Objective: colorectal cancer (CRC) is a common cancer with high mortality. This study aimed to investigate the role of microRNA (miR)-132-3p on proliferation, invasion and migration of CRC cells. Materials and Methods: qRT-PCR and Western blot analyses were used to determine the expression of miR-132-3p and forking box (FOX) protein 2 (FOX2) in CRC cell line Caco-2. The expression of miR-132-3p and FOX was regulated using miR inhibitor and siRNA, and the viability and migration ability of the transfected cells were assessed. Cell cycle dependent kinase (CDK) 1, cyclin D1, matrix metalloproteinase (MMP)-2 and MMP-9 were detected using Western blots. The dual luciferase reporter gene assay was used to verify the targeting of miR-132-3p to FOX2. Results: Compared with control cells, FOX2 and miR-132-3p expressions were decreased or increased significantly (P<0.05), respectively in Caco-2 cells. Up-regulation of miR-132-3p effectively inhibited the proliferation, migration and invasion of Caco-2 cells, and suppressed the expression of FORX2, cyclin-dependent kinase 1 (CDK1), cyclin D1, MMP-2 and MMP-9. Luciferase reporter gene assays revealed that FOX2 expression was negatively regulated by miR-132-3p. Knockdown of FOX2 using siRNA significantly reduced the proliferation and migration of Caco-2 cells, down-regulated the expression FOXP2 as well as CDK1, cyclin D1, MMP-2 and MMP-9. Since FOXP2 is targeted by miR-132-3p, it is likely that miR-132-3p-mediated reduction of proliferation and migration of Caco-2 cells was achieved via reduced translation of FOXP2 mRNA. Conclusions: miR-132-3p inhibits the proliferation, migration and invasion of CRC cells. This is likely achieved via negative regulation of the targeted FOX2 expression. This role may be further explored for therapeutic applications in CRC.

Key words: colorectal cancer, FOX2, migration, miRNA, miR-132-3p, proliferation

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Abbreviations: CDK, cell cycle dependent kinase; CRC, colorectal cancer; FOX, forking box; FOX2, forking box protein 2; MMP, matrix metalloproteinase

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths worldwide with an annual incidence of 1.4 million new cases and 694,000 deaths (Siegel et al., 2018; Sung et al., 2021). About 15% CRC patients are in metastatic stages (stage IV) once diagnosed and the average survival rate is only 2.5 years (Scholtefield et al., 2002). Despite the use of various screening and therapeutic methods based on various prognosis factors such as tumor node, metastasis (TNM) staging, tumor differentiation grade, vessel invasion, performance status and biomarkers, there are still a great number of therapeutic failures and metastasis in CRC patients (Howe et al., 1993). Like other tumors, genetic and epigenetic changes in oncogenes and/or tumor suppressor genes are shown to trigger the occurrence, progression and metastasis of CRC (de Rosa et al., 2015). A better understanding of the molecular mechanisms is crucial to identify new targets and develop new therapeutic avenue for the disease.

After their discovery, micro-RNAs (miRNAs) have been shown to play important roles in cancer. One of the most important features of miRNAs is that they may have multi-targets consisting of up to 200 mRNAs in a coordinated manner (Krek et al., 2005). Increasing evidence indicates that deregulated miRNAs’ expression is often associated with the progression and metastasis of CRC. Due to their stability and abundance, miRNAs have been proposed as a new class of biomarkers for cancers, including CRC (Hayes et al., 2014; Mitchell et al., 2008; Yiu & Yiu, 2016). Specific miRNAs are also involved in CRC progression and metastasis (Balacescu et al., 2018). Since alterations in Wnt/β-catenin, EGFR, TGFβ and TP53 signaling pathways are shown to change the survival, proliferation, invasion and metastasis of CRC, many studies have been conducted to establish the miRNAs-mRNA interaction networks related to these pathways (Mohammadi et al., 2016; Rahmani et al., 2018). For example, restoration of miR-152 expression was found to inhibit cell proliferation, survival, and migration through the suppression of AKT-ERK pathway in CRC (Ghazanchaei et al., 2018); miR-675-5p targets SNAIL and miR-320c targets SOX4, FOXM1, FOQX1 to induce epithelial to mesenchymal transition (EMT) in CRC (Costa et al., 2017; Vishnuvalaji et al., 2016). Recently, miR-375 was found to regulate MMP2 and several EMT-associated genes, including SNAIL and that downregulation of miR-375 promotes proliferation, invasion and migration of CRC cells (Cui et al., 2016).

MiR-132-3p is found expressed abnormally in gastric cancer, osteosarcoma, ovarian cancer and other tumors (Li et al., 2018; RWu et al., 2014), affecting the proliferation, invasion and migration of tumor cells. However, the expression of miR-132-3p in CRC and its impact on CRC are not clear. On other hand, long non-coding RNA (lncRNA) SNHG5 could sponge miR-132-3p in CRC tissue to promote proliferation, metastasis, migration and to inhibit apoptosis in CRC cells (He et al., 2020; Zhang et al., 2019). In addition, circRNA dedicator of cytokinesis 1 (circ_DONEK1) was also found to silence miR-132-3p, resulting in repressed cell growth, migra-
tion, and invasion of human CRC cell lines (HCT116 and SW480) (Zhang et al., 2021), suggesting that miR-132-3p may have a role in CRC. Furthermore, bioinformatics analysis showed that in adenoid cystic carcinoma (AdCC), miR-132-3p is a potential regulator and may interact with the forkhead box P2 gene FOXP2 (Liu et al., 2021), which was recently shown to express abnormally in a number of tumors (Li et al., 2019; Yao et al., 2018). For example, FOXP2 expression is upregulated in triple negative breast cancer (TNBC) and cell lines. Silencing FOXP2 inhibits the growth, invasion and metastasis of TNBC cells in vitro and in vivo (Wu et al., 2018) and down-regulation of FOXP2 could lead to reduced proliferation and migration of cancer cells via TGFβ/SMAD signaling pathway (Chen et al., 2018; Qin et al., 2019; Zhong et al., 2017). FOXP2 is a member of a large FOX family of transcription factors with important functions in multiple biological processes, such as cell cycle control, cell differentiation, proliferation and development (Hannenhalli & Kaestner, 2009; Myatt & Lam, 2007). The human FOX gene family contains at least 43 members, among them some genes such as FOXA1 and FOXM1 are up-regulated in esophageal and lung cancer (Katoh & Katoh, 2004). The dysfunction of FOX genes can alter cell differentiation, metastasis and progression of osteosarcoma (Zhang et al., 2017a). FOXP2 is one of the first discovered FOX genes implicated in a speech and language disorder and it influences many human traits (Nudel & Newbury, 2013). Accumulating evidence now indicates that dysregulated FOXP2 may also play an instrumental role in oncogenesis (Campbell et al., 2010a; Myatt & Lam, 2007; Yan et al., 2015a), but the results are still controversial (Zhang et al., 2017b).

In the present study, we aimed to determine the expression of miR-132-3p in CRC cells, the impact of miR-132-3p on cancers biological features such as proliferation, invasion and migration of CRC cells and the underlying mechanisms, particularly related to FOXP2. The findings would provide insights for developing new therapeutic strategies for CRC.

MATERIALS AND METHODS

Cells

Human colorectal cancer cell line CACO-2 and primary colonic epithelial cells were purchased from Sigma-Aldrich (St Louis, USA) and Cell Biologics (Chicago, USA) and cultured in minimum essential medium eagle (EMEM, M2279, Sigma-Aldrich) at 37°C in 5% CO₂ incubator.

Reagents and equipment

Fetal bovine serum (FBS) and RPMI1640 medium were purchased from Hyelone, USA; Trizol reagent, reverse transcription kit and PCR kit were purchased from Jingmei Biotech, Shenzhen, China; primers were designed and prepared by Simgon Biotech, Shanghai, China; RIRA lysis buffer (cat. no. T1081) was purchased from Solarbio, Beijing; antibodies against CDK1, cyclin D1, matrix metalloproteinase (MMP)-2 and MMP-9 were purchased from Santa Cruz, USA; horseradish peroxidase (hrp)-conjugated secondary antibodies were purchased from Boster Biotech, Wuhan, China; tetramethyl thiazole blue (MTT) and dimethyl sulfoxide were purchased from Sigma-Aldrich, USA; lipofectamine 2000 kit was purchased from Invitrogen, USA; dual luciferase activity assay kit was purchased from Promega, USA. BCA protein assay kit (cat. no. CW00148) was obtained from CWBIO, Beijing.

Cell culture and transfection

CACO-2 cells and colonic epithelial cells were grown in EMEM with 10% FBS at 37°C in 5% CO₂ incubator and harvested at the logarithmic growth phase for detection of gene expression using reverse transcription-quantitative (qRT-PCR) and Western blot analysis. For transfection, cells were grown to 60% confluency and transfected using lipofectamine 2000 according to the supplier's instructions with 0.1µg each of miRNA-132-3p control (miR-con), miR-132-3p mimics (miR-132-3p), siRNA-FOX2 control (si-con), siRNA-FOX2 (siFOX2). The transfected cells were cultured for another 48 h in EMEM with 10% FBS at 37°C in 5% CO₂ incubator and harvested for subsequent analysis.

qRT-PCR

Cells were homogenized in Trizol reagent using a homogenizer (Teleedy Tekmar, OH). RNA was isolated from either chloroform phase separation with an Ambion Ribopure kit (Life Technologies, NY) or directly from Trizol reagent with a Direct-Zol minipurp kit (Zymo Research, CA). Extracted RNA were subsequently reverse transcribed into cDNA using iScript supermix (Bio-Rad, CA) and RT-qPCR was performed using TaqMan miRNA Assays (Applied Biosystems) for miRNA and using TaqMan probes for FOX2 (Life Technologies, NY), respectively. Normalization was done with RNU6b miRNA for miRNA and β-actin for FOXP2. The PCR was carried out in a total volume of 10 µl containing 1.5 µl of diluted and pre-amplified cDNA, 10 µl of TaqMan Master Mix and 1 µl of fluorescence probe. The cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 45 cycles, each one consisting of 15 s at 95°C and 1 min at 60°C. Samples were run in triplicate and the mean relative expression levels were calculated using previously described protocol (Livak & Schmittgen, 2001). The primer sequences were miR-132-3p upstream: 5’-CGTAACAGTCCAGCAGCCTATTG, downstream: 5’-TCCCGTGTGCTGGAGTCTGC; FOXP2 upstream: 5’-CGCGGATCCTCATTCCAGATC; downstream: 5’-CGCAGATCCTCATTTCCAGAT; U6 upstream: 5’-CTCCGCTTCGGCAGACA, downstream: 5’-AACGCTTCACGAATTTGCGT; β-actin upstream: 5’-CTCGCTTCGGCAGACA, downstream: 5’-AACGCTTCACGAATTTGCGT; β-actin upstream: 5’-CTCGCTTCGGCAGACA, downstream: 5’-AACGCTTCACGAATTTGCGT; β-actin upstream: 5’-CGGCCCTCGCCCTTTGGCGAG; downstream: 5’-CGATGCGCATGCGATGCGAG.

Western blotting

100 µl cells were harvested 48 h after transfection and lysed with RIPA buffer containing protease inhibitors cocktail and quantitated using BCA kit according to the manufacturer's instructions. After denaturing by boiling at 100°C for 5 min, 60 µg of proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes, blocked with 5% non-fat milk in 1X TBST buffer with 0.1% Tween 20 for 4 h at room temperature and then detected by staining with above stated-proper primary antibodies at 4°C overnight and secondary antibodies at 25–26°C for 1 hour before visualization with a chemiluminescence kit. Densitometric analysis was conducted using Image J software, β-actin was used as an internal control.
miRNA and CRC

Cell proliferation assay

The cell proliferation was measured using MTT assay as described (Cheleschi et al., 2018). Briefly, cells were harvested 48 h after transfection and seeded into the wells of 96-well culture plates at the density of $2 \times 10^3$ cells per well containing complete medium. After 24 h of incubation at 37°C in 5% CO$_2$ incubator, each well was added with 20 µl MTT solution and incubated at 37°C in 5% CO$_2$ incubator for 4 h. The optical density at 490 nm was measured after adding dimethyl sulfoxide to dissolve the precipitates. Assays were run in triplicate.

Transwell cell migration and invasion assays

Transwell cell migration and invasion assays were performed based on published protocols (Justus et al., 2014). Briefly, cells were transfected with miRNA mimics or controls for 48 h, pelleted by centrifugation at $500 \times g$ for 10 min at room temperature, and suspended in serum-free medium RPMI1640 medium. $2.0 \times 10^4$ cells were inoculated into the upper chamber of an insert (8-µm pore size; BD Bioscience, USA). The low chamber of the Transwell contained RPMI1640 medium with 10% FBS. The insert membrane was either coated or not coated with Matrigel for the assessment of cell invasion and migration, respectively. After 24 h of incubation at 37°C, the cells remaining on the upper membrane were removed with a cotton wool, whereas the cells that had migrated or invaded through the membrane were stained with 2% crystal violet in 25% methanol/PBS, imaged and counted in five randomly selected fields using an EVOS XL Core inverted microscope (Life Technologies, USA). The experiments were independently repeated three times.

Dual luciferase reporter gene assay

Type (WT) and mutant (MUT) FOXP2 3′ untranslated regions (UTRs) were used to construct reporter constructs (pGL-WT and pGL-MUT) using pGL3 as backbone plasmid. The cells were seeded into the wells of 96 plates, grown to 60% confluence and co-transfected with pGL-WT, pGL-MUT, miR-132-3p and control using lipofectamine 2000 according to the supplier’s instructions. The transfected cells were cultured for another 48 h in EMEM with 10% FBS at 37°C in 5% CO$_2$ incubator, pelleted by centrifugation and lysed with 200 µL buffer at 4°C for 30 min. 70 µL lysate was added with 100 µL luciferase detection solution to determine the luciferase activity according to the manufacturer’s protocol. Renilla luciferase reporter gene was used as an internal control.

Statistical analysis

Data are expressed as the mean±standard error of the mean obtained from at least three independent experiments. Statistical comparisons between groups were assessed using one-way ANOVA with Tukey’s post hoc tests. Statistical analysis was performed using SPSS (21.0, IBM, USA). $P<0.05$ was considered statistically significant.

RESULTS

FOXP2 was upregulated and miR-132-3p was downregulated in CRC cells

We first measured the expression of FOXP2 in human CRC cell line CACO-2 and primary colonic epithelial cells using Western blot analysis. The results showed that compared with colonic epithelial cells, CRC cells had significantly ($P<0.01$) elevated the FOXP2 level (Fig. 1A). We further analyzed the mRNA levels of FOXP2 and miR-132-3p in CACO-2 and in the epithelial cells using qRT-PCR. The results revealed that FOXP2 were upregulated and miR-132-3p were down-regulated significantly ($P<0.01$) in CRC cells when compared with the non-cancer cells (Fig. 1B).

miR-132-3p reduced proliferation of CACO-2 cells and down-regulated CDK1, Cyclin D1 expression

To investigate the biological roles of miR-132-3p, CACO-2 cells were transfected with miR-132-3p mimics and control. Compared with miR-con, miR-132-3p mimics (miR-132-3p) significantly increased the level of miR-132-3p and decreased the proliferation of CACO-2 cells,

Table 1. Expression of miR-132-3p, CDK1, Cyclin D1 and growth of CACO-2 cells after transfection with miR-132-3p

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell density (OD495 nm) after</th>
<th>Relative expression</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>miR-con</td>
<td>0.42±0.05</td>
<td>0.72±0.15</td>
</tr>
<tr>
<td>miR-132-3p</td>
<td>0.12±0.02</td>
<td>0.22±0.05</td>
</tr>
</tbody>
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* denotes $P<0.01$ vs miR-con
as measured by OD values over 72 h period after transfection (P<0.01, Table 1). In addition, CDK1 and Cyclin D1 were down-regulated in the CACO-2 cells following transfection with miR-132-3p (P<0.01, Table 1).

MiR-132-3p reduced migration and invasion of CACO-2 cells

Using the Transwell well assays, migration and invasion of CACO-2 cells were assessed. The results showed that after transfection with miR-132-3p, the cell migration and invasion were significantly reduced compared to miR-con (P<0.01, Fig. 2A). Western blot assay of EMC-related proteins showed that MMP-2 and MMP-9 were significantly downregulated after transfection with miR-132-3p compared to miR-con (P<0.01, Fig. 2B).

Mir-132-3p targeted FOXP2

Bioinformatics analysis suggested that there is a sequence complementary to miR-132-3p in the 3'UTR of FOXP2 (Fig. 3A), implying that FOXP2 might be a target of miR-132-3p. To verify this, dual luciferase activity assays were conducted using wildtype and mutant 3'UTR sequences of FOXP2. The results showed that co-transfection of miR-132-3p with pGL-WT resulted in a significant reduction of luciferase activity, while no reduction was observed when pGL-MUT was used (Fig. 3B), confirming that miR-132-3p targets FOXP2 specifically. Furthermore, transfection of miR-132-3p reduced the level of FOXP2 in CACO-2 cells, while miR-con did not alter the level (Fig. 4).

Downregulating FOXP2 expression attenuated the proliferation and migration of CACO-2 cells

To assay the impact of FOXP2 on the migration and invasion assays of CACO-2 cells, we knocked down the expression of FOXP2 using siRNA. Compared with si-con, transfection of CACO-2 cells with siRNA-FOXP2 significantly reduced the level of FOXP2 as well as the cyclin-dependent kinase 1 (CDK1), CyclinD1, MMP-2 and MMP-9 (P<0.01, Fig. 5A). Transwell assays showed that the proliferation and migration of CACO-2 cells were significantly lower after siRNA-FOXP2 transfection than with si-con (P<0.01, Fig. 5B).

DISCUSSION

MiRNAs play an important regulatory role in cell proliferation, apoptosis, migration and invasion of malignant tumors and are potential targets for the treatment of malignant tumors (Ganju et al., 2017; Mishra et al., 2016). Our study showed that miR-132-3p is downregulated and FORX2 is upregulated in CRC cells; upregulation of miR-132-3p reduces the proliferation, migration and invasion of CACO-2 cells. These changes are likely mediated via regulation of FORX2, cell-cycle related proteins and metastasis-related proteins. Our findings indicate that miR-132-3p may be further explored as potential gene therapy agent for CRC.

In an early study, it was found that the expression of miR-132-3p is significantly decreased in gastric cancer tissue, and the inhibition of miR-132-3p expression activates epidermal growth factor receptor (EGFR), and extracellular signal-regulated kinase (ERK), and serine / threonine kinase (AKT) signaling pathways via targeting Mucin 13, leading to an increased proliferation and migration of gastric cancer cells (He et al., 2017). Consistent with the study, we found that miR-132-3p expres-
sion is downregulated in CRC cells as compared with non-cancer cells.

On other hand, FOXP2 was found elevated in CRC cells. FOXP2 is one of the first discovered genes implicated in a speech and language disorder and influences many human traits (Nudel & Newbury, 2013). While it encodes a transcription factor involved in speech and language acquisition, accumulating evidence now indicates that dysregulated FOXP2 may also play an instrumental role in oncogenesis and several FOXP2 family members are directly involved in cancer initiation, maintenance and progression (Campbell et al., 2010b; Myatt & Lam, 2007; Yan et al., 2015b). Its expression is down-regulated in breast cancer (Cuiffo et al., 2014) and hepatocellular carcinoma (Yan et al., 2015a), and upregulated in lymphomas (Campbell et al., 2010b; Wong et al., 2016), neuroblastomas (Khan et al., 2015) and ERG fusion-negative prostate cancers (Stumm et al., 2013), although its expression has not been reported for CRC. Bioinformatics analysis showed that miR-132-3p is a potential regulator of adenoid cystic carcinoma where FOXP2 is involved (Liu et al., 2021). FOXP2 was recently found expressing abnormally in a number of tumors (Li et al., 2019; Yao et al., 2018). For example, FOXP2 expression is upregulated in triple negative breast cancer (TNBC) and cell lines, silencing FOXP2 inhibits the growth, invasion and metastasis of TNBC cells in vitro and in vivo (Wu et al., 2018) via TGFβ/SMAD signaling pathway (Chen et al., 2018; Qin et al., 2019; Zhong et al., 2017). With dual luciferase activity assay, the luciferase activity was found specifically reduced once wildtype FOXP2 3’UTR sequence was used, but not the MUT sequence (which does not pair with miR-132-3p), suggesting that FOXP2 is a specific target of miR-132-3p. This interaction between FOXP2 and miR-132-3p could result in the reduction of FOXP2 through mRNA translation repression or decay (Fabian et al., 2010).

As a consequence, upregulation of miR-132-3p after transfection with miR-132-3p mimics reduces the expression of FOXP2 in CRC cells, leading to reduced cell proliferation, migration and invasion of CRC cells. Similar anti-cancer activity was observed when FOXP2 expression was knocked down with siRNA-FOXP2, suggesting that FOXP2 may be an oncogene, although further studies are needed to elucidate the underlying mechanism.

To assess the molecular mechanisms related to the changes in cell proliferation and migration, we determined the proteins related to cell division (CDK1 and Cyclin D1) and metastasis (MMP-2 and MMP-9). These proteins were down-regulated after transfection with miR-132-3p or after silencing FOXP2, suggesting that miR-132-3p and FOXP2 might act on the same downstream pathways. CDK1 is a driver and a regulator of cell division; down-regulation of CDK1 might reduce the overall capacity of protein synthesis and impair cell’s dividing ability (Haneke et al., 2020; Michowski et al., 2020). Cyclin D1 expression is required for cancer cell survival and proliferation likely via the inactivation of the RB tumor suppressor, and cyclin D1 depletion could facilitate cellular senescence in cancer cells (Laphanwat et al., 2018). Cyclin D1 protein plays an important role in regulating the progress of the cell during the G1 phase of the cell cycle and is amplified in approximately 20% of mammary cancers (Barnes & Gillett, 1998). It was shown that down-regulation of cyclin D1 could result in G0/G1 cell cycle arrest and inhibit cancer cell proliferation (Zheng et al., 2020). Vitex rotundifolia fruit (a traditional medicine for treating inflammation, headache, migraine) was found to inhibit the proliferation of human CRC cells through down-regulating cyclin D1 and CDK4 via proteasomal-dependent degradation and transcriptional inhibition (Song et al., 2018).

MMP-2 is a 72-kDa zinc- and calcium-dependent endopeptidase. It has intracellular and extracellular functions ranging from the modulation of extracellular matrix remodeling to cell growth and migration, angiogenesis and inflammation (Fernandez-Patron et al., 2016). MMP-2 might be a prognostic marker in advanced gastric cancer patients with disseminated metastasis (Noh et al., 2011). MMP-9 level was significantly elevated in gastric cancer patients when compared with control subjects (Wu et al., 2007). Earlier study showed that dysregulating MMPS such as MMP-2, MMP-9 and MT1-MMP by ACY-241 and JQ1, would suppresses proliferation and metastasis of head and neck squamous cell carcinomas and down-regulation of MMP-2/MMP-9 by myricetin, a natural polyphenol, could suppress breast cancer metastasis (Ci et al., 2018). All these findings confirmed that miR-132-3p and FOXP2 exert a biological effect via acting on CDK1, cyclin D1, MMP-2 and MMP-9. Further studies are needed to elucidate the molecular mechanisms underlying the interaction between miR-132-3p and FOXP2 and these proteins. Furthermore, findings from the present study were derived from one cell line and should be further validated in more cell lines or tissue.

CONCLUSIONS

miR-132-3p is down-regulated in CRC cells and transfection of CRC cells inhibits the expression of FOXP2

![Figure 5. Expression of FOXP2, CDK1, CyclinD1, MMP-2 and MMP-9 in CACO-2 cells and migration and invasion of CACO-2 cells after transfection with siRNA-FOXP2.](Image 64x474 to 291x735)
and proliferation, migration and invasion of CRC cells, which is likely achieved via negative regulation of FOXP2 expression. Down-regulation of FOXP2, which is confirmed to a target gene of miR-132-3p, also results in a reduced proliferation, migration and invasion of CRC cells. Therefore, miR-132-3p may be further examined to be a potential biomarker for CRC.

Declarations

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

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