MicroRNA-150 inhibits the proliferation and metastasis potential of colorectal cancer cells by targeting iASPP

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Abstract. In the present study, the function of miR-150 and its downstream target iASPP in the growth and metastasis of colorectal cancer (CRC) cells was investigated. The expression of miR-150 and iASPP was first investigated in clinical CRC samples. Subsequently, the effects of miR-150 overexpression and iASPP inhibition on cell viability, cell cycle distribution, apoptosis, migration and invasion were detected with CCK-8, flow cytometry, scratch and Transwell assays. The interaction between miR-150 and iASPP was confirmed using a dual-luciferase assay. Subsequently, the key role of iASPP in the anti-CRC function of miR-150 was assessed by inducing the expression of the gene in miR-150 overexpressed SW480 cells. In clinical samples, the level of miR-150 was downregulated, while iASPP was induced. Enforced expression of miR-150 decreased the viability, induced G1 cell cycle arrest and apoptosis, and inhibited the migration and invasion of SW480 cells. Knockdown of iASPP exerted a similar effect on SW480 cells to that of the overexpression of miR-150. Dual-luciferase assay demonstrated that miR-150 directly bound to iASPP and inhibited its transcription. The function of miR-150 depended on the inhibition of iASPP; induced expression of iASPP in miR-150-knockdown SW480 and HCT116 cells restored cell viability, migration and invasion while inhibiting G1 cell cycle arrest and apoptosis. Increased expression of miR-150 suppressed viability, proliferation, migration and invasion of SW480 cells. Furthermore, iASPP was a direct target of miR-150 and played a key role in its anti-CRC function. miR-150 may be a promising predictor of prognosis in CRC patients.

Introduction

Colorectal cancer (CRC) remains the fourth most common cause of cancer-related deaths worldwide (1), with more than 1.2 million new cases being identified annually (2). Although most cases of CRC are sporadic, 20-30% of individuals with CRC carry inherited mutations in key tumor suppressors, such as APC and TP53 (3,4). Compared with other solid malignancies, CRC is characterized by slow development, which renders the tumor curable and preventable. Additionally, the survival rate of patients with CRC is critically dependent on the tumor stage at diagnosis. Thus, early diagnosis of CRC has become a central subject in the field of CRC studies. The identification of robust molecular indicators associated with the proliferation and tumor-node-metastasis (TNM) potential of CRC can lead to avoiding understaging of the tumor and help to pinpoint patients with early-stage CRC. In recent years, studies regarding microRNA (miR) have indicated the potential of this non-coding RNA type in categorizing the subtype and prognosis of CRC (5-7).

miR can suppress mRNA translation of targeted genes by binding to the 3’ untranslated region (UTR) of target messenger RNA (mRNA) (8). Dysregulation of miRs has been demonstrated to be associated with tumorigenesis of various human organs, including the colorectum (9,10). Liu et al (6) revealed that miR-124 upregulation reduced cell viability and proliferation of CRC cells in vitro. In another clinical study, Sarlinova et al (11) reported significantly upregulated expression of miR-21 and miR-221 and downregulation of miR-150 in blood samples of CRC patients. As one of the most studied miRs in various types of cancer (12-14), the positive correlation between the expression of miR-150 and the survival of CRC patients has been long revealed (15,16). Furthermore, the dysregulation of miR-150 in CRC was subsequently studied by Wang et al (7) and Feng et al (17), who revealed the antagonistic effect of miR-150 on the oncogenesis and progression of CRC via targeting MUC4 and c-Myb. Given that the above-mentioned studies markedly indicated that miR-150 is a tumor suppressor gene in CRC, it is reasonable to further explore the mechanism driving the antitumor function of miR-150 in CRC.

Inhibitor of apoptosis stimulating protein of p53 (iASPP) belongs to the ASPP family (18). This factor can inhibit the normal function of p53, which leads to oncogenesis in human organs (19,20). Furthermore, iASPP can also negatively regulate the p65 subunit of nuclear factor-κB (NF-κB), which plays a vital function in inflammation and apoptosis (21). Therefore, suppressing the function of iASPP may serve as a promising...
therapeutic strategy for the prevention and treatment of CRC. Based on bioinformatic analysis, iASPP is a potential target of miR-150 and regulation of iASPP by miR-150 may influence the biological features of CRC cells. To verify our hypothesis in the present study, we challenged the expression of miR-150 in clinical samples and then detected the effect of miR-150 induction/iASPP inhibition on the viability, apoptosis and mobility of CRC cells. The findings outlined in the present study confirmed the direct regulation of iASPP by miR-150, which would impair the growth and metastasis potential of CRC.

Materials and methods

Reagents and chemicals. Antibodies against iASPP (cat. no. ab115605) and GAPDH (cat. no. KC-5G5) were purchased from Abcam (Cambridge, UK) and Kangcheng Bio (Beijing, China), respectively. Hoechst staining kit (cat. no. H1399) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). IgG-HRP antibody (cat. no. BA1054) was purchased from Wuhan Boster Biological Technology Ltd. (Wuhan, China).

Patient and CRC specimen collection. CRC specimens were collected from 30 patients (from July 2015 to October 2016) in The Chinese People’s Liberation Army General Hospital. The specimens were fixed and prepared in paraffin sections. The patients enrolled in the present study were diagnosed with primary CRC and had detailed clinicopathological and prognostic information. Screening, inspection and data collection were approved by the Ethics Committee of The Chinese People’s Liberation Army General Hospital, and a written informed consent form was signed by all subjects. The procedures performed adhered to the Declaration of Helsinki.

Immunohistochemistry. Immunohistochemistry was used to detect the expression of iASPP protein in tissues, according to the operation guide of immunohistochemistry. Paraformaldehyde (4%) fixed tissues and paraffin embedded sections for antigen retrieval were used. Samples were then incubated overnight at 4°C with iASPP antibodies (1:400). After being washed with PBS, samples were incubated for 50 min at RT with IgG-HRP antibodies (1:500). Then the samples were observed and images were captured by an optical microscope (CX41-23C02; Olympus Corp., Tokyo, Japan).

Cell culture. Human CRC cell lines FHC, HCT116, HCT8, HT29, H1299 and SW480 were obtained from Shanghai Bioleaf Biotech, Co., Ltd. (Shanghai, China). The cells were cultured in minimum essential medium (MEM, M2279; Bioleaf Biotech, Co., Ltd., (Shanghai, China). The cells were cultured in minimum essential medium (MEM, M2279; Sigma-Aldrich, St. Louis, MO, USA) with 15% fetal bovine serum (FBS; 10099-141; Gibco, Carlsbad, CA, USA) and 1% (v/v) antibiotics mix and maintained in an atmosphere of 95% air and 5% CO2 at 37°C. The expression level of miR-150 was determined using reverse transcription real-time PCR (qPCR) as described in the following sections. The cell line with the lowest expression level of miR-150 was selected for subsequent assays and, based on the results of qPCR, SW480 cell line had the lowest expression level of miR-150 (Fig. 1D) and was employed as an in vitro model for CRC.

Construction of vector, sequences of siRNA and transfection. Specific siRNA targeting iASPP (5'-AGTTCATGTC CAGAAAGTCCC-3') and non-targeting siRNA (5'-ACGU GACACGUUCGGAGAATT-3') were used to knockdown the expression of iASPP. Coding sequences were cloned through amplification reaction using primers (iASPP forward, 5'-GGGGTACCATGGACAGCGAGGCATTCC-3' and iASPP reverse, 5'-CCGCTCGAGCTAGACTTTACTCCTTTGAG GCTTTCAC-3'). Subsequently, the PCR product (2487 bp) was ligated to the pcDNA3.0 plasmid, and recombinant plasmid was confirmed by sequencing after digestion with KpnI/XhoI. SW480 cells were transfected with different vectors using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific). The coding sequence of iASPP was ligated into the pcDNA plasmid to form the pcDNA-iASPP vector for overexpression of the gene.

Experimental design and grouping. To detect the function of miR-150 in the oncogenesis of CRC, SW480 cells were divided into two groups: i) NC group, SW480 cells transfected with NC mimics; and ii) mimics group, SW480 cells transfected with miR-150 mimics. Each group was represented by at least five replicates.

To elucidate the key role of iASPP in the progression of CRC, SW480 cells were divided into three groups: i) Blank group, SW480 cells; ii) NC group, SW480 cells transfected with pcDNA-NC plasmid; and iii) siRNA group, SW480 cells transfected with pcDNA-siiASPP plasmid. Each group was represented by at least five replicates.

The interaction between miR-150 and iASPP was further assessed with four groups: i) blank group, SW480 cells; ii) NC group, SW480 cells transfected with NC mimics; iii) mimics group, SW480 cells transfected with miR-150 mimics; and iv) mimics+pcDNA group, miR-150 stably overexpressed in SW480 cells transfected with pcDNA-siAASPP plasmid.

Dual-Luciferase assay. The direct regulating function of miR-150 on the 3’UTR of iASPP was determined with a Dual-Luciferase assay. Luciferase activity was detected by Dual-Luciferase assay kit (E1960; Promega, Madison, WI, USA) after 24 h of transfection and co-transfection of Renilla luciferase plasmid, used as the internal control for transfection efficiency.

Real-time PCR. Total RNA in the cells was extracted by RNA purification using RNA Extraction kit (9109; Takara Bio, Inc., Otsu, Japan) accordingly. β-actin and U6 were selected as the internal reference genes. cDNA templates were achieved by using Super MMLV Reverse Transcriptase (DB1-2342; DBI Bioscience, Shanghail, China), and the final RT-qPCR reaction mix contained 10 µl Bestar® SYBR Green qPCR Master Mix, 0.5 µl of each primer (miR-150, forward 5'-ACACTCCAG CTGGGTTCTCCAAACCTTTGACC-3' and reverse, 5'-CCTGGAAGCAGTGCTGA-3'), 5'ACTGTGGTCGTCGGA-3'; iASPP, forward, 5'-GAAAGTCCAGCTGGTCTCCAAACCTTTGACC-3' and reverse, 5'-GCGCTCGAGCTAGACTTTACTCCTTTGAGGCTTTCAC-3'); and GAPDH, forward, 5'-CTCGCCGTCTCGCCTTC-GCAACA-3' and reverse, 5'-AGCGCTTTACAGGATAATTTCAGGT-3'; and GAPDH, forward, 5'-TGTCCGCTCATGG GTGTGAC-3' and reverse, 5'-ATGGCAGGATGCTTGG TCAT-3'), 1 µl cDNA template and 8 µl RNase-free H2O.
Amplification was performed as follows: a denaturation step at 94°C for 2 min, followed by 40 cycles of amplification at 94°C for 20 sec, 58°C for 20 sec and 72°C for 20 sec. The reaction was stopped at 25°C for 5 min. The relative expression levels were detected and analyzed by Exicycler™ 96 (Bioneer Corp., Daejeon, Korea) based on the formula of $2^{-\Delta\Delta \text{ct}}$.

**Cell Counting Kit-8 (CCK-8) assay.** CCK-8 assay was performed to detect cell viability. Briefly, SW480 cells (1x10^5 cells/ml) that underwent different treatments were seeded into one well of a 96-well plate and incubated for 72 h. Every 24 h, 10 µl of CCK-8 solution was added to every well and incubated at 37°C for a minimum of another 4 h. The OD values were detected at 450 nm and employed as the representative of cell viability.

**Flow cytometry.** Cell cycle distribution was determined using flow cytometry. Cells were stained with propidium iodide (PI) in the dark for 20 min at room temperature. The results were analyzed using a FACS flow cytometer (BD Accuri C6; BD Biosciences, San Jose, CA, USA).

**Hoechst staining.** DNA damage in cell nuclei was detected using a Hoechst staining method after cells were transfected for 24 h. Cells were stained with Hoechst 33258 (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions.

**Scratch assay.** To evaluate cell mobility, a scratch assay was performed on the transfected cells. Cells at a density of 2x10^4 cells/well were incubated in one well of a 24-well plate. After marking reference points, cells were cultured to confluence at 37°C for 48 h. Then, a cell-free line was made (regarded to as a scratch), and debris at the edges was removed. Twenty-four hours after the scratch was made, gap distances between the midline were assessed using an optical microscope (Olympus Corp.) in reference to the reference points.

**Transwell assays.** Transwell assays were performed to detect the mobility of SW480 cells. In brief, cells at a density of 2x10^4 cells/well were incubated in the upper chamber (Corning Costar, Cambridge, MA, USA) after a 24-h serum-free incubation, and the chamber was pre-coated with 40 µl Matrigel (0.8 µg/µl) at 37°C for 2 h. Then, the system was placed at 37°C for 24 h and, subsequently, cells in the upper surface were completely removed. The lower surfaces of the chamber were stained for 5 min using 1% (w/v) crystal violet and the cell number was recorded using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

**Western blotting.** Total cellular protein was extracted using the protein lysate. Western blot analysis was conducted according to Lin et al (22). The membranes were incubated with primary antibodies against iASPP (1:4,000) and GAPDH (1:10,000) for 1 h at room temperature. Secondary HRP-conjugated IgG
antibodies (1:20,000) were then added and incubated for 45 min at room temperature. The blots were developed and the results were recorded in the Gel Imaging System (ScanWizard Bio; Microtek International, Inc., Taipei, Taiwan). Then, data were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc.).

Statistical analysis. Data were expressed as the mean ± standard deviation (SD). Student’s t-test was performed with a significance level of 0.05. Statistical analyses and graphing were performed using GraphPad Prism 6.01 (GraphPad Software, Inc., Chicago, IL, USA).

Results

The expression of miR-150 is induced and the expression of iASPP is upregulated in clinical CRC samples. The expression status of miR-150 was determined with qPCR validation in clinical CRC samples and corresponding para-carcinoma tissues from patients (Table I). As displayed in Fig. 1, the level of miR-150 in CRC samples was lower than that in para-carcinoma samples and the difference was statistically significant (Fig. 1B; P<0.05). On the contrary, the expression of iASPP at the mRNA level was induced in CRC samples (Fig. 1C). Based on the detection in clinical samples, it was inferred that miR-150 had an anti-CRC effect during the progression of the tumor. Furthermore, miR-150 expression was assessed, and lower expression was detected in SW480 and HCT116 cells, and relatively higher expression presented in SW620 and HT29 cells compared with the FHC cell line (Fig. 1D). The expression of iASPP was also investigated using immunohistochemistry in clinical tissues from CRC patients. As displayed in Fig. 1A, the expression of iASPP was relatively higher in tumor tissue than para-carcinoma tissue.

Augmented expression of miR-150 suppresses cell viability and induces cell apoptosis and G1 cell-cycle arrest in SW480 cells. The effect of miR-150 overexpression on normal cell features was determined to validate the antitumor effect of miR. Being induced by transfection of specific mimics, the upregulated expression of miR-150 significantly decreased the OD450 value in the mimics group at 72 h (Fig. 2A), representing impaired viability of SW480 cells. Furthermore, the overexpression of miR-150 induced G1 cell cycle arrest and cell apoptosis in the mimics group, as a larger proportion of cells were distributed in the G1 phase (Fig. 2B) and more Hoechst-positive cells (Fig. 2C) were detected in cells transfected with miR-150. Detection focusing on the proliferation potential of SW480 cells confirmed the conclusion that miR-150 is an anti-CRC molecule that influences CRC cells both by decreasing cell proliferation and inducing cell apoptosis.

Augmented expression of miR-150 impairs the migration and invasion ability of SW480 cells. The effect of miR-150 overexpression was further assessed by detecting its effect on the metastasis potential of SW480 cells. For cells subjected to

| Characteristics | Total | Low | High |
|-----------------|-------|-----|------|
| Age (years)     |       |     |      |
| <60             | 18    | 10  | 8    |
| ≥60             | 12    | 5   | 7    |
| Sex             |       |     |      |
| Male            | 17    | 9   | 8    |
| Female          | 13    | 6   | 7    |
| Tumor size (cm) |       |     |      |
| <5              | 11    | 9   | 2    |
| ≥5              | 19    | 6   | 13   |
| TNM stage       |       |     |      |
| I+II            | 13    | 10  | 3    |
| III+IV          | 17    | 5   | 10   |

CRC, colorectal cancer; TNM, tumor-node-metastasis.

Figure 2. miR-150 decreases cell viability and induces cell cycle arrest and apoptosis in SW480 cells. (A) CCK-8 assay demonstrated that miR-150 decreased the OD_{450} value at 48 and 72 h. (B) Flow cytometry assay revealed that a higher number of cells distributed in the G1 phase was recorded in the mimics group. (C) Hoechst staining exhibited that DNA damage was observed in the mimics group. NC, negative control; Mimics, miR-150 mimics. *P<0.05 vs. NC.
the scratch assay, induced expression of miR-150 resulted in a delayed closure rate of the gap (wider gap width) (Fig. 3A). Furthermore, impaired invasion ability was also detected in SW480 cells with overexpression of miR-150 (Fig. 3B), as less cells penetrating the membranes were recorded in the mimics group. The results of the scratch and Transwell assays together indicated the inhibitory effect of miR-150 on the metastasis potential of CRC cells.

iASPP is directly regulated by miR-150 and plays a promoting role in the oncogenesis of CRC. Based on bioinformatic analysis, iASPP was a potential target of miR-150. In the present study, a possible interaction between the two indicators was determined using a dual-luciferase assay. The results indicated that only cells transfected with miR-150 mimics and wild-type iASPP 3′UTR demonstrated a decreased relative luciferase activity (Fig. 4), which indicated a direct and specific modulating effect of miR-150 on the iASPP gene.

To confirm the results that iASPP promoted the progression of CRC, the effects of iASPP knockdown on cell viability, cell apoptosis, cell cycle distribution and cell migration and invasion were also assessed. The expression of iASPP was confirmed by western blot analysis after transfection with siRNA (Fig. 5A). The results indicated that iASPP knockdown exhibited a similar effect on SW480 cells to that of miR-150 overexpression: in the siRNA group, cell viability was decreased (Fig. 5B), G1 cell cycle arrest and apoptosis were induced (Fig. 5C and D) and cell migration and invasion were impaired (Fig. 6).

miR-150 exerts its suppressing effect on CRC cells by targeting iASPP. Given the direct regulating function of miR-150 in the transcription of iASPP, it was hypothesized that the antitumor effect of miR-150 on CRC was dependent on the suppressed function of iASPP. Therefore, the expression of iASPP was induced in miR-150 overexpressed SW480 and HCT116 cells (Figs. 7A and 9A, respectively). Subsequently, the growth and metastasis potential of SW480 and HCT116 cells in different groups were assessed. Based on the results, induced expression of iASPP counteracted the effect of miR-150 overexpression on SW480 and HCT116 cells. Concerning growth potential, induced iASPP expression increased cell viability, relieved cells from G1 cell cycle arrest and inhibited cell apoptosis in the miRNA+pcDNA group when compared with the miRNA group (Figs. 7B-D and 9B-D). Additionally, upregulated iASPP levels also improved the metastatic potential of SW480 and HCT116 cells, as a faster closure rate and more cells penetrating the membrane were detected in the miRNA+pcDNA group as demonstrated by scratch and Transwell assays (Figs. 8 and 9E-G). It was hypothesized that, without the low level of iASPP, the suppressing function of miR-150 on CRC cells was confounded, which indicated that the anti-CRC function of miR-150 was exerted via the inhibition of iASPP.
Discussion

The suppressed expression of miRs in colorectal cancer (CRC) may represent a novel therapeutic avenue for the treatment of CRC (5,23). Among all reported downregulated miRs in CRC, the potential of miR-150 as a biomarker for diagnosing and predicting CRC has been reported in several studies (7,17). In the present study, the data confirmed the decreased level of miR-150 in clinical CRC samples. Furthermore, reintroduction of miR-150 markedly suppressed viability, induced apoptosis and inhibited migration and invasion of CRC cells in vitro. The effect of miR-150 on SW480 cells depended on the function of iASPP, the overexpression of which blocked the impairments of miR-150 mimics on SW480 cells. The present study clearly indicated that miR-150 was capable of predicting and suppressing CRC, a finding that deserves further investigation.

Dysregulation of miR-150 has been reported in diverse tumor types. However, the exact function of miR-150 varies by tumor type. For gastric, breast and lung cancer, miR-150 plays a role in promoting the progression of cancer (13,24,25). Conversely, for pancreatic cancer, miR-150 is able to suppress the growth of the tumor by targeting MUC4 (26). The possible involvement of miR-150 in CRC was first reported by Ogata-Kawata et al (27) however, in their study, serum exosomal levels of miR-150 were markedly higher in primary CRC patients than in healthy controls. With emerging attention being paid to the function of miR-150 in CRC, several other researchers have consistently revealed a reduced level of miR-150 in CRC tumors (5,15,17), which indicated that miR-150 is a biomarker associated with CRC prognosis (15). In the present study, qPCR validation of clinical CRC and para-carcinoma samples confirmed the conclusion that miR-150 was suppressed in CRC tissues. Subsequently, induced miR-150 in human CRC cell line SW480 further verified the inhibitory effect of miR-150 on the growth and metastatic potential of CRC cells. To further uncover the
downstream pathways involved in the antagonizing effect of miR-150 on CRC, we also detected the effects of interaction between miR-150 and iASPP on CRC cells.

iASPP acts as a negative regulator of the tumor suppressor p53 and its overexpression has been associated to poor prognosis and survival in some types of cancers (18,21). The gene can suppress apoptosis by deactivating the function of p53 on the promoters of proapoptotic genes (21). In addition to regulating p53, iASPP has been also proven to inhibit the transcription of RelA/p65 and reduce inflammation (28). Induced expression of miR-124 in CRC cells attenuated cell viability, proliferation and colony formation via inhibition of iASPP protein expression and forced overexpression of iASPP rescued CRC cells from the inhibitory effect of miR-124 (6). Therefore, targeted suppression of iASPP may serve as the mechanism by which its upstream miR prevents oncogenesis.
of CRC. In the present study, we proved that overexpression of iASPP contributed to the enhanced growth and metastasis of CRC cells. By performing a dual-luciferase assay, iASPP was validated to be a direct target of miR-150 in CRC cells and induced expression of miR-150 restricted the expression of iASPP both at the mRNA and protein levels. However, the impairment of miR-150 in SW480 cells was partially obscured by re-expression of iASPP. Collectively, these results indicated that miR-150 can act as a suppressor on the growth and metastasis of CRC and this effect was exerted by direct inhibition of the expression of iASPP.

In conclusion, the present study provided more evidence supporting the anti-CRC function of miR-150. The low expression miR-150 in clinical samples highlighted the possibility that the molecule may predict poor prognosis in CRC patients. In addition, the suppressed viability, proliferation, migration and invasion in SW480 cells due to enforced expression of miR-150 supported the treatment potential of miR-150 in CRC. The present study also revealed a key role of iASPP in the oncogenesis of CRC, which can be targeted by miR-150. The findings of the present study provided a supplementary mechanism underlying the suppressing effect of miR-150 on CRC and offered a therapeutic target for future exploration.

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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

CL and XD conceived and designed the study. CL and SX performed the experiments. CL and LC wrote the study. CL

Figure 9. The suppressing effect of miR-150 on HCT116 cells depends on the overexpression of iASPP. (A) The expression of iASPP was assessed after HCT116 cells were transfected with mimics or/and iASPP. (B) Cell proliferation was reversed after the cells were transfected with pcDNA-iASPP compared with the mimics group. (C) Flow cytometry assay indicated that a lower number of cells distributed in the G1 phase was recorded in the mimics+pcDNA group compared with the mimics group. (D) Hoechst staining demonstrated that less Hoechst-positive cells were observed in the mimics+pcDNA group compared with the mimics group. (E-G) The inhibitive effect on cell migration and invasion was reversed in the mimics+pcDNA group compared with the mimics group. Blank, SW480 cells; NC, negative control, Mimics, miR-150 mimics. *P<0.05, **P<0.01 vs. NC, #P<0.05 vs. mimics.
and XD reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All procedures were approved by the Chinese People’s Liberation Army General Hospital from May 2015 to October 2016.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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