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Original Research Article

MiR-378a/FSCN1 regulatory axis inhibits tumor stemness and increases the cytotoxicity of chemotherapeutic drugs in colorectal cancer cells

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Abstract

Purpose: *To evaluate the effect of miR-378a/FSCN1 axis on tumor stemness and aerobic glycolysis in colorectal cancer cells (CRC).*

Methods: *Abnormal expressions of miR-378a and FSCN1 in CRC tissues were analyzed through TCGA database. Cell viability and apoptosis were determined using 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide (MTT) assay and flow cytometry, respectively, while expression of miR-378a was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). Furthermore, Western blotting assay was used to assess protein expressions. The miR-378a target was evaluated using ENCORI and confirmed by luciferase assay. Aerobic glycolysis was evaluated by determining glucose uptake, lactate production and lactate dehydrogenase (LDH) activity.*

Results: *Downregulation of miR-378a and upregulation of FSCN1 were observed in both CRC tissues and cell lines ($p < 0.05$). Overexpression of miR-378a and repression of FSCN1 reduced cell viability and tumor sphere formation, and induced cell apoptosis. Protein expression of SOX2, KLF4, Bmi1 and Oct-4 were downregulated by either the overexpression of miR-378a or repression of FSCN1 ($p < 0.01$). Glucose uptake, lactate production and LDH activity were inhibited by either overexpression of miR-378a or repression of FSCN1, while cytotoxicity of Dox and 5-Fu was increased by upregulation of miR-378a or downregulation of FSCN1 ($p < 0.05$). The predictive results of ENCORI demonstrated that FSCN1 was the direct target of miR-378a, and this was confirmed by luciferase assay results ($p < 0.005$). All the effects of miR-378a in CRC were reversed by overexpression of FSCN1 ($p < 0.05$).*

Conclusion: *This study has shown that miR-378a suppresses tumor stemness and increases the cytotoxicity of chemotherapeutic drugs by directly targeting FSCN1, resulting in the prevention of CRC tumorigenesis. Thus, these findings suggest a new approach to the chemotherapeutic management of CRC.*

Keywords: *miR-378a, FSCN1, Tumor stemness, Aerobic glycolysis, Colorectal cancer cells*

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INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related death, with an increasing incidence in developing countries [1]. In 2020, there were 5.55 million new CRC cases in China [2]. According to the Chinese Society of Clinical Oncology guidelines, surgery is the most

common treatment for early CRC. Radiotherapy, chemotherapy, targeted therapy and immunotherapy are recommended for advanced and metastatic CRC in order to prolong the survival of patients. However, the patient outcomes were still poor, with 2.86 million deaths caused by CRC in China in 2020 alone, and resistance to chemotherapy identified as one of the treatment limitations to CRC therapy [2]. Novel drugs are indeed needed to improve the prognosis of CRC patients.

Abnormal energy metabolism such as aerobic glycolysis, is a hallmark of cancer development, metastasis and drug resistance [3]. Aerobic glycolysis is usually accompanied with high glucose uptake, high lactate production and high lactate dehydrogenase (LDH) activity, which result in changes in the tumor microenvironment, thus providing "fuel" for cancer cell growth, angiogenesis and immunosuppression [4]. Therefore, the reduction of aerobic glycolysis may prevent tumor growth and metastasis. In addition, cancer stem cells (CSC) may facilitate tumor, initiate tumorigenesis and promote treatment resistance [5]. The inhibition of tumor stemness is a potential therapeutic strategy for cancer therapy.

It has been reported that miRNAs regulate cancer progression and treatment resistance [1,6]. For example, miR-378a-5p prevents cell proliferation by targeting cyclin-dependent kinase 1 in CRC, indicating a tumor suppressive effect [7]. By inhibiting AMPKα2-FTO-m6A/MYC signaling pathway, miR-96 promoted tumor occurrence and progression in CRC, which aggressively accelerated tumor growth [8].

Fascin actin-bundling protein 1 (FSCN1) is a globular filamentous actin-binding protein belonging to the fascin family [9]. FSCN1 was overexpressed in CRC [10]. A previous study revealed that FSCN1 can be directly suppressed by miR-133 and miR-145 [11,12]. However, there are no studies on the relationship between miR-378a and FSCN1 on the one hand, and the effects of miR-378a and FSCN1 on tumor stemness and aerobic glycolysis in CRC. The purpose of this study was to evaluate the effects of miR-378a/FSCN1 axis on CRC.

EXPERIMENTAL

Cell culture and transfection

Human normal colonic epithelial cells (NCM460 cells) and human colorectal cancer cell (SW48, HCT116, LoVo and DLD-1 cells) were obtained from Shanghai Huzhen Biotechnology Co. Ltd. (Huzhen, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10 % fetal bovine serum (FBS, Senbeijia, China), and 1 % penicillin-streptomycin solution (100 ×, Procell, China). All cells were cultured at 37 °C in an incubator with 95 % air and 5 % CO_2.

Doxorubicin (Dox) and 5-fluorouracil (5-Fu) were purchased from Merck KGaA (Germany) and dissolved in nano-pure water to produce a 10 mM stock solution. Small interfering RNA of FSCN1 (si-FSCN1) and its silenced RNA (si-NC), miR-378a mimic and its negative control (mimic NC) were purchased from Beijing OriGene Biotechnology Co. Ltd (OriGene, China) and dissolved in nano-pure water as the stock solution (1 μg/mL). HCT116 cells were cultured in 6-well plate at a density of 5×10^5 cells/well overnight. The culture medium was replaced with serum-free medium. An aliquot (10 ng/μl) of siRNA or miRNA (50 μl) was then transfected into the cells using Lipofectamine 2000 (Invitrogen), following the manufacturer's protocol. The cells were then cultured with DMEM containing 10% FBS for another 24h for further analysis.

MTT assay

Cell proliferation was evaluated using 3 commercial MTT Assay Kit (Abcam, UK) following the manufacturer's protocol. The cells were then transferred into a 96-well plate at the density of 5×10^3 cells per well after transfection and incubated for 24 h. The culture medium was removed. Each well was added with 50 μL of FBS-free medium and 50 μl MTT reagent at 37 °C, and kept for for 3 h. An aliquot (150 μl) of MTT solution was added to each well, and the plate shaken for 15 min in the dark. Colorimetric determination was carried out at 570 nm using a Varioskan LUX multimode microplate reader (Thermo Fisher).

Flow cytometry

Cell apoptosis was recorded using flow cytometry. After transfection, the cells were resuspended and washed using phosphate buffered saline (PBS, Solarbio, China), and then stained using Annexin V-FITC/PI Apoptosis Detection Kit (Yeasen, China). About 20 000 cells were collected for apoptosis analysis using CytoFLEX S Cytometer (Beckman, USA).

Tumor sphere formation assay

Tumor stemness was evaluated using tumor sphere formation assay. After transfection,

HCT116 cells were cultured in FBS-free DMEM containing 1 % L-glutamine (Sigma-Aldrich), 20 ng/ml basic fibroblast growth factor (Thermo Fisher, USA), 20 ng/mL recombinant human epidermal growth factor (Thermo Fisher) and 2 % B-27 Serum-Free Supplement (50×, Baiaolaibo, China). At day 10, tumor sphere formation was examined under an inverted microscope (Nikon, Japan), and the cells were then collected subjected to Western blotting analysis.

Determination of aerobic glycolysis

Aerobic glycolysis was determined according to the changes in glucose uptake, lactate production and the activity of LDH. These measurements were achieved by using the commercial kits (Abcam, United Kingdom) in accordance with the manufacturer's protocol.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol RNA Isolation Reagents (Invitrogen). The RNA concentration was determined using Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher). Reverse transcription was conducted using 100 ng of RNA through a High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher). The cDNA was amplified using SYBR™ Green PCR Master Mix (Thermo Fisher) through a QuantStudio™ 7 Pro Real-Time PCR System (Thermo Fisher). The relative RNA expression was quantified using the $2^{-\Delta\Delta CT}$ method. The expression of miR-378a was performed using TaqMan microRNA assays (Thermo Fisher). The primary sequences (Sigma-Aldrich) used in this study were shown in Table 1.

Table 1: The primary sequences used in this study

Gene name	Forward (5'-3')	Forward (5'-3')
U6	AAAGCAAATCA TCGGACGACC	GTACAACACATT GTTTCCTCGGA

Western blotting

After treatment, the cells were collected and lysed using RIPA Lysis and Extraction Buffer (Thermo Fisher) so as to extract total proteins. The proteins were quantified using BCA Protein Assay Kit (Abcam), and 25 μg of the total protein was loaded and electrophoresed using 10 % ExpressPlus™ PAGE Gels (GenScript USA). The separated proteins were then transferred to nitrocellulose membranes (Invitrogen) from gels at 100 V and then blocked using QuickBlock™ Blocking Buffer (Beyotime) for 1 h at room temperature. The membranes were probed with primary antibodies at 4 °C overnight, and then with secondary antibodies for 2 h at room temperature. Finally, the protein bands were developed using Pierce™ ECL Western Blotting Substrate (Thermo Fisher). Images were obtained using GelView 6000Plus Imaging Systems (Bltlux, China). The primary antibodies (Abcam) used in the present study were: FSCN1 (ab220195, 1:5000 dilution), SOX2 (ab171380, 1:2000 dilution), KLF4 (ab215036, 1:1000 dilution), Bmi1 (ab126783, 1:5000 dilution), Oct-4 (ab200834, 1:5000 dilution) and GAPDH (ab8245, 1:10000 dilution).

Luciferase assay

Recombinant vectors, pMIR-Report Luciferase vectors (Thermo Fisher), containing the full sequence of FSCN1 (WT-FSCN1) or its mutant sequence (MUT-FSCN1) were obtained from Thermo Fisher Scientific Inc. (Thermo Fisher). The vectors contained WT-FSCN1 or MUT-FSCN1 and were co-transfected with mimic NC or miR-378a mimics into HCT116 cells. The transfected cells were then cultured for 14 h. Luciferase activity was measured using Pierce™ Firefly Luciferase Flash Assay Kit (Thermo Fisher).

Statistical analysis

All the data are presented as mean ± SEM, and analyzed using GraphPad Prism 6.0 software (GraphPad, USA). Differences between the two groups were compared using Student's *t*-test, while the difference among ≥ 3 groups was determined by one-way ANOVA. Bonferroni test was used for further statistical analysis when the datasets contained ≥ 3 groups. $P < 0.05$ was considered statistically significant.

RESULTS

Downregulation of miR-378a and upregulation of FSCN1 in CRC tissues and cells

To investigate the dysregulation of miR-378a in tumor and normal tissue, data from the TCGA database were analyzed and the results demonstrated that a low expression of miR-378a and a high expression of FSCN1 were observed in primary CRC tissues in relation to normal tissues (Figure 1 a). miR-378a expression was lower and mRNA expression of FSCN1 was higher in CRC cells than in NCM460 cells (Figure 1 b). Protein expression of FSCN1 was also upregulated in CRC cells (Figure 1 c). These results indicated that miR-378a was repressed while FSCN1 was overexpressed in CRC. Since

HCT116 had the lowest miR-378a expression and highest FSCN1 expression, were chosen for the experiments that followed.

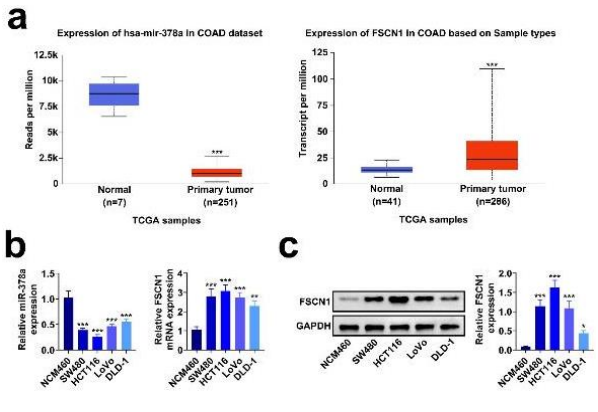

Figure 1: Downregulation of miR-378a and upregulation of FSCN1 in CRC cells. (a) Results from TCGA database showed the downregulation of miR-378a and the upregulation of FSCN1 in CRC tissues; (b) downregulation of miR-378a and upregulation of FSCN1 mRNA was observed in CRC cell lines (c) Upregulation of FSCN1 proteins was observed in CRC cell lines. *P < 0.05 versus (vs) normal or NCM460; **p < 0.01 vs normal or NCM460; ***p < 0.005 vs normal or NCM460. ***Key:*** CRC: colorectal cancer; FSCN1: Fascin actin-bundling protein 1

Overexpression of miR-378 or suppression of FSCN1 inhibited cell proliferation and aerobic glycolysis, and promoted cell apoptosis in CRC cells

To investigate the roles of miR-378a and FSCN1 in CRC, miR-378a was overexpressed in HCT116 cells using miR-378a mimics (Figure 2 a) and the protein expression of FSCN1 was downregulated by using si-FSCN1 (Figure 2 b). Cell viability was reduced by both miR-378a mimics and si-FSCN1 (Figure 2 c). The percentage of apoptotic cells was increased in cells transfected with miR-378a mimics or si-FSCN1 (Figure 2 d). Moreover, glucose uptake, lactate production and LDH activity were reduced by both miR-378a mimics and si-FSCN1 (Figure 2 e). These results indicate that the overexpression of miR-378a and the inhibition of FSCN1 suppressed cell survival and aerobic glycolysis.

Overexpression of miR-378 or suppression of FSCN1 increased the cytotoxicity of chemotherapeutic drugs in CRC cells

Cell survival rate was determined using MTT assay after treatment with cytotoxic chemotherapeutic drugs. Overexpression of miR-378a further reduced cell survival rate after treatment with Dox and 5-Fu in a dose-dependent manner (Figure 3). Cell survival was reduced by Dox and 5-Fu in a concentration-dependent manner, and the downregulation of FSCN-1 further reduced the cell survival rate (Figure 3). Therefore, the cytotoxicity of chemotherapeutic drugs was enhanced by the upregulation of miR-378a or downregulation of FSCN1.

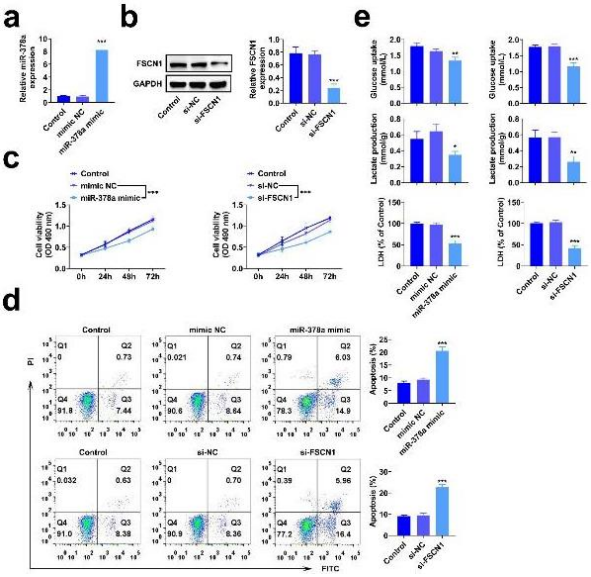

Figure 2: Overexpression of miR-378 or suppression of FSCN1 inhibited cell proliferation and aerobic glycolysis, and enhanced cell apoptosis in CRC cells. (a) miR-378a was overexpressed after transfection with miR-378a mimics; (b) FSCN1 was downregulated by si-FSCN1; (c) cell viability was reduced by both miR-378a mimics and si-FSCN1; (d) cell apoptosis was enhanced by both miR-378a mimics and si-FSCN1; (e) aerobic glycolysis was blocked by both miR-378a mimics and si-FSCN1. *P < 0.05 vs mimic NC or si-NC; **p < 0.01 vs mimic NC or si-NC; ***p < 0.005 vs mimic NC or si-NC. (mimic NC: negative control of miR-378a mimics; si-FSCN1: small interfering RNA of FSCN1; si-NC: scramble RNA of si-FSCN1; LDH: lactate dehydrogenase)

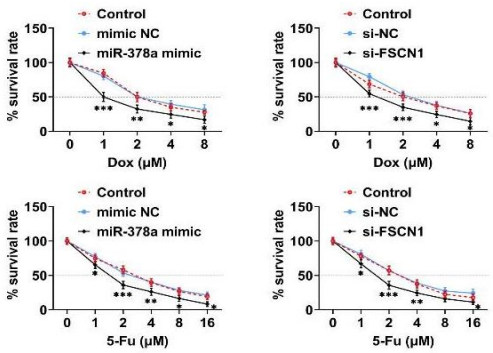

Figure 3: Overexpression of miR-378 or suppression of FSCN1 increased the cytotoxicity of Dox and 5-Fu in CRC cells. *P < 0.05 vs mimic NC or si-NC; **p < 0.01, vs mimic NC or si-NC; ***p < 0.005, vs mimic NC or si-NC. (Dox: Doxorubicin (Dox); 5-Fu: 5-fluorouracil)

Overexpression of miR-378 or suppression of FSCN1 reduced tumor stemness in CRC cells

Tumor stemness was determined using tumor sphere formation assay. The results demonstrated that the capability of tumor sphere formation was weakened by the overexpressions of miR-378a or the repression of FSCN1 (Figure 4 a). The upregulation of miR-378a inhibited the protein expressions of SRY-Box Transcription Factor 2 (SOX2), Kruppel-like factor 4 (KLF4), Bmi1 and Octamer-binding transcription factor 4 (Oct-4), while expression of these proteins was also suppressed by downregulation of FSCN1 (Figure 4 b). Thus, overexpression of miR-378 or suppression of FSCN1 reduced tumor stemness in CRC cells.

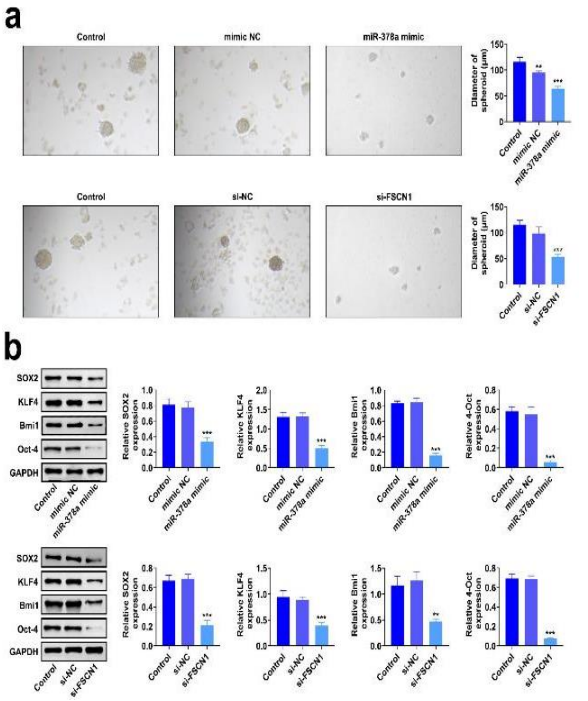

Figure 4: Overexpression of miR-378 or suppression of FSCN1 reduced tumor stemness in CRC cells. (a) Tumor sphere formation was inhibited by miR-378a mimics and si-FSCN1; (b) protein expressions of SOX2, KLF4, Bmi1 and Oct-4 was downregulated by miR-378a mimics and si-FSCN1. ***P* < 0.01, vs mimic NC or si-NC; ****p* < 0.005, vs mimic NC or si-NC. (SOX2: SRY-Box Transcription Factor 2; KLF4: Kruppel-like factor 4; Oct-4: Octamer-binding transcription factor 4)

FSCN1 was the direct target of miR-378a, but overexpression of FSCN1 reversed the effects of miR-178a in CRC cells

The target of miR-378a was predicted via ENCORI (https://starbase.sysu.edu.cn/). The predictive results demonstrated that there was a complementary sequence between miR-378a and 3'-untranslated regions (3'-UTR) of FSCN1 (Figure 5 a). Consistent with this prediction, luciferase activity was reduced by miR-378a mimics in the WT-FSCN1 group but not in MUT-FSCN1 group (Figure 5 b). The protein expression of FSCN1 was reduced by miR-378a mimics (Figure 5 c). The co-transfection of miR-378a mimics and FSCN1 plasmids rose along with the expression of FSCN1, compared to cells transfected with miR-378a mimics alone (Figure 6 a). The co-transfection of miR-378a mimics and FSCN1 plasmids reversed the inhibition of cell viability and the promotion of cell apoptosis induced by miR-378a mimics (Figure 6 b and c). miR-378a-induced reduction of glucose uptake, lactate production and LDH activity were also reversed by the overexpression of FSCN1 (Figure 6d).

Overexpression of FSCN1 inhibited the miR-378a-induced cytotoxicity of Dox and 5-Fu (Figure 6 e). The capability of tumor sphere formation inhibited by miR-378a mimics was recovered while miR-378a mimics and FSCN1 plasmids were being co-transfected into the HCT116 cells (Figure 6 f). miR-378a-induced the downregulation of SOX2, KLF4 and Bmi1, and Oct-4 was blocked by overexpression of the FSCN1 (Figure 6 g). Therefore, FSCN1 was the direct target of miR-378a and could reverse the effects of miR-378a in CRC cells.

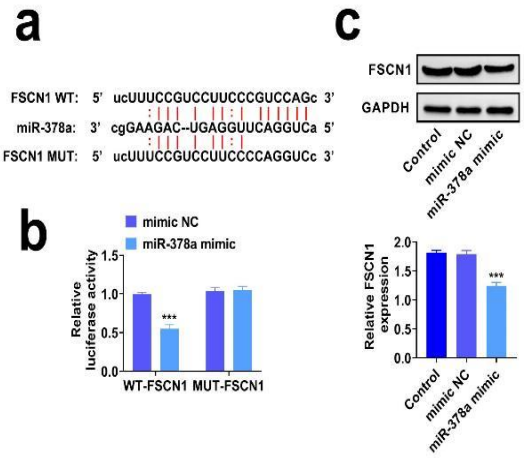

Figure 5: FSCN1 was the direct target of miR-378a. (a) Complementary sequence between miR-378a and 3'-UTR of FSCN1; (b) miR-378a reduced luciferase activity in cells co-transfected with WT-FSCN1 and downregulated FSCN1 mRNA expression; (c) miR-378a downregulated FSCN1 protein expression. ***P* < 0.01, vs mimic NC; ****p* < 0.005, vs mimic NC. (WT-FSCN1: recombinant vectors pMIR-Report Luciferase containing the full sequence of FSCN1; MUT-FSCN1: recombinant vectors pMIR-Report Luciferase containing the mutant sequence of FSCN1)

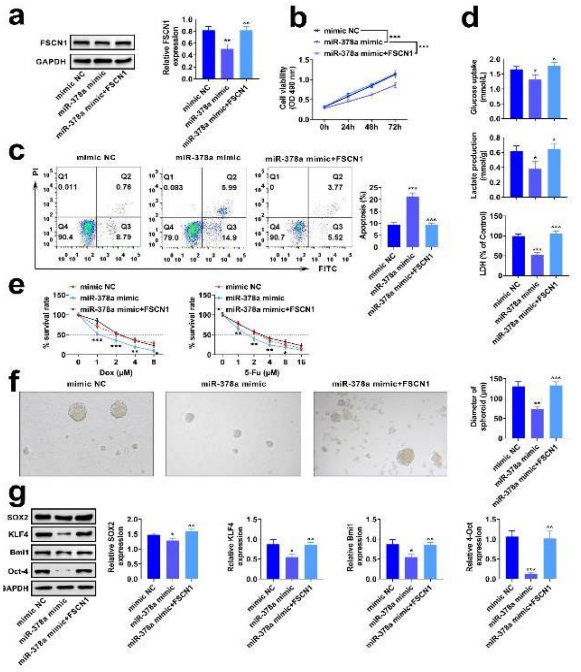

Figure 6: Overexpression of FSCN1 reversed the effects of miR-178a on CRC cells. (a) Overexpression of FSCN1 reversed miR-378a-induced downregulation of FSCN1; (b) overexpression of FSCN1 reversed miR-378a-induced cell viability; (c) overexpression of FSCN1 reversed miR-378a-induced cell apoptosis; (d) overexpression of FSCN1 reversed miR-378a-induced inhibition of aerobic glycolysis (e) the overexpression of FSCN1 inhibited miR-378a-induced cytotoxicity of Dox and 5-Fu; (f) overexpression of FSCN1 reversed miR-378a-induced prevention of tumor sphere formation; (g) overexpression of FSCN1 reversed miR-378a-induced downregulation of SOX2, KLF4, Bmi1 and Oct-4. *P < 0.05, vs mimic NC; **p < 0.01, vs mimic NC; ***p < 0.005, vs mimic NC; ^ p < 0.05, vs miR-378a mimic; ^^ p < 0.01 vs miR-378a mimic; ^^^ p < 0.005 vs miR-378a mimic

DISCUSSION

The pathophysiology of CRC is complicated, and it is well acknowledged that CRC is recurrent and often develop resistance to drugs [13]. It is therefore required that new mechanisms and new targets should be identified to provide new therapeutic targets for CRC treatment. In the present study, the upregulation of miR-378a and the downregulation of FSCN1 were observed in CRC tissues and cell lines. Further experiments demonstrated that miR-378a inhibited cancer stemness and increased the cytotoxicity of chemotherapeutic drugs by targeting FSCN1, thereby preventing CRC tumorigenesis. These results suggest that miR-378a is a tumor suppressor, while FSCN1 is a tumor contributor, thus providing a new understanding of CRC progression as well as a potential therapeutic target for anti-cancer research.

A previous study has demonstrated that miR-378a repressed glucose metabolism and reduced lactate production through the repression of glucose transporter 1 in prostate cancer [14]. In this study, miR-378a blocked aerobic glycolysis, leading to the inhibition of cancer cell survival, potentially preventing CRC progression. FSCN1 was reported to promote tumor growth [15], but no report has revealed the effects of FSCN1 on aerobic glycolysis. For the first time, FSCN1 was proven to enhance aerobic glycolysis. The presence of CSC enhances the aggressiveness of the tumor, including tumor growth, recurrence and treatment resistance [5]. The inhibition of tumor stemness is crucial in cancer treatment. The high expressions of SOX2, KLF4, Bmi1 and Oct-4 are key features of CSC [16,17]. The overexpression of miR-378a and inhibition of FSCN1 downregulated the protein expressions of SOX2, KLF4, Bmi1and Oct-4. These results indicate that miR-378a inhibited tumor stemness, confirming that miR-378a played an anti-tumor role in CRC.

It has been reported that miR-378a downregulated and inhibited cell proliferation in prostate cancer [14]. In accordance with this previous finding, miR-378a was also lowly expressed in CRC, and the overexpression of miR-378a induced cell apoptosis and blocked of cell proliferation, indicating that miR-378a plays the tumor suppressive role in CRC. Dox and 5-Fu are used in CRC treatment [18]. MiR-378a mimics further reduced the cell survival rate in cells treated with Dox or 5-Fu, suggesting that miR-378a played an anti-tumor role in CRC. As mentioned above, FSCN1 was highly expressed in tumor tissues, including CRC [10], and positively promoted cancer metastasis [15]. Data from the present study is consistent with the findings of previous studies, further confirming that miR-378a and FSCN1 may be useful biomarkers for CRC diagnosis.

The predictive results from ENCORI showed that there was a complementary sequence between miR-378a and FSCN1, and luciferase assay proved that FSCN1 was the direct target of miR-378a. All the effects of miR-378a on CRC were reversed by overexpression of FSCN1. Therefore, miR-378a inhibited tumor stemness and aerobic glycolysis by directly binding to 3'-UTR of FSCN1.

CONCLUSION

Data from this study show that miR-378a is upregulated and FSCN1 downregulated in CRC tissues and cell lines. Both miR-378a

overexpression and FSCN1 inhibit tumor stemness and aerobic glycolysis, resulting in the enhancement of cell apoptosis and reduction of cell proliferation in CRC. Further experiments confirmed that FSCN1 is the direct target of miR-378a, and that all the effects of miR-378a are attenuated through the overexpression of FSCN1. These results showed that miR-378a prevented tumor stemness and increased the cytotoxicity of chemotherapeutic drugs by binding to 3'UTR of FSCN1, thereby providing the prognostic biomarkers and potential therapeutic targets for new therapies of CRC. However, *in vivo* experiments should be conducted in animal models to confirm the regulatory effects of miR-378a/FSCN1 axis on CRC.

DECLARATIONS

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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. Taizhe Zhang and Jie Du designed the experiments, Sandang Li and Chuanming Zheng carried them out; Zhenjie Wang analyzed and interpreted the data, and Fuchen Xie prepared the manuscript with contributions from all the co-authors.

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