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Regular paper

WIF1 was downregulated in cervical cancer due to promoter methylation

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Wnt inhibitory factor 1 (WIF1) is frequently downregulated in a variety of cancer due to promoter methylation. However, the methylation status of the WIF1 promoter in cervical cancer remains unclear. This study aimed to elucidate the mechanism by which WIF1 promoter methylation contributes to cervical cancer development. The expression of WIF1 in cervical cancer tissues was examined by immunohistochemistry. The methylation status of the WIF1 promoter in cervical cancer cells was detected by methylation specific PCR. WIF1 mRNA levels and protein levels were detected by PCR and Western blot analysis. We found that WIF1 expression was low in cervical cancer tissues compared to adjacent normal cervical tissues. The WIF1 promoter was methylated in the cervical cancer SiHa cell line but not in the normal cervical epithelial cell line Ect1. Correspondingly, WIF1 mRNA levels and protein levels were significantly lower in SiHa cells than in Ect1 cells. Treatment with 5-aza-2-deoxycytidine (AZA) led to the upregulation of WIF1 mRNA and protein levels in SiHa cells, but the effects were abrogated by treatment with WIF1 siRNA. In addition, AZA treatment induced apoptosis and inhibited the invasion of SiHa cells, and the effects were abrogated by WIF1 siRNA. The protein levels of survivin, c-myc and cyclinD1 were significantly lower in SiHa cells treated with AZA, but their levels were upregulated after treatment with WIF1 siRNA. In conclusion, the methylation of the WIF1 promoter leads to the downregulation of WIF1 and the activation of Wnt/β-catenin signaling in cervical cancer cells. WIF1 is a tumor suppressor that is inactivated in cervical cancer.

Keywords: WIF-1; Wnt/β-Catenin; cervical cancer; methylation

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Abbreviations: AZA, 5-aza-2-deoxycy-tidine; DMSO, dimethyl sulfoxide; WIF1, Wnt inhibitory factor 1

INTRODUCTION

Cervical cancer is the second most common malignant tumor in women worldwide, and persistent infection with high-risk human papillomavirus is the main cause of cervical cancer (Halim *et al*., 2021; Sundaram *et al*., 2021). Although comprehensive treatment options such as surgery, radiotherapy and chemotherapy can improve the efficacy of cervical cancer therapy, the prognosis of patients with advanced stage or relapse of cervical cancer is poor. Therefore, it is important to further investigate the mechanism of cervical cancer development to improve current treatment strategies (Gao *et al*., 2020).

The Wnt/β-Catenin signaling pathway is an important pathway that promotes tumorigenesis (Paul & Dey, 2008; Zhu *et al*., 2021). Wnt inhibitory factor 1 (WIF1) gene is located at 12q14 and encodes a secreted protein that binds to Wnt and acts as a Wnt antagonist to inhibit Wnt/β-catenin signaling (Mazieres *et al*., 2004). *WIF1* has been shown to inhibit the proliferation of different cancer cells (Kim *et al*., 2007; Tang *et al*., 2009). Notably, *WIF1* is frequently downregulated in a variety of cancer due to promoter methylation, indicating that WIF1 is a tumor suppressor (Paluszczak *et al*., 2015; Karamitrousis *et al*., 2020; Zhang *et al*., 2014).

DNA methylation is one important DNA epigenetic modification in eukaryotic cells. DNA methylation transferase leads to the covalency binding of methyl groups provided by S-adenosine methionine (SAM) to specific bases. Aberrant DNA methylation, especially for tumor suppressors, could silence their expression and contribute to the development and progression of cancers including cervical cancer (Lai *et al*., 2010; Zummeren *et al*., 2018; van Leeuwen *et al*., 2019). Consequently, DNA methyltransferase inhibitor 5-aza-2-deoxycytidine (AZA) has been utilized to inhibit DNA methylation of tumor suppressors for cancer treatment (Donia *et al*., 2021). However, the status of methylation of WIF1 promoter in cervical cancer remains unclear. Therefore, in this study, we aimed to detect whether the WIF1 promoter is methylated in cervical cancer and elucidate the mechanism by which WIF1 promoter methylation contributes to cervical cancer development.

MATERIALS AND METHODS

Clinical samples

The clinical samples were from 5 patients with cervical squamous cell carcinoma confirmed by pathological examinations in the Fourth Hospital of Hebei Medical University from December 2019 to June 2020. All patients signed written informed consent. This study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (Approval No. 2019035).

Immunohistochemistry

The streptomycin avidin-peroxidase method was used to detect the expression of WIF1 in clinical samples. The cervical cancer tissues and adjacent cervical tissues were cut into 5 μm thin sections. Antigen retrieval was performed by the incubation of the sections in 10 mM citrate buffer (pH 6.0), and the sections were heated at 100°C for 1 h to block endogenous peroxidase. Next, the sections were incubated with WIF1 antibody (Abcam, Cat# ab71204, Cambridge, MA, USA; 1:500 dilution) for 1 h at 37°C, washed with phosphate buffered saline (PBS), and then incubated with SP-9001 kit (Zhong Shan Golden Bridge, Beijing, China) to visualize the staining. PBS instead of the primary antibody was used as the negative control.

Cell culture

Human cervical cancer cell line SiHa and normal ectocervical cell line Ect1/E6E7 were purchased from American Type Culture Collection and cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS, Thermo Fisher, CA, USA). The incubator condition was 37°C with 5% CO_2. AZA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted in dimethyl sulfoxide (DMSO). SiHa cells were treated with 1 μM AZA or DMSO as the control for 48 h. In addition, SiHa cells were transfected with siRNA for WIF1 or scramble siRNA as control (Sangon Biotech, Shanghai, China) using Lipofectamine 3000 (Thermo Fisher, CA, USA). Cells were collected 48 h after transfection for further analysis.

Transwell invasion assay

The treated cells were added to the upper chamber of the Transwell (Corning Costar; Oneonta, USA), while the lower chamber was filled with 600 μl of RPMI 1640 medium containing 10% FBS. After incubation for 24 h, the cells were fixed with 95% methanol, stained with crystal violet for 20 min, and photographed under the microscope to count the number of invaded cells.

Flow cytometry

The apoptosis of treated cells was examined by using an Annexin V/FITC apoptosis kit (BD Biosciences, USA) following the manufacturer's protocol. The stained cells were immediately analyzed using FACS Calibur System (Becton-Dickinson). The number of positively stained cells was counted to calculate the apoptosis ratio.

Methylation-specific PCR (MSP)

Genomic DNA was extracted from SiHa and Ect1 cells using a DNeasy kit (Qiagen, Germany) following the manufacturer's protocols. Bisulfite modification of genomic DNA was performed by using a methylation kit (Zymo Research, Orange, CA, USA). MSP was performed with bisulfite-treated genomic DNA as a template and the following primers: unmethylation specific primers 5'-TTGTGGGTGTTTTATTGGGT-3' (upstream) and 5'-AACAAAACC AACAATCAACA-3' (downstream); methylation specific primers 5'-TCGCGGGCGTTTTATTGGGC-3' (upstream) and 5'-AACGAAACCAACAATCAACG-3' (downstream).

RT-PCR

Total RNA was extracted from cells and cDNA was synthesized from total RNA using a reverse transcription kit. PCR was performed with cDNA as a template and the following primers: WIF1 5'-CCGAAATGGAGGCTTTTGTA-3' (upstream) and 5'-TGGTTGAGCAGTTTGCTTTG-3' (downstream). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize WIF1 mRNA levels.

Western blot analysis

Total protein was extracted from cells using RIPA buffer and protein concentration was determined by using a bicinchoninic acid assay. Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked in 5% non-fat milk and then incubated with primary antibodies for WIF1 (1:800), survivin, c-myc, CyclinD1 and GAPDH (all from Abcam, Cambridge, UK). The membranes were further incubated with secondary antibodies (Abcam, Cambridge, UK), and detected by chemiluminescence. Densitometry analysis of the bands was performed using Image-J software with GAPDH as a loading control.

Statistical analysis

The data were presented as the mean± standard deviation (S.D.) and analyzed using SPSS statistical software (IBM Corp., Chicago, IL, USA). Comparisons between groups were performed using Student's *t*-test. The difference was considered significant for $p < 0.05$.

RESULTS

WIF1 expression was low in cervical cancer tissues

First, we compared WIF1 expression in cervical cancer tissues and adjacent normal cervical tissues by immunohistochemistry. For negative control, we could not detect WIF1 expression in normal cervical tissues because PBS was used instead of WIF1 antibody (Fig. 1A). When we used WIF1 antibody, we detected strong nuclear staining of WIF1 in normal cervical tissues (Fig. 1B). In contrast, we detected weak nuclear staining of WIF1 in cervical cancer tissues (Fig. 1C). These results indicated that WIF1 expression was low in cervical cancer.

WIF1 promoter was methylated in cervical cancer cells

To elucidate how WIF1 is downregulated in cervical cancer tissues, we used cervical cancer cell as the model. First, we detected the methylation status of the WIF1 promoter in cervical cancer cells by MSP. Compared to normal cervical epithelial cells Ect1, WIF1 promoter was methylated in cervical cancer SiHa cells (Fig. 2A). RT-PCR showed that WIF1 mRNA levels were significantly lower in SiHa cells than in Ect1 cells (Fig. 2B).

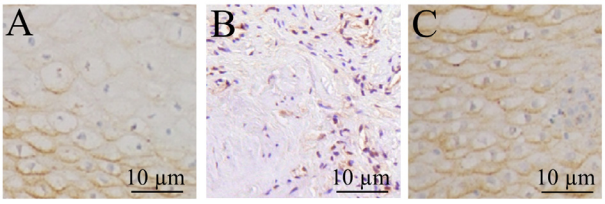

Figure 1. The expression of WIF1 in cervical cancer tissues. (**A**) WIF1 was barely detected in adjacent cervical tissues stained with PBS instead of WIF1 antibody as a negative control. (**B**) WIF1 was strongly stained in the nuclei of adjacent normal cervical cells. (**C**) WIF1 was weakly stained in the nuclei of cervical squamous carcinoma cells.

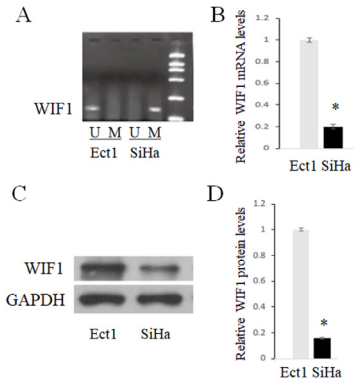

Figure 2. WIF1 promoter was methylated and WIF1 was downregulated in cervical cancer cells.
(**A**) MSP analysis of WIF1 promoter in SiHa and Ect1 cells. U: unmethylated; M: methylated. (**B**) RT-PCR analysis of WIF1 mRNA levels in SiHa and Ect1 cells. (**C**) Western blot analysis of WIF1 protein levels in SiHa and Ect1 cells. GAPDH was loading control. (**D**) Densitometry analysis of WIF1 protein levels in SiHa and Ect1 cells. Data were expressed as the mean ± S.D. (n=3). **P*<0.05 compared to Ect1 cells.

Furthermore, we detected WIF1 protein levels in SiHa cells and Ect1 cells (Fig. 2C). The results showed that WIF1 protein levels were significantly lower in SiHa cells than in Ect1 cells (Fig. 2D). Collectivity, these data indicated that WIF1 promoter was methylated and WIF1 was downregulated in cervical cancer cells.

AZA upregulated WIF1 expression in cervical cancer cells

To examine whether demethylation of the WIF1 promoter can restore WIF1 expression in cervical cancer cells, we treated SiHa cells with the demethylation agent AZA. Compared to SiHa cells treated with DMSO as control, WIF1 mRNA levels were significantly higher in SiHa cells treated with AZA. However, the upregulation of WIF1 mRNA levels by AZA was abrogated by treatment with WIF1 siRNA (Fig. 3A).

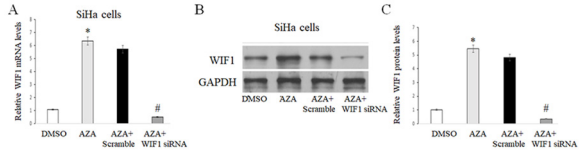

Figure 3. AZA upregulated WIF1 expression in cervical cancer cells.
(**A**) RT-PCR analysis of WIF1 mRNA levels in SiHa cells treated with DMSO, AZA (5-aza-2-deoxycytidine), AZA+ scramble siRNA, AZA + WIF1 siRNA. (**B**) Western blot analysis of WIF1 protein levels in SiHa cells treated with DMSO, AZA, AZA+ scramble siRNA, AZA + WIF1 siRNA. GAPDH was loading control. (**C**) Densitometry analysis of WIF1 protein levels in SiHa cells treated with DMSO, AZA, AZA+ scramble siRNA, AZA + WIF1 siRNA. Data were expressed as the mean ± S.D. (n=3). **P*<0.05 for the AZA group compared to the DMSO group. #*P*<0.05 for AZA + WIF1 siRNA group compared to AZA group.

Next, we examined WIF1 protein levels in SiHa cells in different treatment groups (Fig. 3B). Densitometry analysis showed that WIF1 protein levels were significantly higher in SiHa cells treated with AZA than in cells treated with DMSO as control. However, the upregulation of WIF1 protein levels by AZA was abrogated by treatment with WIF1 siRNA (Fig. 3C). These results confirmed that AZA targeted WIF1 promoter to upregulate WIF1 expression in cervical cancer cells.

AZA inhibited malignant behaviors of cervical cancer cells

To examine the effects of AZA on cervical cancer cell behaviors, we performed flow cytometry and found that AZA treatment increased the apoptosis of SiHa cells, while the effect of AZA on apoptosis was abrogated by WIF1 siRNA (Fig. 4A). Quantitative analysis showed that apoptosis percentage was significantly higher in SiHa cells treated with AZA than in cells treated with DMSO but was significantly lower in SiHa cells treated with both AZA and WIF1 siRNA than in cells treated with AZA alone (Fig. 4B).

Transwell invasion assay showed that AZA treatment inhibited the invasion of SiHa cells, while the ef-

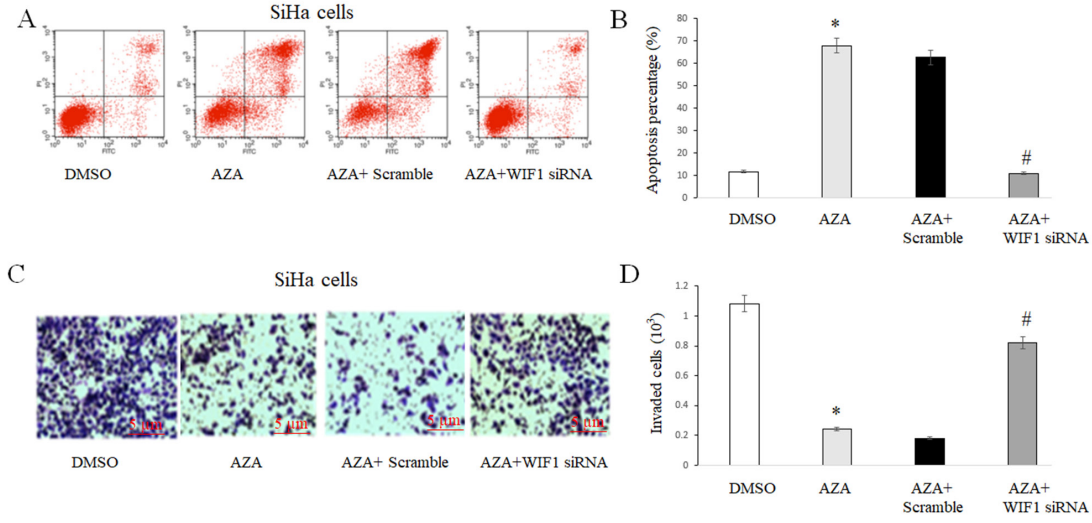

Figure 4. AZA induced the apoptosis and inhibited the invasion of cervical cancer cells.
(**A**) Flow cytometry analysis of apoptosis in SiHa cells treated with DMSO, AZA, AZA+ scramble siRNA, AZA + WIF1 siRNA. (**B**) Quantitative analysis of apoptosis percentage in SiHa cells treated with DMSO, AZA, AZA+ scramble siRNA, AZA + WIF1 siRNA. (**C**) Transwell invasion assay of SiHa cells treated with DMSO, AZA, AZA+ scramble siRNA, AZA + WIF1 siRNA. (**D**) Quantitative analysis of the number of invaded SiHa cells treated with DMSO, AZA, AZA+ scramble siRNA, AZA + WIF1 siRNA. Data were expressed as the mean ± S.D. (n=5). **P*<0.05 for the AZA group compared to the DMSO group. #*P*<0.05 for AZA + WIF1 siRNA group compared to AZA group.

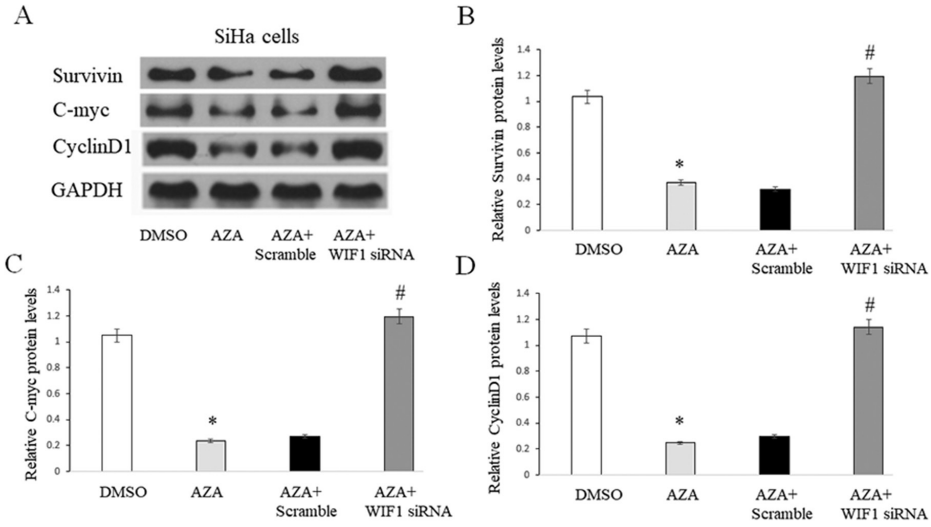

Figure 5. AZA inhibited the expression of Wnt/β-catenin target genes in cervical cancer cells.
(**A**) Western blot analysis of survivin, c-myc and CyclinD1 protein levels in SiHa cells treated with DMSO, AZA, AZA+ scramble siRNA, AZA + WIF1 siRNA. GAPDH was loading control. (**B**) Densitometry analysis of survivin protein levels in SiHa cells treated with DMSO, AZA, AZA+ scramble siRNA, AZA + WIF1 siRNA. (**C**) Densitometry analysis of c-myc protein levels in SiHa cells treated with DMSO, AZA, AZA+ scramble siRNA, AZA + WIF1 siRNA. (**D**) Densitometry analysis of cyclinD1 protein levels in SiHa cells treated with DMSO, AZA, AZA+ scramble siRNA, AZA + WIF1 siRNA. Data were expressed as the mean ± S.D. (n=3). *P<0.05 for the AZA group compared to the DMSO group. #P<0.05 for AZA + WIF1 siRNA group compared to AZA group.

fect of AZA on invasion was abrogated by WIF1 siRNA (Fig. 4C). Quantitative analysis showed that the number of invaded cells was significantly lower in SiHa cells treated with AZA than in cells treated with DMSO but was significantly higher in SiHa cells treated with both AZA and WIF1 siRNA than in cells treated with AZA alone (Fig. 4D). Taken together, these results indicated that AZA induced the apoptosis and inhibited the invasion of cervical cancer cells.

AZA inhibited the apoptosis of cervical cancer cells via the inhibition of the Wnt pathway

To investigate how AZA inhibited the apoptosis of cervical cancer cells, we detected protein levels of survivin, c-myc and cyclinD1, which are Wnt pathway target genes and regulate cell proliferation and apoptosis (Fig. 5A). Densitometry analysis showed that protein levels of survivin, c-myc and cyclinD1 were significantly lower in SiHa cells treated with AZA than in cells treated with DMSO as control. However, protein levels of survivin, c-myc and cyclinD1 were significantly higher in SiHa cells treated with both AZA and WIF1 siRNA than in cells treated with AZA alone (Fig. 5B–D).

DISCUSSION

In this study, we demonstrated that WIF1 was downregulated in cervical cancer tissues and cells due to the methylation of the WIF1 promoter. AZA abrogated the methylation of the WIF1 promoter and upregulated WIF1 expression. Consequently, AZA inhibited the invasion and induced the apoptosis of cervical cancer cells. Furthermore, AZA inhibited the expression of survivin, c-myc and cyclinD1, which may be the mechanism by which AZA inhibited tapoptosis and promoted the proliferation of cervical cancer cells.

The development and progression of cervical cancer is a complex process that involves both genetic and epigenetic mechanisms (Albulescu *et al.*, 2021; Wang *et al.*, 2021). DNA methylation, especially the methylation of tumor suppressor genes, is one of the most important types of epigenetic modification that contribute to tumorigenesis (van Leeuwen *et al.*, 2019). Recent studies have shown that promoter methylation of WIF1 promoted the development of a variety of tumors (Zhang *et al.*, 2014). However, the role of WIF1 promoter methylation in cervical cancer remains unclear.

Using normal cervical epithelial cells as control, we showed that the WIF1 promoter was methylated in SiHa cervical cancer cells. Consistently, both mRNA and protein levels of WIF1 were significantly downregulated in SiHa cells compared to normal cervical epithelial cells. However, AZA treatment led to the upregulation of WIF1 at both mRNA and protein levels, and the effects of AZA on WIF1 could be abrogated by WIF1 siRNA. Taken together, these data indicate that the downregulation of WIF1 in cervical cancer cells is due to promoter methylation.

Wnt/β-catenin signaling plays an important role in tumorigenesis. The activation of Wnt/β-catenin signaling leads to the translocation of β-catenin from the cytoplasm into the nuclei where it interacts with transcription factor TCF/LEF to drive the transcription of downstream target genes such as survivin, c-myc and CyclinD1 (Koushyar *et al.*, 2022). Among a variety of targets of Wnt/β-catenin signaling, survivin is a known anti-apoptosis protein that promotes cell survival. C-myc and CyclinD1 are known to promote cell proliferation and cell cycle progression. The upregulation of survivin, c-myc and CyclinD1 in SiHa cells treated by AZA may explain why the apoptosis percentage was lower in these cells compared to control cells treated with DMSO. In addition, AZA induced apoptosis of SiHa cell was abrogated by WIF1 siRNA. On the other hand, AZA inhibited the invasion of SiHa cells, and SiHa cell invasion could be restored after treatment with WIF1 siRNA. Notably, cancer cell invasion depends on the action of matrix metalloproteinases (MMPs), and MMP9 was recently identified as a target of Wnt/β-catenin signaling

(Ingraham *et al*., 2011; Lee *et al*., 2014; Chen *et al*., 2021). Cervical cancer metastasis remains a big challenge for the effective treatment of cervical cancer in the clinic (Cheng and Huang, 2021). Further studies are needed to identify target genes of Wnt/β-catenin signaling that could be regulated by AZA, which could be novel therapeutic targets for metastatic cervical cancer.

In conclusion, this study provides evidence that the methylation of the WIF1 promoter leads to the downregulation of WIF1 and the activation of Wnt/β-catenin signaling in cervical cancer. AZA treatment reduced the methylation of WIF1 protomer and upregulated WIF1 expression to inhibit Wnt/β-catenin signaling in cervical cancer cells. Consequently, AZA induced apoptosis and inhibited the invasion of cervical cancer cells, which could be rescued by WIF1 siRNA. These findings suggest that WIF1 is a tumor suppressor that is inactivated in cervical cancer, and the development of approaches to restore WIF1 expression is promising for cervical cancer treatment.

Declarations

Availability of data and material. All data and materials are included in this manuscript.

Competing interests. The authors have no conflict of interest.

Authors' contributions. YW, SY, HL, LH, FZ, and XW collected and analyzed the data, and JM designed the study and wrote the manuscript. All authors read and approved the final manuscript.

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