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Original Research Article

MiR-216b modulates cisplatin resistance and stem cell-like features in breast cancer cells by targeting E2F4

Xiaoshen Zhang, Lun Chen*
Department of Breast Surgery, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou 510120, Guangdong, China

For correspondence:* ***Email:* *caolun41755667554@163.com*

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Abstract

Purpose: *To study the influence of miR-216b expression in breast cancer (BC), and the underlying mechanism(s) of action.*
Methods: *Viability of BC cell lines was determined using CCK-8 as well as colony formation assays, while the abundance of mRNAs of key genes was assessed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Western blot assay was utilized to measure protein expressions of E2F4 and stem cell markers. Bioinformatics and dual-luciferase procedures were deployed to predict the targets of miR-216b on 3'-UTR of E2F4.*
Results: *MiR-216b was reduced in BC tissues and mammary carcinoma cell line, MCF-7, relative to those in paracancerous tissue and normal breast cell line, MCF-10A ($p < 0.05$). Compared with miR-NC group, cell viability, median inhibitory concentration (IC_{50}), and potential to form colonies in cisplatin-insensitive cell line MCF-7/DDP were reduced by miR-216b. Moreover, MiR-216b suppressed stem cell characteristics and decreased the expressions of ALDH1 and Oct-4 in BC cells. Inhibition of miR-216b resulted in significant increase in microsphere formation and expressions of ALDH1 and Oct-4, when compared with NC-inhibitor group. In addition, E2F transcription factor 4 (E2F4) was identified as the downstream gene of miR-216b. Knockdown of E2F4 annulled the influence of miR-216b on insensitivity to cisplatin and stem cell likeness of MCF-7/DDP.*
Conclusion: *MiR-216b induces increase in BC response to cisplatin and suppressed stem cell characteristics by regulating E2F4. This finding may be useful for developing strategies aimed at reversing cisplatin insensitivity in BC.*

Keywords: *Anti-tumor, Cisplatin resistance, Breast cancer, microRNA-216b, E2F transcription factor 4*

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INTRODUCTION

According to the World Health Organization, breast carcinoma (BC) accounts for 25 % of the incidence and 15 % of the mortality rate of female cancers worldwide [1]. Even with optimized treatment regimens that have improved outcomes, some breast cancer patients may develop varying degrees of drug resistance during treatment [2]. As a result, a better knowledge of BC development and medication resistance might lead to the development of new BC therapies.

Target genes are expressed by microRNAs (miRNAs) [3]. Many miRNAs are expressed abnormally in tumors, and they are involved in disease progression [4]. In addition, miRNAs serve as tumor suppressors or oncogenes in BC. For example, some miRNAs (miR-335 and miR-126) inhibit the growth of BC [5], whereas others such as miR-210 and miR-373, promote BC proliferation [6].

Research on tumor drug resistance has also focused on miRNAs. In recent years, evidence shows that miR-216b is involved in a wide range of cancers. It has been linked to chemosensitivity of lung adenocarcinomas, and it is linked to cisplatin insensitivity in ovarian carcinoma [7]. On the other hand, miR-216b impact on BC cell stemness and drug resistance is yet to be determined. The present study was carried out to investigate if there is a link between BC pathogenesis and miR-216b. It was hypothesized that miR-216b may exert a tumor-suppressive effect on BC, based on the aforementioned studies.

METHODS

Ethics statement

The ethical authority of The First Affiliated Hospital of Guangzhou Medical University gave approval for this investigation (approval no. 20200526). This study met the criteria outlined in Helsinki Declaration [8]. The patients and/or family members submitted written and signed informed permissions. Tumor specimens and matched para-cancerous samples were taken from 50 BC subjects after surgery, from July 2020 to December 2020. Cancer patients who were not able to undergo surgery were treated with chemotherapy. Cisplatin-resistant (CR) BC patients were considered as those in whom cisplatin-based chemotherapy failed to shrink the primary tumors or metastases. In contrast, BC patients whose primary tumor size or metastasis was inhibited after receiving several courses of chemotherapy with cisplatin, were considered cisplatin-sensitive (CS).

Cell lines and treatment

Mammary carcinoma MCF-7 cell line and healthy mammary MCF-10A from ATCC were cultured in RPMI 1640 containing 10 % FBS (Invitrogen). The medium was maintained at 37 ℃ in 5 % CO_2. To induce chemo-resistance in cell lines, continuous gradient exposure to cisplatin was extensively employed, leading to the production of cisplatin-resistant BC cell lines [9]. A one-year cisplatin treatment with gradient cisplatin concentrations (0.05 to 1 mg/mL) was used to produce cisplatin-resistant and MCF-71/DDP cell lines from MCF-71.

Evaluation of cell viability

A 24-well plate was seeded with BC cells (3000 cells per well), and the CCK-8 assay was carried out as previously reported [10]. For colony formation assay, a 12-well plate was seeded with cells (1000 cells per well). On day 14, the cell colonies formed were subjected to fixation, staining, and counting.

Transfection of cells

MiR-216b mimetics, miR-216b inhibitors, si-E2F4, and corresponding control miRNAs were made. Lipofectamine 2000 for transfection and harvesting of BC cells was supplied by Invitrogen, Waltham, MA, USA.

Luciferase assay

To construct the E2F4 3'UTR (wt) and E2F4 3'UTR (mt) plasmids, miR-216b target sites from wild-type or mutant E2F4 were amplified. Then, miR-216b mimetics, miR-NC, wild-type E2F4 3'UTR, and mutant E2F4 3'UTR were transfected into MCF-7 cells. The level of luciferase activity at 48 h was measured using Dual-Luciferase Detection kit (Promega Corporation).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

TRIzol (Takara, Japan) was used to isolate total RNA, which was reverse-transcribed to cDNA using the Reverse Transcription System (Takara). On the ABI 7700 System, Takara SYBR green qPCR (Life Technologies, Pleasanton, CA, U.S.) was utilized in 35 cycles at 95 ℃ (10 min), 95 ℃ for 45 sec, 55 ℃ for 45 sec, and 72 ℃ for 90 sec. The internal controls were U6 and GAPDH. The mRNA expression levels were computed using $2^{-\Delta\Delta Ct}$ method. Information on primer sequences is shown in Table 1.

Western blot assay

The primary markers of stem cells (ALDH1 and Oct-4) and E2F4 were produced by Abcam. Total protein extraction was done with RIPA buffer, and protein concentration was measured with BCA kits (Invitrogen, America). Then, equal amounts of proteins were resolved on SDS-polyacrylamide gel electrophoresis, followed by electro-transfer to nitrocellulose films. Thereafter, the membranes were sealed in 5 % skimmed milk powder, followed by incubation with 1º

immunoglobulins overnight at 4 °C, and further incubation with anti-mouse 2° immunoglobulin. The bands were subjected to enhanced chemiluminescence (cat. no. 32106; Pierce Biotechnology Incorporated).

Table 1: Sequences of primers for miR-216b, E2F4, ALDH1, Oct4, CD44, and CD44

Name of primer	Sequence (5'-3')
miR-216b (F)	AAA UCU CUG CAG GCA AAU GUG A
miR-216b (R)	AAA UCU CUG CAG GCA AAU GUG A
U6 (F)	TGCGGGTGCTCGCTTCGGCAGC
U6 (R)	GTGCAGGGTCCGAGGT
E2F4 (F)	TGCCACCACCTGAAGATTT
E2F4 (R)	GGAGTGAGCTGAGGACTATTTG
ALDH1 (F)	GCCATAACAATCTCCTCTGCTC
ALDH1 (R)	TCTCCCAGTTCTCTTCCATTTC
Oct4 (F)	TTGAGGCTCTGCAGCTTAG
Oct4 (R)	GCCGGTTACAGAACCACAC
GAPDH (F)	AACTTTGGCATTGTGGAAGG
GAPDH (R)	GGATGCAGGGATGATGTTCT

Statistics

Results are expressed as mean ± standard deviation (SD) of 3 independent repeat tests. Paired comparison was done using *t*-test while multi-group comparison was done by ANOVA. Differences were assumed significant at $p < 0.05$.

RESULTS

Cellular and tissue MiR-216b expressions were down-regulated

Figure 1 a shows significantly lower expressions of miR-216b in BC tissues, unlike healthy controls. Figure 1 b shows that the expression of miR-216b in cisplatin-sensitive patients was higher than that in cisplatin-resistant patients ($p < 0.05$). Moreover, in Figure 1 c, miR-216b level was significantly reduced in the MCF-7 group, when compared to MCF-10A, while miR-216b expression was significantly reduced in MCF-7/DDP, when compared to MCF-7 ($p < 0.05$; Figure 1 d).

Overexpressed miR-216b sensitized BC to cisplatin *in vitro*

Figure 2 a shows that miR-216b mimics boosted miR-216b level in MCF-7/DDP cell, relative to miR-NC ($p < 0.01$). Furthermore, overexpressed miR-216b significantly reduced the IC_{50} of cisplatin in cisplatin-insensitive BC cells (Figure 2 b). Furthermore, miR-216b mimetics reduced the capacity of cisplatin-resistant BC cells to form colonies when exposed to cisplatin ($p < 0.01$; Figure 2 c).

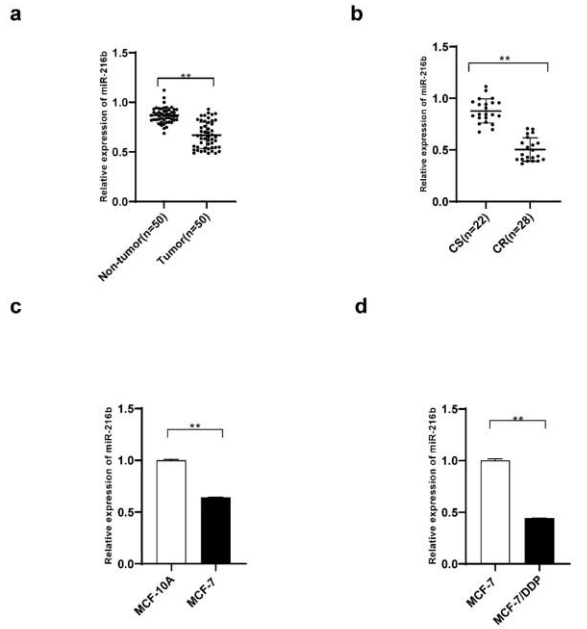

Figure 1: Cellular and BC tissue miR-216b levels were reduced. (a) miR-216b levels in BC and matching healthy controls. (b) miR-216b levels in BC tissues from CR and CS subjects. (c) miR-216b levels in BC cells and healthy cells. (d) miR-216b expression in MCF-7/DDP cells

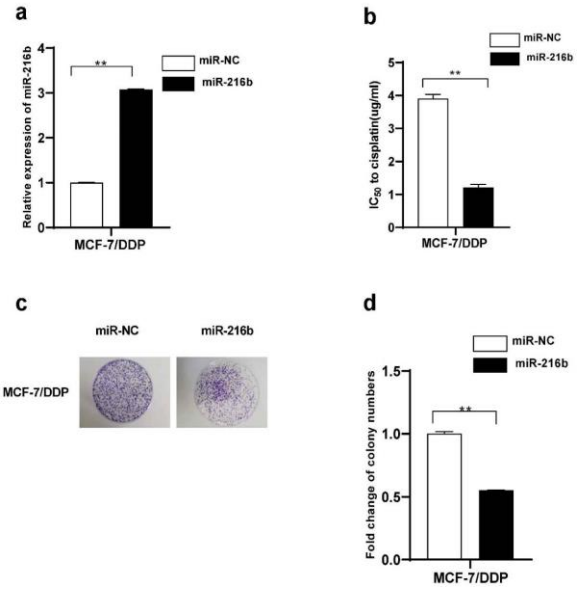

Figure 2: Enhanced levels of miR-216b accentuated the effect of cisplatin on cisplatin-insensitive BC cells. (a) Relative concentrations of miR-216b. (b) IC_{50} values of cisplatin in MCF-7/DDP subjected to transfection for 48 h. (c) Colonies of transfected MCF-7/DDP cells after treatment with cisplatin (1 mg/mL). *$P < 0.05$; **$p < 0.01$, vs miR-NC

Stem cell-resembling features of breast carcinoma cells were regulated by miR-216b

Figure 3 a demonstrates that miR-216b inhibition increased microsphere formation in MCF-7 cells ($p < 0.01$). Furthermore, the miR-216b inhibitor increased the transcription and translation of related markers in MCF-7 cells ($p < 0.01$; Figures 3 b and c).

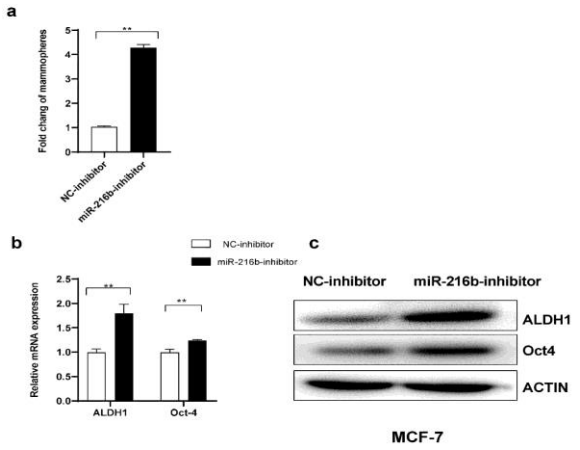

Figure 3: Stem-cell characteristics in BC were modulated by miR-216b. (a) miR-216b attenuation enhanced microsphere features in MCF-7. (b & c) miR-216b knockdown upregulated transcription (b) and translation (c) of stem cell indices in MCF-7. *$P < 0.05$; **$p < 0.01$, compared to NC-inhibitor group

MiR-216b directly targeted E2F4 in breast cancer cells

Through TargetScan, it was predicted that E2F4 3'UTR has a binding site for miR-216b (Figure 4 a). In MCF-7 cells containing E2F4 3'UTR (WT) plasmid, miR-216b mimetics lowered luciferase expression ($p < 0.01$; Figure 4 b), whereas miR-216b mimetics produced no significant impact following transfection of BC cells using E2F4 3'UTR mutant plasmid (Figure 4 b). Moreover, when miR-216b was overexpressed, transcription and translation of E2F4 were downregulated in MCF-7 cells (Figures 4 c and e, whereas miR-216b suppressor enhanced mRNA and protein expressions of E2F4 (Figures 4 d and f; $p < 0.01$).

Knockdown of E2F4 annulled the influence of miR-216b on cisplatin insensitivity and stem-cell characteristics

In MCF-7/DDP cells, miR-216b inhibitor significantly elevated mRNA and protein expressions of E2F4, whereas si-E2F4 reversed these effects (Figures 5 a and b). Results from CCK-8 test showed that miR-216b inhibitor significantly raised the proliferation of MCF-7/DDP cells, while si-E2F4 reversed this effect (Figure 5 c; $p < 0.01$). Furthermore, as shown in Figure 5 d, miR-216b inhibitor increased the IC_{50}, while si-E2F4 inhibited it ($p < 0.01$). Furthermore, miR-216b inhibitor increased the colonies of MCF-7/DDP cells, while si-E2F4 reversed this effect (Figure 5 e, $p < 0.01$). Moreover, miR-216b inhibitor significantly increased the transcription and translation of stem cell markers, while si-E2F4 reversed these effects (Figure 5 f and g; $p < 0.01$).

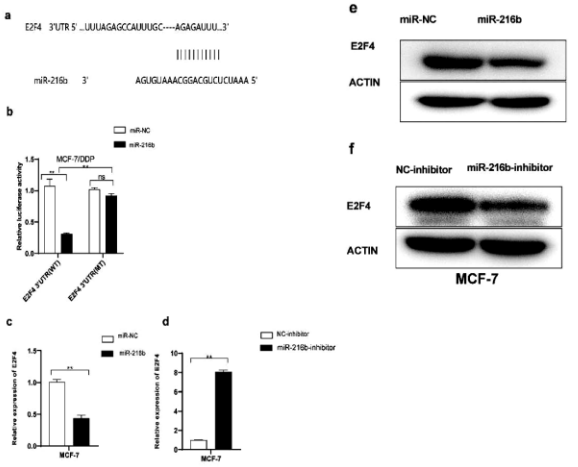

Figure 4: miR-216b directly targeted E2F4 in BC. (a) Scheme depicting site of interaction of miR-216b with E2F4 3'UTR, relative to miR-216b and WT E2F4 3'UTR or mutated postulated zones in E2F4 3'UTR (TMED3 30UTR [MT]). (b) Enzyme activities in MCF-7 after transfection, as measured using luciferase assay. (c & d) Transcription of E2F4 was determined with qRT-PCR in cells treated with miR-216b or miR-216b inhibitor. (e & f) Protein expression of E2F4, as measured using western blotting. *$P < 0.05$; **$p < 0.01$, relative to miR-NC or NC-inhibitor group

DISCUSSION

Tumor cell resistance often occurs in chemotherapy for patients with mid to late-stage cancer. Cisplatin is a frequently-used chemotherapeutic agent for breast carcinoma patients. However, it may become less effective due to the development of resistance to cisplatin in breast cancer cells. There are several investigations on the role of miRNAs in tumor development, and the correlation between miRNA-mediated post-transcriptional gene silencing and tumor drug resistance has attracted a lot of attention. A study reported decreased miRNA-216b levels in ovarian cancer cells [10]. Moreover, miR-216b inhibited the multiplication and invasiveness of renal clear cell cancer cells, suggesting that miR-182 is an oncogenic factor [11].

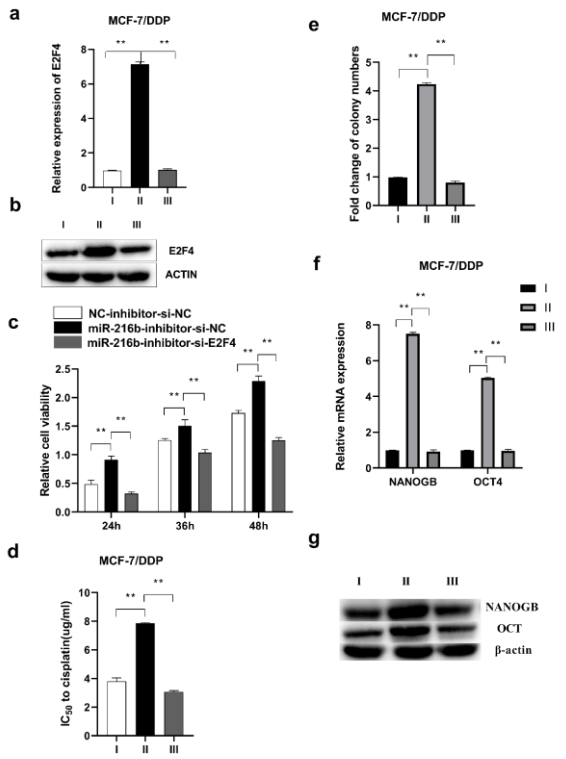

Figure 5: Attenuation of E2F4 annulled the influence of miR-216b on cisplatin insensitivity in BC as well as stem cell characteristics. (a) The mRNA and (b) protein (b) expressions of E2F4 were measured after transfection of cells. (c) Viability of cells following transfection (1 mg/mL). (d) IC_{50} values of cisplatin after 48 h exposure in MCF-7/DDP cells following transfection. (e) Effect of cisplatin on colony formation in cells following transfection. (f & g) The mRNA (f) and protein (g) expression levels of stem cell markers in transfected MCF-7/DDP cells. *P < 0.05; **p < 0.01

In another study, miR-216b inhibited autophagy, promoted apoptosis and inhibited proliferation by targeting ATG5, and it increased cisplatin efficacy in BC cells [12]. However, it is not known whether it modulates the efficacy of cisplatin in mammary tumor. In this study, miR-216b was lowly expressed in BC tissue samples, relative to matched healthy tissues. Furthermore, there was reduced level of miR-216b in BC specimens from subjects sensitive to cisplatin. Results from qRT-PCR revealed significantly lower miR-216b level in BC cells than in healthy cells. Furthermore, the miR-216b level in MCF-7/DDP cells that were resistant to cisplatin was lower than that in MCF-7 cells. As a result, it may be hypothesized that miR-216b might have an anti-tumor influence on BC growth and resistance to cisplatin.

Therefore, the function of miR-216b in cisplatin resistance in BC was investigated further using MCF-7/DDP cells. The outcomes demonstrated that miR-216b mimics increased sensitivity to cisplatin in BC cells that are resistant to cisplatin, as seen in survival of MCF-7/DDP cells, colony-forming ability, and IC_{50}.

Tumor stem cells (TSCs) are self-renewing cells that exist in tumor tissue and can differentiate into different families of tumor cells to form the whole tumor [13]. The tumorigenicity, differentiation potential, self-renewal and drug resistance of TSCs are key to the failure of tumor therapy, and the induction of differentiation of TSCs to inhibit or even reverse their stem cell properties has become a frontier in tumor biology and tumor therapy [14]. Studies have shown that the malignant phenotypic characteristics of breast cancer, such as development, recurrence, metastasis, radiation resistance and drug resistance, are associated with breast cancer stem cells [15]. In a spheroid colony formation experiment, miR-216b expression was inversely related to the ability of BC cells to form microspheres. Furthermore, overexpression of miR-216b suppressed the transcription and translation of genes related to stem cell markers. As a consequence, miR-216b inhibited stem cell-like capabilities of BC cells. Furthermore, distinct signaling networks may influence the growth of BC and the self-renewal of breast CSCs. Therefore, more research is needed to determine which signaling pathways are impacted by miR-216B in BC.

MicroRNAs are non-coding RNAs of about 18 – 25 NTs, which act as promoters or inhibitors of tumor growth by silencing or degrading mRNAs [16]. Results showed that miR-216b is involved in key processes in breast cancer development and progression. The E2F4 is the most important member of the E2F family of transcription factors. It is expressed at the highest levels in organisms, and it accounts for a significant portion of the E2F family protein activity [17]. The function of E2F4 in organisms is complex and controversial. In the past, most researchers believed that E2F4 was a blocker in organisms, but in recent years, an increasing number of studies have found that it can also be present as an activator [18]. In this study, bioinformatics was used to predict the relevant target gene of miR-625-5p. By dual luciferase reporter gene experiments, it can be concluded that E2F4 is a direct target of miR-216b. Furthermore, miR-216b mimics reduced E2F4 protein and mRNA expressions in BC cells, but its inhibitors increased E2F4 expression. As a result of these findings, it was speculated that the inhibitory roles of miR-216b on BC were linked to changes in E2F4 expression. Transfection of miR-216b suppressor and si-E2F4 into MCF-7/DDP cells was done in order to investigate the aforementioned hypothesis. Additional investigations revealed that miR-216b

inhibitor greatly increased chemoresistance and stem cell characteristics of MCF-7/DDP. In addition, miR-216b inhibitor increased cell viability, IC_{50}, and marker expression in stem cells. All impacts of the miR-216b suppressor were reversed by si-TE2F4.

Taken together, these results showed that a decrease in miR-216b expression and a rise in E2F4 expression aided the resistance of BC cells to cisplatin. Furthermore, miR-216b suppressed BC cells by directly targeting and adversely regulating E2F4. As a result, the current study suggests that restoration of lost function through miR-216b replacement treatment, could be a strong therapeutic option for BC. Furthermore, these promising findings indicate that miR-216b may be employed as a novel therapeutic target for BC. However, more research into its anti-tumor properties in BC *in vivo* should be undertaken.

Limitations of the study

Being an *in vitro* investigation, this study did not involve *in vivo* research with animal experiments. In addition, this study did not explore the downstream pathway of E2F4.

CONCLUSION

The results obtained in this study indicate that miR-216b inhibits cisplatin insensitivity and stem cell characteristics of BC *via* specific interaction with E2F4. This finding is considered useful for developing strategies aimed at reversal of cisplatin insensitivity in BC.

DECLARATIONS

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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. Xiaoshen Zhang and Lun Chen conceived and designed the study, collected, analyzed and interpreted the experimental data, drafted the manuscript and revised the manuscript for important intellectual content. Both authors read and approved the final manuscript.

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