Mechanism of miR-107-targeting of regulator of G-protein signaling 4 in hepatocellular carcinoma

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Abstract. The aim of the present study was to investigate the mechanism of microRNA (miR)-107 in targeting regulator of G-protein signaling 4 (RGS4) in hepatic carcinoma. SK-HEP-1 cells were transfected with miR-107 mimics and control mimics. Reverse transcription-quantitative PCR was performed to determine the miR-107 expression levels, and following miR-107 upregulation, MTT, colony formation, transwell and wound-healing assays were performed to assess cell proliferation, colony-forming ability, invasion and migration, respectively. In addition, the effect of miR-107 upregulation on the cell cycle and apoptosis in SK-HEP-1 cells was evaluated using flow cytometry. Western blot analysis was performed to measure the protein expression levels of RGS4, epidermal growth factor receptor (EGFR), CXC chemokine receptor type 4 (CXCR4) and matrix metalloproteinase (MMP)-2 and -9. Expression level changes and the association between miR-107 and RGS4 in HCC cells were assessed using dual luciferase analysis. The results indicated that the overexpression of miR-107 in HCC cells suppressed cellular proliferation, invasion, migration and colony-forming ability, but promoted apoptosis and G1 phase arrest. Furthermore, miR-107 mimics notably increased the protein expression level of RGS4, but significantly downregulated that of EGFR, CXCR4 and MMP-2 and -9. Together, these findings suggest that targeting this potential mechanism of miR-107 may be beneficial in the treatment of patients with HCC.

Introduction

Hepatocellular carcinoma (HCC) accounts for ~95% of all primary liver cancers. HCC is one of the solid tumors with the poorest prognosis, with the 6th highest incidence and 3rd highest mortality rates worldwide (1). Although various new treatments have been applied in the clinic, the incidence and mortality rates of HCC have not adequately improved (2). Patients with HCC have a higher recurrence rate even after curative therapy, and a five-year survival rate of only 11% (3). The poor prognosis of patients with HCC highlights the need to explore the mechanisms of disease progression. Understanding the molecular pathogenesis of HCC, particularly the mechanisms of tumorigenesis, is important for developing novel biomarkers and treatment strategies.

MicroRNAs (miRNAs) are endogenous, small single-stranded, non-coding RNAs of ~20-25 nucleotides in length. miRNAs regulate translation by binding to specific sequences in the 3'-untranslated region (3'-UTR) of target mRNAs (4,5). Accumulating evidence suggests that miRNA deregulation contributes to a wide range of human diseases, including cancer (6). In terms of the onset and prognosis of human cancers, miRNAs are able to regulate oncogenes or tumor suppressor genes during tumorigenesis, in a target-dependent manner (7). It is important to clarify the function of miRNAs in tumor pathogenesis and progression, as miRNAs may regulate a variety of critical biological processes, including cell differentiation, proliferation, cell cycle distribution, apoptosis, migration and tumor cell drug resistance (8-10).

Studies have indicated a preference for the detection of miRNA (miR)-107 over a-fetoprotein (AFP) for the early diagnosis of HCC (11), and the combination of serum AFP and ultrasound surveillance is the most widely used strategy for screening and detecting HCC in high-risk groups (12). miR-107 is located on chromosome 10 and has been found to be abnormally expressed in various tumors (13-15). Evidence has supported the hypothesis that reduced expression levels of miR-107 are associated with the growth, migration and invasion of various cancer types, including HCC (16,17). Furthermore, previous research has revealed that miR-107 enhances the viability, migration and invasion of U2OS cells, which may be associated with the activation of the MEK/extracellular signal-regulated kinase and nuclear factor-kB signaling pathways via targeting of the tumor suppressor gene tropomyosin 1 (18). It was also reported that miR-107 may inhibit glioma cell proliferation by targeting sal-like protein 4 (19), and that miR-107 overexpression inhibited colon cancer cell proliferation by targeting hypoxia inducible factor-β (20). These reports demonstrate the complex nature of miR-107 activity. Therefore, it was speculated that depleted plasma expression...
levels of miR-107 may be associated with tumor progression and poor outcome in patients with HCC.

Regulator of G-protein signaling (RGS) proteins are expressed in the majority of cell types, tissues and organ systems. RGS proteins have been implicated in a variety of physiologies and pathologies (21), including hematopoi-

esis (22), cancer migration and invasion (23), and synaptic signaling plasticity in brain-anxiety disorder (24). RGS4 belongs to the B/R4 subfamily (25) of the RGS protein family, which is characterized by a conserved 120-amino acid RGS region flanking the short amino and carboxyl termini (26). RGS4 is an intracellular protein primarily recognized for its GTPase activating function, which by stimulating Gα-bound GTP hydrolysis, inactivates the Gα subunits of the heterotrimeric G protein, and subsequently inhibits G-protein coupled receptor (GPCR) signaling (27). Data has shown that RGS4 induces G1/M arrest in the breast cancer cell cycle, and therefore, the signal transduction pathway initiated by RGS4 may be associated with breast cancer cell proliferation (28). RGS4 has been extensively studied in the central nervous and circulatory systems (29), and its involvement in cancer is also increasingly being investigated. Given that RGS proteins serve important roles in tumorigenesis, it was hypothesized that optimizing the function or overexpression of RGS proteins in tumor tissues may be an effective strategy for tumor therapy.

Additionally, as a tumor suppressor, RGS4 inhibited tumor invasiveness and metastasis by regulating matrix metallo-

proteinases (MMPs) and epithelial mesenchymal transition (EMT)-associated markers (30). Previous studies have also demonstrated that MMP-2 and MMP-9 are involved in the development and metastasis of cutaneous melanoma (29,31,32), and have shown that epidermal growth factor receptor (EGFR) serves a key role in the occurrence and development of primary liver cancer (33). CXC chemokine receptor type 4 (CXCR4) is one of the most widely expressed chemokine receptors in tissues, serving an important role in cell growth and invasion, and the metastasis of various malignant tumors. There is evidence to indicate the activation of CXCR4/stromal cell-derived factor 1 promoted prostate cancer cell invasion through MMP-9, and that the overexpression of RGS4 can block the increased invasive and metastatic influences of EGFR and CXCR4 (30). It has also been shown that RGS4 overexpression can completely reverse CXCR4-mediated, and partially reverse EGFR-mediated invasion and metastasis. This suggests that CXCR4, as a GPCR, may be directly negatively regulated by RGS4.

In the present study, it was discovered that the RGS4 3′-UTR contained a potential binding site for miR-107 in the putative target sequence, and a previous study has confirmed that the expression level of miR-107 was lower in 30 HCC samples relative to their corresponding adjacent liver tissues (17). However, the role of miR-107 in the progression of HCC, and the direct targets of miR-107 in the regulation of HCC remain undefined. Therefore, the present study investigated the potential role of miR-107 in RGS4 expression and the tumor characteristics of HCC.

Materials and methods

Cell lines and culture. The HCC cell line SK-HEP-1 was purchased from the American Type Culture Collection (ATCC), and maintained in 25 cm² flasks containing Dulbecco’s modified eagle’s medium (DMEM; ATCC) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator (5% CO₂ and 95% humidity).

**Lentivirus transduction of miR-107.** miR-107 mimics and control mimics were transduced into SK-HEP-1 cells. Lentiviruses expressing miR-107 mimics (5′-AGCAGCAUU GUACAGGGCUAUCA-3′) and control mimics (5′-UUCUCC GAACGUGUCAGUTT-3′) were purchased from Shanghai Sun Biotechnology Co., Ltd. In total, 100 pmol lentivirus and 8 µg/ml polybrene were added to the culture medium at a multiplicity of infection of 10-15. After incubation overnight, the medium was replaced, and the cells were maintained for another 5-7 days to stabilize the lentiviral transduction. Subsequently, the transduction efficiency was verified by reverse transcription-quantitative (RT-q) PCR. The groups were designated a as the negative control (NC), miR-107 mimics and control mimics groups.

**RT-qPCR analysis.** TRIZol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to isolate the total RNA from the miR-107-SK-HEP-1 cells, and cDNA was synthesized with the oligo(dT)₁₄ primer. The RT reaction was performed by incubating a reaction mixture containing 0.5 µg RNA, 100 pmol random hexamer primer (Applied Biosystems; Thermo Fisher Scientific, Inc.), 50 units reverse transcriptase (Applied Biosystems; Thermofisher Scientific, Inc.), 20 units RNase inhibitor (Promega Corporation), and 1 mM dNTP (Thermo Fisher Scientific, Inc.) in a total 20 µl volume. The RT conditions were as follows: 25°C for 15 min, 45°C for 30 min and 94°C for 5 min. Then, qPCR was performed using a Light Cycler system with LightCycler FastStart DNA Master PLUS SYBR®-Green I (Roche Diagnostics) under the following conditions: Initial denaturation at 94°C for 10 min, followed by 40 cycles at 94°C for 10 sec and 60°C for 1 min, and 72°C 30 sec for elongation. The qPCR primers used were as follows: miR-107 forward, 5′-ATA CGGCTGAGTGGCATGTGTCAGTAAT-3′ and reverse, 5′-ATACCGCTCGAGTTCATGCTCAACTCCT-3′; U6 forward, 5′-CTCGTTGACACGTTCAG-3′ and reverse, 5′-AACGCTTACGAGTTGTCAT-3′. U6 was used as a reference gene for miR-107 quantification, and the relative quantitative 2⁻ΔΔCq method (34) was used for data analysis. The experiments were repeated three times.

**MTT assay.** HCC cell proliferation was assessed using an MTT assay. SK-HEP-1 cells (1x10⁴ cells/well) in the exponential growth phase were seeded into 96-well plates. Then, 1 mg/ml MTT solution was added to each well and further incubated for 4 h at 37°C. To dissolve the formazan crystals, 100 µl dimethyl sulfoxide was added to each well and the absorbance at 570 nm was measured using a microplate reader.

**Dual-luciferase reporter assay.** SK-HEP-1 cells (1x10⁴ cells/well) were seeded into 96-well plates, and a pGL3 firefly luciferase reporter gene vector (Promega Corporation) with the 3′-UTR-wild-type (WT) or 3′-UTR-mutant (MUT)
fragment of human RGS4 cDNA, containing a putative target site for miR-107, was co-transfected into SK-HEP-1 cells with the miR-107 mimics and negative controls at a 100 nM final concentration using lentiviral transfection. Lipofectamine™ 3000 transfection reagent (Thermo Fisher Scientific, Inc.) was used for all transfections following manufacturer's instructions. At 48 h post-transfection, the luciferase activity of the cell extracts, which was normalized with Renilla luciferase, was measured using the Dual-Luciferase Reporter Assay System (Promega Corporation) and a fluorescence microscope, following the manufacturer's protocol. Experiments were independently repeated ≥3 times.

Colony formation assays. At 24 h post-transfection, SK-HEP-1 cells were resuspended in serum-free DMEM containing 1% N2, 2% B27, 20 ng/ml human fibroblast growth factor-2 and 20 ng/ml EGF (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, cells were seeded in 6-well ultra-low attachment plates (300 cells/well). Following 9 days of incubation in 5% CO2 at 37°C with fresh medium replaced every 3 days, the supplement was discarded and the cells were then washed with phosphate-buffered saline (PBS). The cells were then fixed with 4% paraformaldehyde for 15 min at room temperature and stained with Giemsa stain (Beijing Solarbio Science & Technology, Co., Ltd.) for 20 min. Colonies were counted under a light inverted microscope (TS100; Nikon Corporation) and each assay was performed in triplicate.

Wound-healing migration assay. A wound-healing assay was employed to assess the migrational capacity of SK-HEP-1 cells following transfection. The SK-HEP-1 cells were plated into 24-well plates (2 ml, 2.5x10⁴ cells/well) and cultured in serum-free medium for 24 h to obtain a monolayer. When the cell confluence had reached 80%, a sterile pipette tip held perpendicular to the bottom of the well, was used to scratch the cell surface to create a wound. Following removal of the debris with the tip of the pipette, the culture was replenished with fresh medium and cells were incubated at 5% CO2 and 37°C for 24 h. Images of the migrated cells were captured at 24 h under a light inverted microscope (TS100; Nikon Corporation) and analyzed using ImageJ (v1.8.0; National Institutes of Health).

Transwell invasion assay. A Transwell assay was performed to identify the invasion ability of SK-HEP-1 cells after transfection with miR-107 mimics. Transwell culture inserts precoated with Matrigel (8-mm pore size; BD Biosciences) were placed into upper chambers at 37°C for 30 min. A total of 150 µl cell suspension (2.5x10⁴ cells) suspended in serum-free medium was added to the upper chamber and 500 µl RPMI-1640 medium containing 10% FBS was placed into lower chambers of 24-wells culture plates. Then, the non-invaded cells on the upper surface of the membrane were removed with a cotton swab after incubation at 37°C for 24 h. After fixation with methanol, cells on the lower surface of membrane were stained with 0.005% crystal violet at room temperature in PBS for 1 h, and the number of migrated or invaded cells in 10 random fields was counted under a light inverted microscope (TS100; Nikon Corporation; magnification, x200).

Flow cytometry analysis. After 48 h of transfection with miR-107 mimics, SK-HEP-1 cells were centrifuged at 200 x g for 10 min and fixed with 70% ice-cold ethanol for 24 h at 4°C. Next, the cells were harvested and washed twice with cold PBS and then stained with Annexin V and 7-aminomycin D (7-AAD; BD Biosciences). Following the addition of 5 µl Annexin V and 5 µl 7-AAD with RNaseA (Sigma-Aldrich; Merck KGaA), the cells were incubated in the dark for 30 min at 4°C. The apoptotic cells and the cell cycle distribution were detected using a FACS Calibur flow cytometer (BD Biosciences) and Cell Quest Software (v3.1; BioMedica, Diagnostics, Inc.) following the manufacturer's instructions. All experiments were performed three times.

Western blotting. RIPA lysis buffer (Gibco; Thermo Fisher Scientific, Inc.) was used to extract the total protein from the cells, and the protein concentration was determined using the bicinchoninic acid method. Proteins (40 µg) were separated by 8-15% SDS-PAGE and transferred onto PVDF membranes (Thermo Fisher Scientific, Inc.). GAPDH was used as the internal control. Anti-RGS4 (ab97307; 1:1,000), -EGFR (ab52894; 1:1,000), -CXCR4 (ab1670; 1:500), -MMP-2 (ab37150; 1:500), and -MMP-9 (ab119906; 1:500) were purchased from Abcam. The PVDF membranes were then blocked with 5% non-fat milk in 20 mM Tris-HCI, 137 mM NaCl and 1% Tween-20 (pH 7.6; Invitrogen; Thermo Fisher Scientific, Inc.) for 2 h at room temperature. After that, the membranes were incubated with the primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. Subsequently, the bands were developed with Immobile Western Chemiluminescence HRP Substrates (EMD Millipore). The images were captured using a Luminescence/Fluorescence Imaging System (GE Healthcare), and the signal intensities were quantified using ImageJ analysis software version 1.51j8 (National Institutes of Health). This experiment was performed in triplicate.

Statistical analysis. All data are presented as the mean ± standard deviation obtained from at least three independent experiments. The expression levels of miRNA and mRNA were quantified using the 2^-ΔΔCt method. Statistical analysis was performed using SPSS software version 21.0 (IBM Corp.) and GraphPad Prism version 5.01 (Graph-Pad Software, Inc.). Significant differences between two groups were determined using the Student’s t-test, while differences among several groups were assessed by one-way analysis of variance followed by Dunnett’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-107 expression level is increased in SK-HEP-1 cells after transfection. After transfecting with miR-107 mimics and control mimics, the expression level of miR-107 was determined in SK-HEP-1 cells using RT-qPCR. The results indicated a marked rise in miR-107 expression levels in the miR-107 mimics group compared with the NC and control mimics groups ("P<0.01; Fig. 1A). The result also confirmed the successful transfection of the miR-107 mimics plasmid.
Overexpression of miR-107 represses the growth, migration, invasion and colony formation of HCC cells. To evaluate the biological functions of miR-107 in HCC cells, SK-HEP-1 cells were infected with miR-107-expressing adenoviruses and the effects on cell growth, colony formation, migration and invasion were analyzed. The results of the MTT assay showed that the overexpression of miR-107 significantly reduced cell proliferation, compared with the NC and control mimics groups (P<0.05, at 48 h; P<0.01, at 72 h; Fig. 1B). In addition, the number of SK-HEP-1 cell colonies formed was significantly reduced by miR-107 overexpression (P<0.001; Fig. 2A and B). Transwell and wound-healing assays indicated that both the invasion (P<0.001; Fig. 2C and D) and migration (P<0.05; Fig. 3A and B) capacities of SK-HEP-1 cells were significantly repressed by miR-107 overexpression. The results indicated that miR-107 regulated the proliferation, migration, invasion and colony formation of HCC cells.

The RGS4 3'UTR contains a putative miR-107 binding site that is required for the miR-107-mediated regulation of RGS4 expression. To verify whether the miR-107 binding site is required for regulating RGS4 expression, two luciferase reporter plasmids were constructed, a wild-type 3'-UTR (RGS4-3'-UTR-WT) and a mutant (RGS4-3'-UTR-MUT). To further investigate whether the predicted binding site of miR-107 to the RGS4 3'-UTR was responsible for this regulation, the 3'-UTR of RGS4 was cloned downstream of a luciferase reporter gene (WT-RGS4). It was observed that the overexpression of miR-107 remarkably reduced the luciferase activity of RGS4-3'-UTR-WT (P<0.01; Fig. 4), but not RGS4-3'-UTR-MUT when compared with the NC and control mimics groups. From these results, it may be concluded that the sequence 5'-AUG CUG C-3' within the RGS4 3'-UTR is required for miR-107-mediated regulation of RGS4 expression in HCC cells.

Overexpression of miR-107 enhances the expression of RGS4, and reduces that of EGFR, CXCR4 and MMP-2 and -9. To further confirm that miR-107 regulates the expression of RGS4, EGFR, CXCR4, MMP-2 and MMP-9, SK-HEP-1 cells were transfected with miR-107 mimics and control mimics, and the protein expression levels were detected by western blot analysis. The results showed that enhancing miR-107 expression significantly promoted GRS4 expression, compared with the NC and control mimics groups, whereas enhanced miR-107 significantly repressed EGFR, CXCR4, MMP-2 and MMP-9 expression (P<0.01; Fig. 5).

Effects of miR-107 overexpression on SK-HEP-1 apoptosis and cell cycle distribution. To explore the possible mechanisms of miR-107 overexpression in SK-HEP-1 cells, the effect of miR-107 mimics on the apoptotic rate and cell cycle distribution was determined. 7-Amino-actinomycin D (7-AAD) has a high DNA binding constant and is efficiently excluded by cells in early apoptosis, while it can enter cells in late apoptosis and necrotic cells to stain their nuclei. Therefore, the matching use of Annexin V and 7-AAD can be used to distinguish cells between early and late apoptosis and dead cells. As shown in Fig. 6, flow cytometry indicated that the early apoptotic rate was notably increased following miR-107 overexpression (P<0.001; Fig. 6). Furthermore, the overall level of apoptosis was increased. Cells treated with miR-107 mimics were arrested in S phase, and S phase injury repair was not completed. (P<0.05 and P<0.01; Fig. 7). There was no significant difference in the G2 phase population size among the miR-107 mimics, mimics control and NC groups. These results suggested that the overexpression of miR-107 promoted cell proliferation by inhibiting the cell cycle and inducing apoptosis.

Discussion

Globally, HCC is one of the most common human malignancies, and its incidence in China is the highest among the Asian countries (35). The number of patients diagnosed with HCC each year is >700,000, and >600,000 mortalities are associated with malignant HCC (36). In previous years, several studies have shown that miRNAs are involved in the progression and metastasis of tumors (37). miRNA-107 is regarded as a tumor suppressor and may be downregulated in breast cancer, possibly through the regulation of its downstream target, brain-derived neurotrophic factor (38). Imamura et al (39) identified downregulated tumor suppressor miRNAs in the plasma (including miR-107) for the treatment of pancreatic cancer, using a comprehensive miRNA array-based approach.
Similarly, other studies have shown that miR-107 acted as a tumor suppressor in several different tumor types, including breast cancer, non-small cell lung cancer and head and neck squamous cell carcinoma (40-42). In the present study, the overexpression of miR-107 suppressed cell proliferation, invasion, migration and colony-forming ability, promoted apoptosis and caused G1 arrest, indicating that miR-107 acts as a tumor suppressor in hepatic carcinoma cells.

Moreover, \textit{in vitro} experiments revealed that the overexpression of miR-107 enhanced RGS4 expression. The G protein-signaling pathway serves a key role in the development and regeneration of normal liver tissue, and an important role in the development of HCC. Reportedly, altered levels of RGS4 expression are associated with several human diseases, including cancer (43). A number of studies have shown that specific members of the RGS family are also involved in the...
occurrence of various tumors types. RGS2 inhibition contributes to the development of bladder cancer (44). Furthermore, RGS5 and RGS10 are involved in the development of ovarian cancer (45), and RGS6 and RGS16 are both associated with the EGF-mediated apoptosis and proliferation of breast cancer cells (46). RGS17 can promote the proliferation of lung cancer cells through the cyclic adenosine monophosphate (cAMP)-protein kinase-cAMP response element-binding protein signal transduction pathway (47). Additionally, RGS22 can also inhibit the migration of pancreatic cancer cells (48), and numerous studies have demonstrated that RGS4 may regulate GPCR signaling during breast cancer cell proliferation (49).

In addition, RGS4 may inhibit the signal pathway by downregulating the Gi-coupled receptors protease-activated receptor 1 and CXCR4 signal transduction, which were associated with the migration and invasion of breast cancer cells (23). RGS4 is also present in NSCLC cells, and its overexpression reduces the invasion and migration of tumor cells by inhibiting MMP-2 and-9 and reversing EMT (30). In addition, MMP-2 and -9 were confirmed to be involved in the development and metastasis of cutaneous melanoma (31,32). In human cancer cells, the function of kinase-independent EGFR is to prevent autophagic cell death (50). Activated EGFR is involved in the regulation of cell survival, proliferation and differentiation. The present study showed that an increase in the expression level of RGS4 protein resulted in a corresponding reduction in the migration of SK-HEP-1 cells.
in migration, invasion and colony-forming ability of HCC cells. Therefore, it was hypothesized that miR-107 serves as a tumor suppressor and negatively regulates RGS4, which in turn alters the expression levels of EGFR, CXCR4 and MMP-2 and -9. These findings suggest that miR-107 may be a promising therapeutic target, and support its involvement in the regulation of RGS4 overexpression in HCC.

In conclusion, the present study indicated that miR-107 directly regulates RGS4 expression by targeting the RGS4 3’-UTR, thereby suppressing the migration and invasion of human HCC cells, and promoting apoptosis by reducing the expression levels of EGFR, CXCR4 and MMP-2 and -9. To the best of our knowledge, this is the first report to demonstrate that miR-107 is depleted in HCC cells, and that it may act as a biomarker and therapeutic target for HCC. These findings support the accumulating evidence that miR-107 participates in the progression of hepatic carcinoma by modulating RGS4 expression.
Figure 6. Upregulation of miR-107 promotes apoptosis in SK-HEP-1 cells. The percentages of apoptotic cells (in Q2 and Q4) are displayed in the bar graph. Experiments were performed three times and the values are expressed as the mean ± standard deviation. ***P<0.001 vs. NC and control mimics. NC, negative control; miR, microRNA; Q, quadrant.

Figure 7. Upregulation of miR-107 promotes changes in the cell cycle distribution of SK-HEP-1 cells in the G1, S and G2/M phases. Compared with the NC and control mimics groups, SK-HEP-1 cells transfected with miR-107 mimics were arrested in S phase, and S phase injury repair was not completed. Furthermore, the overall level of apoptosis was increased. *P<0.05, **P<0.01 vs. NC and control mimics. NC, negative control; miR, microRNA.
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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

DX designed the research, wrote the manuscript, and performed cell culture and molecular biology experiments; HXG designed the research and critically revised the manuscript for intellectual content. Both authors have read and approved the final manuscript.

Ethics approval and consent for publication

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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