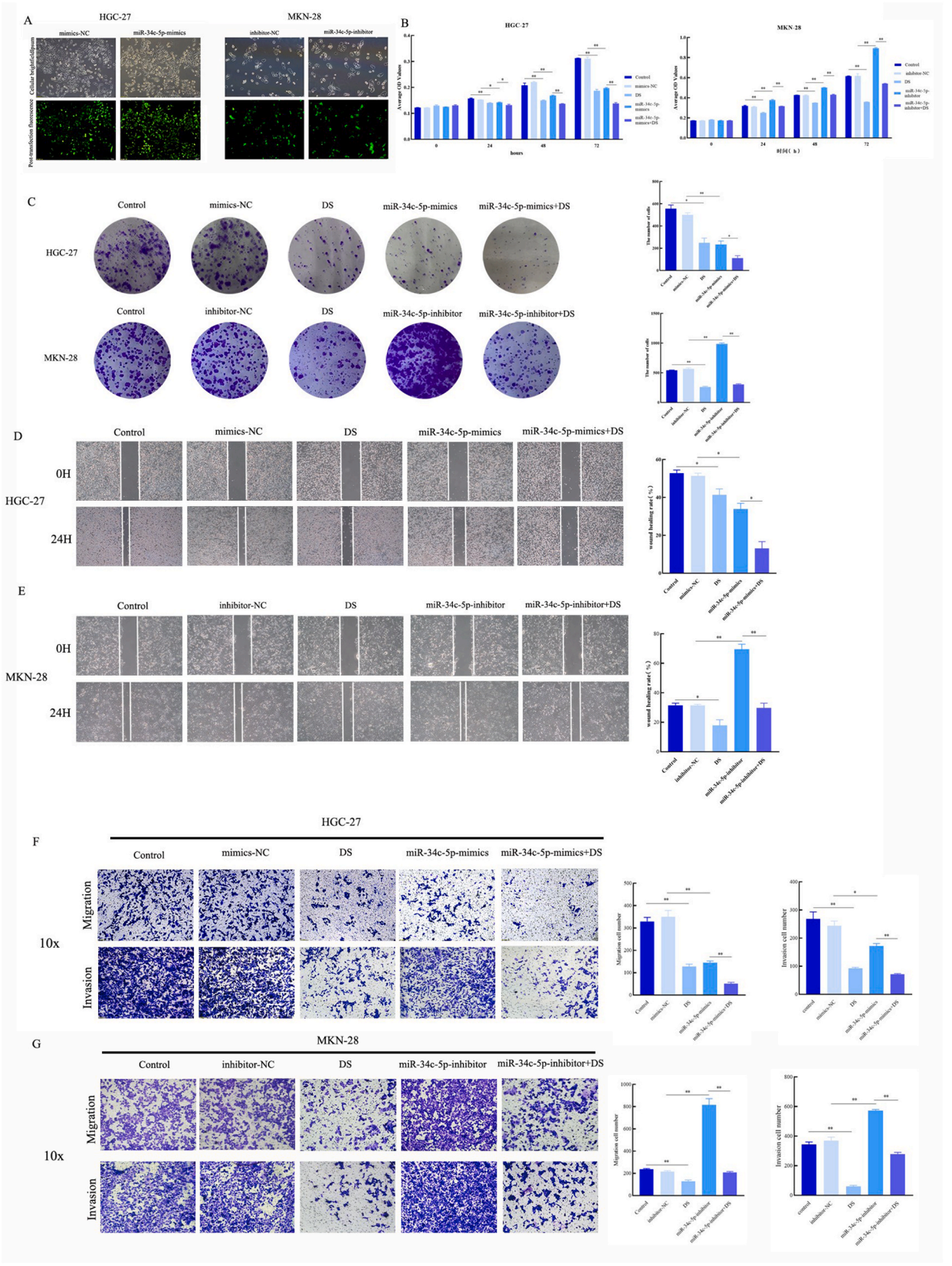
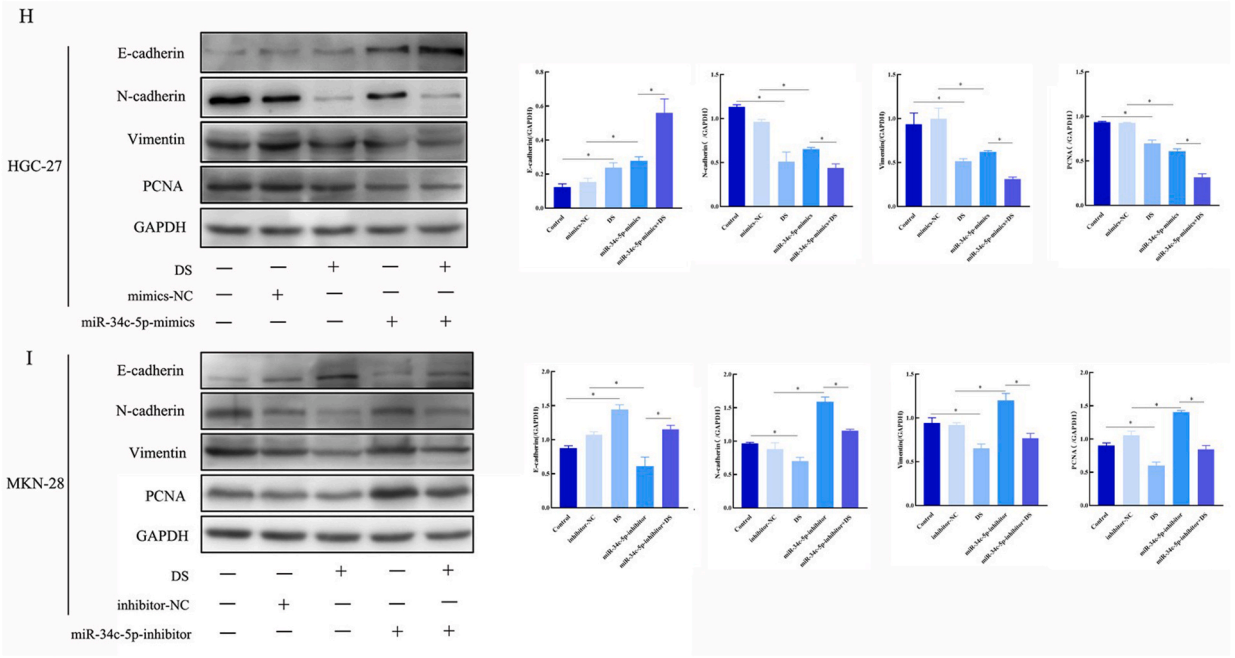

A
HGC-27
MKN-28
mimics-NC
miR-34c-5p-mimics
inhibitor-NC
miR-34c-5p-inhibitor
Cellular brightfield
Post-transfection fluorescence
B
HGC-27
MKN-28
Average OD Value
hours
时间(h)
Control
mimics-NC
inhibitor-NC
DS
miR-34c-5p-mimics
miR-34c-5p-inhibitor
miR-34c-5p-mimics+DS
miR-34c-5p-inhibitor+DS
C
HGC-27
MKN-28
Control
mimics-NC
inhibitor-NC
DS
miR-34c-5p-mimics
miR-34c-5p-inhibitor
miR-34c-5p-mimics+DS
miR-34c-5p-inhibitor+DS
The number of cells
D
HGC-27
0H
24H
wound healing rate(%)
E
MKN-28
F
HGC-27
10x
Migration
Invasion
Migration cell number
Invasion cell number
G
MKN-28

Fig. 2. DS inhibits proliferation, invasion and migration of gastric cancer cells by regulating miR-34c-5p. (A) Transfection efficiency by inverted fluorescence microscopy. (B) CCK-8 assays to detect the proliferation ability of each group of cells. (C) Colony formation assays to detect the proliferation ability of each group of cells. (D, E) Wound healing assays to detect the migration ability of each group of cells. (F, G) Cell migration and invasion capacity were assessed by transwell assay. (H, I) Western blot was performed to detect E-cadherin, N-cadherin, Vimentin, PCNA protein expression levels in each cell group. (The original image is provided in the Supplementary file). *$P < 0.05$, **$P < 0.01$.



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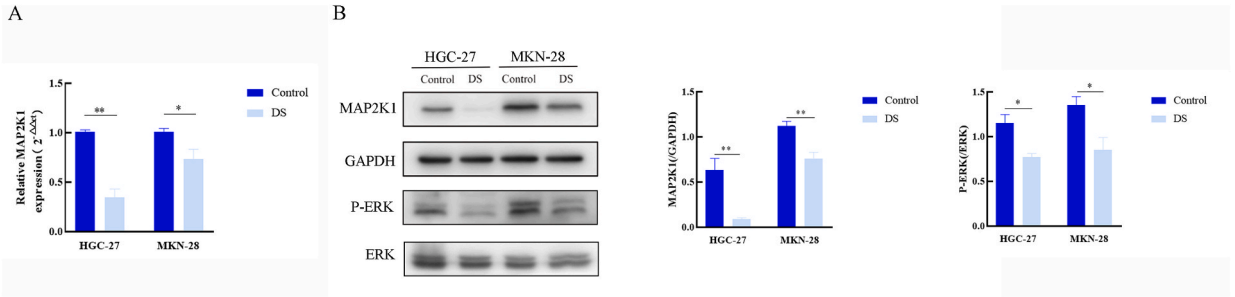


Fig. 3. DS down-regulates MAP2K1 expression and inhibits activation of the ERK signaling pathway in gastric cancer. (A) Detection of MAP2K1 mRNA expression by RT-qPCR. (B) Western blot analysis of MAP2K1, P-ERK, and ERK protein expression levels. (The original image is provided in the Supplementary file). *$P < 0.05$, **$P < 0.01$.

3.4. miR-34c-5p mediates DS regulation of MAP2K1

We then explored whether miR-34c-5p could mediate the regulation of MAP2K1 by DS and thus influence the malignant biological behavior of gastric cancer. We first co-transfected HGC-27 cells with the over-expression plasmid PCMV-MAP2K1 and miR-34c-5p mimics, and then co-transfected MKN-28 cells with MAP2K1 siRNA and miR-34c-5p-inhibitor and treated them with DS, and detected the relative expression of MAP2K1 by RT-qPCR. The results showed that the expression of MAP2K1 was significantly higher in the miR-34c-5p-mimics + MAP2K1 group than in the miR-34c-5p-mimics group, while the relative expression of MAP2K1 was suppressed by DS treatment. The expression of MAP2K1 was reduced in the miR-34c-5p-inhibitor + si-MAP2K1 group compared with the miR-34c-5p-inhibitor group, and the expression of MAP2K1 was even lower with DS treatment (Fig. 4A). In vitro experiments confirmed that tumor growth and metastasis were promoted in the miR-34c-5p-mimics + MAP2K1 group compared to those in the miR-34c-5p-mimics group, while tumor growth and metastasis were partially reversed in the DS + miR-34c-5p-mimics + MAP2K1 group. In contrast, tumor growth and metastasis were inhibited in the miR-34c-5p-inhibitor + si-MAP2K1 group compared with the miR-34c-5p-inhibitor group, whereas the DS + miR-34c-5p-inhibitor + si-MAP2K1 group showed enhanced inhibition of tumor

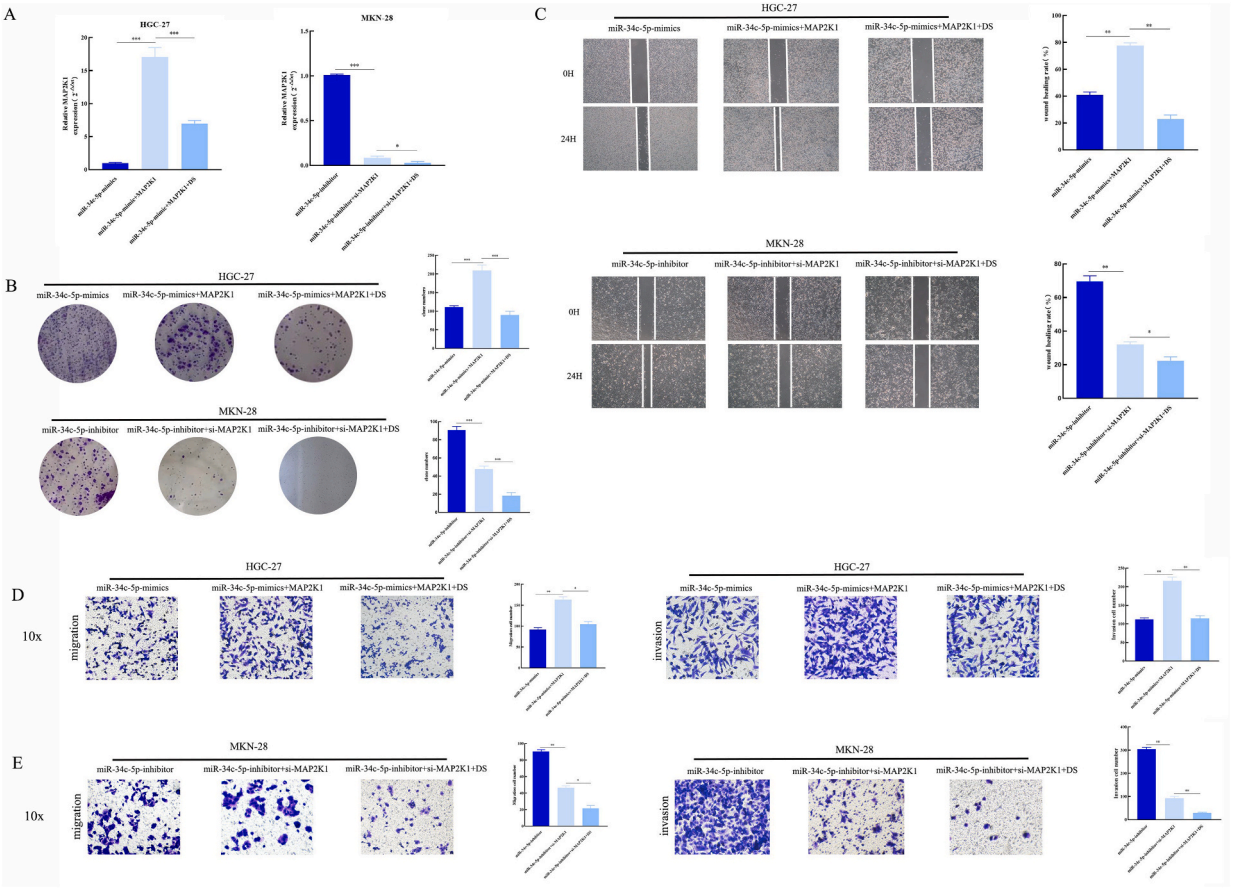


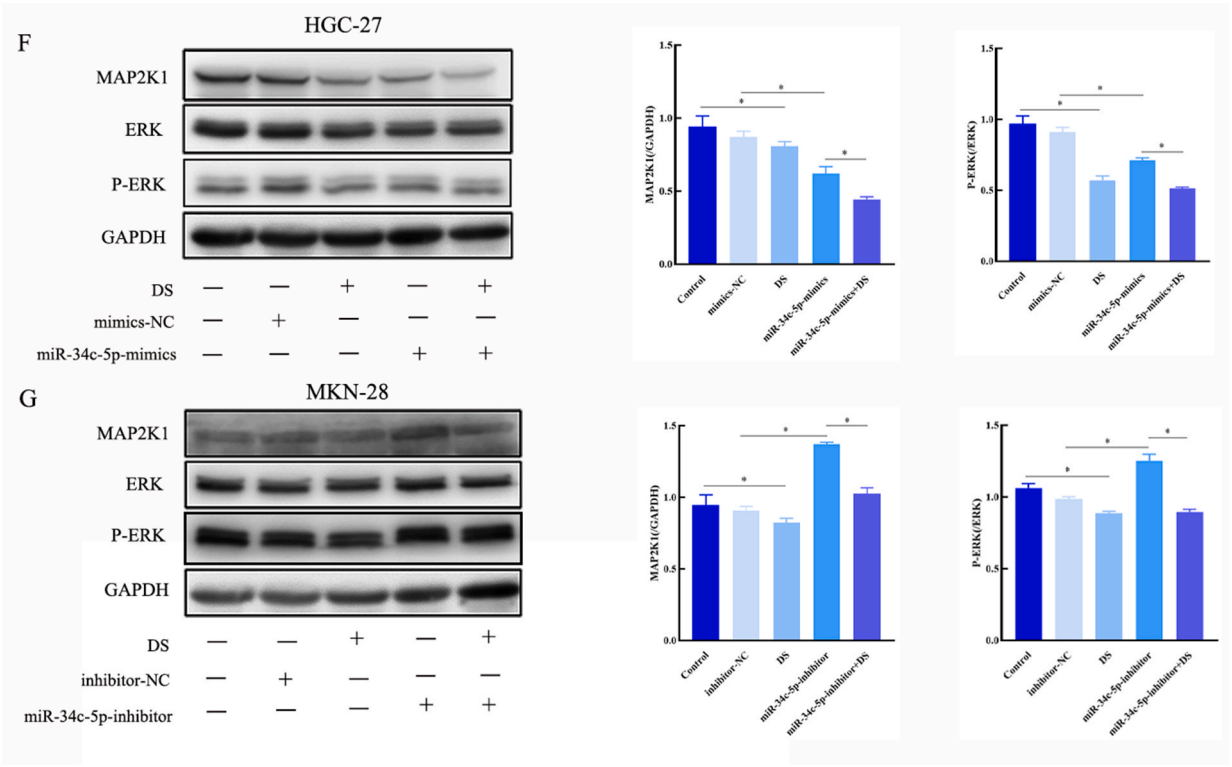
Fig. 4. MiR-34c-5p mediates DS regulation of MAP2K1. (A) RT-qPCR was used to detect the expression level of MAP2K1. (B) The ability of HGC-27 and MKN-28 cells to proliferate was tested using clone formation assays. (C) The migration ability of HGC-27 and MKN-28 cells was tested using a wound healing assay. (D, E) Migration and invasion ability of HGC-27 and MKN-28 cells were tested using Transwell assays. (F, G) Western blot assays to detect MAP2K1, ERK, P-ERK expression. (The original image is provided in the Supplementary file). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

growth and metastasis(Fig. 4B-E). In conclusion, these results suggest that miR-34c-5p can mediate the regulation of MAP2K1 by DS.

To further determine whether the regulation of the ERK signaling pathway by DS was mediated by affecting miR-34c-5p expression, we examined the expression of MAP2K1, ERK, and P-ERK by western blotting. The results demonstrated that the expression levels of MAP2K1 and P-ERK were significantly reduced after transfection with miR-34c-5p-mimics compared with those in the mimics-NC group, indicating that over-expression of miR-34c-5p could inhibit the activation of the ERK signaling pathway. In contrast, the expression levels of MAP2K1 and P-ERK were significantly increased after transfection with the miR-34c-5p-inhibitor, indicating that inhibition of miR-34c-5p could activate the ERK signaling pathway. Compared with the miR-34c-5p-mimics group, the expression levels of MAP2K1 and P-ERK in the DS + miR-34c-5p-mimics group were significantly lower, indicating that DS could enhance the inhibitory effect of over-expression of miR-34c-5p on the ERK signaling pathway. In contrast, MAP2K1 and P-ERK expression levels were reduced in the DS + miR-34c-5p-inhibitor group compared to the miR-34c-5p-inhibitor group (Fig. 4F and G), indicating that DS can reverse the activation of the ERK signaling pathway by miR-34c-5p.

3.5. *In vivo results*

An abdominal implantation metastasis model was established in nude mice, and it was found that after DS intervention, the tumor volume and weight of the control group were significantly larger than those of the DS group, and the levels of miR-34c-5p mRNA in the greater omentum and liver tissues of the nude mice were significantly higher than those of the control group (Fig. 5A and B).Western blot experiments showed that compared with the control group, the levels of PCNA were decreased in the greater omentum and liver tissues after DS intervention, Vimentin, N-cadherin, MAP2K1 and P-ERK protein expression levels were reduced and E-cadherin protein expression levels were increased in the greater omentum and liver tissues after DS intervention compared with the control group (Fig. 5C and D). These data further confirmed that DS inhibited the proliferation and metastasis of gastric cancer possibly by up-regulating the expression of miR-34c-5p, which in turn inhibited the activation of the MAP2K1/ERK pathway.



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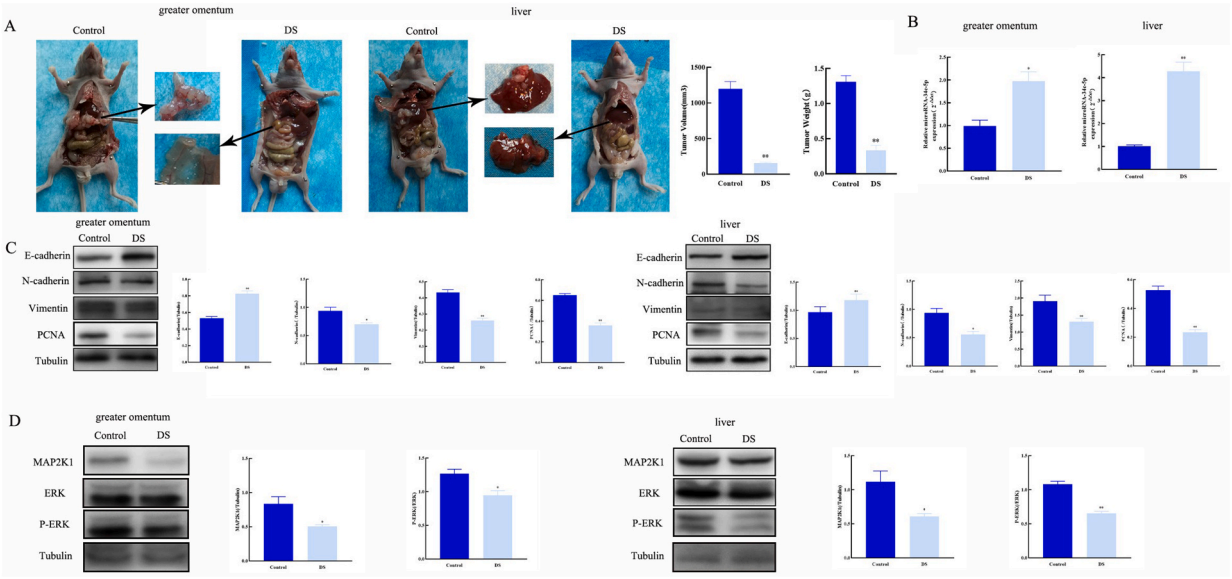

Fig. 5. DS promotes the expression of miR-34c-5p in vivo and inhibits the proliferation and metastasis of gastric cancer as well as the activation of MAP2K1/ERK signaling pathway. (A) Images of abdominal metastases in nude mice. (B) RT-qPCR detection of miR-34c-5p expression in the greater omentum and liver tissue. (C, D) Effect of DS on E-adherin, N-adherin, Vimentin, PCNA, MAP2K1, ERK, P-ERK expression by Western blot. (The original image is provided in the Supplementary file). $^{*}P < 0.05$, $^{**}P < 0.01$.

4. Discussion

Gastric cancer, the fifth most common and fourth deadliest cancer in the world [17], has evolved from a single surgical treatment to a combination of radiotherapy, chemotherapy and immunotherapy with a better understanding of its pathogenesis. Because the low rate of early diagnosis of gastric cancer and the predisposition to abdominal implantation and metastasis in the late stage are the main causes of the high mortality rate, it has become a hot topic in gastric cancer research to explore novel markers for early diagnosis of gastric cancer and to find effective anti-tumor drugs [18–20].

In the development of gastric cancer, miRNAs play an important role and can be used as a new indicator for diagnosis and prognosis [21,22]. For example, miR-130a-3p, miR-484 and miR-1-3p are aberrantly expressed in gastric cancer and can influence the malignant biological behavior of gastric cancer, and they may be used as new molecular markers for the early diagnosis and treatment of gastric cancer [23–25]. MiR-34c-5p, a member of the miR-34c family, is aberrantly expressed in various cancers and can act as an oncogenic or carcinogenic factor involved in tumor development [26–28]. MiR-34c-5p has been shown to inhibit the proliferation, invasion and migration of tumor cells such as renal cell carcinoma, osteosarcoma and lung cancer [29–31]. Our group has demonstrated in previous studies that miR-34c-5p, which is lowly expressed in gastric cancer, can act as an oncogenic factor to inhibit the development of gastric cancer [16].

DS is a large molecular dextran derivative with a relative molecular weight of 5000–500,000 Da, with different molecular weights acting differently [32]. Our research group has found that DS with a molecular weight of 500,000 Da can effectively inhibit the peritoneal implantation metastasis of gastric cancer through multiple pathways and achieve anti-tumor effects. A growing number of researchers have also confirmed that the mechanism of action of anti-tumor drugs applied in cancer is also closely linked to the regulation of miRNAs, but there are few studies on drugs directly acting on miR-34c-5p, and only Wan et al. found that Pien Tze Huang could inhibit the proliferation of colorectal cancer by increasing the expression of miR-34c-5p in 2017 [33]. Therefore, we venture to speculate that DS may be able to inhibit the malignant biological behavior of gastric cancer by regulating the expression of miR-34c-5p.

To test this conjecture, we first used a pre-assayed 0.3 % DS solution to intervene in gastric cancer cells. After 24 h of culture, we found that DS could up-regulate the expression of miR-34c-5p, suggesting that DS inhibits the proliferation, invasion and migration of gastric cancer cells probably by up-regulating the expression of miR-34c-5p. To test this hypothesis, we selected the two cell lines with the lowest and highest miR-34c-5p expression in gastric cancer cell lines for subsequent experiments, and transfected miR-34c-5p inhibitor to MKN-28 cells with the highest expression, and transfected miR-34c-5p mimic to HGC-27 cells with the lowest expression, so that this treatment could maximally validate the miR 34c-5p in gastric cancer. Afterwards, the transfected cells were treated with DS, and it was found that DS inhibited the proliferation, invasion and migration of human gastric cancer cells by up-regulating miR-34c-5p.

To investigate the specific mechanism by which DS regulates miR-34c-5p, our group has screened the TargetScan database for mitogen-activated protein kinase kinase 1 (MAP2K1) as a downstream target gene of miR-34c-5p and validated it using a dual luciferase reporter gene assay [16]. Therefore, in this study, we directly examined the relative expression of MAP2K1 in gastric cancer cells after DS intervention. The results demonstrated that DS significantly inhibits the expression of MAP2K1 in gastric cancer cells. We then found that miR-34c-5p-mediated inhibition of the proliferation, invasion and migration ability of gastric cancer cells could be reversed by MAP2K1 co-transfection, whereas DS intervention reversed the facilitation effect caused by MAP2K1 co-transfection. These results suggest that DS can exert anti-cancer effects by regulating the miR-34c-5p/MAP2K1 axis. The above conclusions are consistent with the previous speculation, while the mechanism of DS in gastric cancer has been further investigated to provide more directions.

MAP2K1, also known as MEK1, is a tyrosine/threonine (Tyr/Thr) bispecific protein kinase that functions as an important factor in the mitogen-activated protein kinase (MAPK) cascade reaction, and extracellular signal-regulated kinase (ERK) is the only protein downstream of MAP2K1 that can be specifically phosphorylated to activate the MEK/ERK signaling pathway and promote tumor development [34–36]. DS intervention or over-expression of miR-34c-5p both reduced the expression of MAP2K1 and P-ERK and inhibited the activation of the MEK/ERK signaling pathway, conversely, inhibition of miR-34c-5p increased the expression of MAP2K1 and P-ERK and activated the MEK/ERK signaling pathway, which could be reversed after DS intervention. Therefore, we deduced that DS may inhibit the activation of MAP2K1/ERK signaling pathway by up-regulating miR-34c-5p to achieve an anti-tumor effects.

Finally, in vivo experiments revealed that gastric cancer can metastasise to the greater omentum and liver, and DS not only up-regulated the expression of miR-34c-5p in gastric cancer, but also inhibited abdominal implantation metastasis, which was consistent with the results of in vitro cellular experiments.

In conclusion, tumor development mainly originates from the mutation of cells into cancer cells under the action of oncogenic factors in vivo, at which time the cells grow out of control, continue to divide and proliferate, and invasive migration occurs after the cancer cells break through the basement membrane, thus causing metastasis of the tumor. Tumor metastasis is a complex process, which involves the degradation of extracellular matrix, epithelial-mesenchymal transition (EMT), angiogenesis, and invasion of surrounding tissues, which is also the main reason for the difficulty in treating gastric cancer patients with poor prognosis [37]. Therefore, in this experiment, we focused on the new anti-tumor drug DS, trying to explore the mechanism of DS inhibiting the proliferation and metastasis of gastric cancer cells, and found a new molecular pathway-miR-34c-5p/MAP2K1/ERK, which will provide a more adequate basic experimental basis for the application of DS in clinical treatment.

Of course, there are some limitations in the present study. Firstly, we should have been included in the in vivo experiments to verify the inhibitory effect of miR-34c-5p on gastric cancer in nude mice subcutaneous tumor formation experiments, due to the influence of the COVID-19 during the experimental period, it was not possible to purchase enough nude mice to conduct the experiments. Secondly, the mechanism of DS inhibiting the development of gastric cancer is still poorly studied. Earlier studies have shown that the ERK

signalling pathway can directly target MMPs to affect the invasion of cancer cells, and that there is a mutual regulatory relationship between miRNAs and lncRNAs, which jointly affects the occurrence of many diseases, therefore, in the future studies, we can further analyse whether DS influences the invasion of gastric cancer by modulating the axis of lncRNAs/miR-34c-5p/MAP2K1/ERK/MMPs. All of the above need to be further explored in future studies.

5. Conclusion

In summary, DS was shown to significantly inhibit the proliferation and metastasis of gastric cancer in vitro and in vivo, and its mechanism of action may be through the miR-34c-5p/MAP2K1/ERK axis, a finding that provides a new approach for the clinical diagnosis and treatment of gastric cancer.

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Ethics approval statement

The current study was approved by the Medical Ethics Review Committee of Ningxia Medical University (Approval No. 2022-G029).

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Data availability statement

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

CRediT authorship contribution statement

Yuan Zhao: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Qian Ma:** Supervision, Resources, Investigation, Formal analysis. **Wenwei Gao:** Supervision, Software, Project administration, Methodology, Investigation. **Zhaojun Li:** Visualization, Validation, Supervision, Resources. **Guangfu Yu:** Supervision, Software, Methodology, Investigation, Formal analysis. **Bing Li:** Visualization, Validation, Supervision, Software. **Yuanyi Xu:** Writing – review & editing, Visualization, Supervision, Software, Resources, Project administration, Funding acquisition. **Yunning Huang:** Resources, Project administration, Methodology, Funding acquisition, Formal analysis.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Yuanyi Xu and Yunning Huang reports article publishing charges was provided by Natural Science Foundation of Ningxia Province. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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