Silencing long intergenic non-coding RNA 00707 enhances cisplatin sensitivity in cisplatin-resistant non-small-cell lung cancer cells by sponging miR-145

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Abstract. The aberrant expression of long non-coding RNAs is closely associated with drug resistance in multiple types of cancer. Long intergenic non-coding RNA 00707 (LINC00707) has previously been reported to be an oncogene able to promote lung adenocarcinoma cell proliferation and metastasis. However, its role in the progression of cisplatin (DDP) resistance in non-small-cell lung cancer (NSCLC) requires further elucidation. In the present study, LINC00707 and microRNA (miR)-145 expression levels were measured using reverse transcription-quantitative PCR (RT-qPCR). MTT and flow cytometric assays were performed to evaluate the IC50 value of DDP and cell apoptosis, respectively. Bcl-2, Bax, multidrug resistance protein 1 (MRP1) and P-glycoprotein (P-gp) mRNA and protein expression were detected using RT-qPCR and western blotting, respectively. The interaction between LINC00707 and miR-145 was explored using a luciferase reporter assay. LINC00707 expression was found to be significantly upregulated in DDP-resistant A549 cells (A549/DDP) cells when compared with that in parental A549 cells. LINC00707 knockdown reduced the IC50 value of DDP, enhanced apoptosis and inhibited Bcl-2, MRP1 and P-gp expression, while promoting Bax expression in A549/DDP cells. miR-145 expression was found to be significantly decreased in A549/DDP cells when compared with A549 cells. LINC00707 directly interacted with miR-145 and negatively regulated its expression. Furthermore, miR-145 downregulation weakened the effect of LINC00707 knockdown in A549/DDP cells. Therefore, silencing of LINC00707 enhanced DDP sensitivity in A549/DDP cells by sponging miR-145, thereby shedding light on LINC00707 and its corresponding molecular mechanisms involved in the progression of DDP resistance in NSCLC cells.

Introduction

Non-small-cell lung cancer (NSCLC) accounts for ~85% of all lung cancer cases, demonstrating a high degree of mortality and poor survival worldwide (1). In recent years, substantial advances have been achieved in NSCLC diagnosis and treatment; however, the 5-year survival rate has remained unchanged at 15% (2). Cisplatin (DDP) is a first-line drug used in NSCLC chemotherapy; however, resistance to chemotherapy drugs administered after surgeries limits the prognosis of patients, as well as the use of DDP in clinical applications (3). Therefore, the molecular mechanisms underlying DDP resistance need to be urgently elucidated in order to improve the survival rate of patients with NSCLC.

Long non-coding RNAs (lncRNAs) are non-protein coding RNAs that are greater than 200 nucleotides in length (3). Numerous studies have indicated that lncRNAs are capable of regulating gene expression at the transcriptional, post-transcriptional and epigenetic levels, and serve an important role in human cancer development, prognosis and drug resistance (4,5). Recent studies have associated certain lncRNAs, including CCAT1 (6) and TRPM2-AS (7), with drug resistance (4,5). Aberrant expression of various lncRNAs has been suggested to contribute to DDP resistance in human cancers (13). miR-145, a tumor suppressor miRNA, has been reported to be downregulated in several types of human cancers, including lung cancer (14). Furthermore, Zhan et al (15) reported that miR-145 promoted multidrug resistance protein 1 (MRP1) mRNA degradation and, therefore, sensitized gallbladder cancer cells...
to DDP. However, whether LINC00707 acts as an miR-145 sponge in order to regulate DDP resistance in NSCLC cells remains to be investigated.

The aim of the present study was to investigate the role and potential regulatory mechanism of LINC00707 in DDP-resistance progression in NSCLC.

Materials and methods

Cell culture and transfection. DDP-resistant A549 cells (A549/DDP) and parental A549 cells were obtained from The Cancer Institute of the Chinese Academy of Sciences. The cells were maintained in RPMI-1640 medium containing 10% FBS (both HyClone; GE Healthcare Life Sciences) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator with 5% CO₂ at 37°C. To maintain the DDP-resistant phenotype, 2 µM DDP (Sigma-Aldrich; Merck KGaA) was also added to the culture media of A549/DDP cells. LINC00707 siRNA (si-LINC00707; 5'-GCA GGA ACAUCAACAUUUU-3'), siRNA negative control (si-NC; 5'-UUCCUGGAUGUCAGUUTT-3'), miR-145 mimic (5'-UCCAGUUUUCCCAGAUAUUUC-3'), miRNA negative control (NC, 5'-UCACACCCUCCUGA AAGAGUAGA-3'), miR-145 inhibitor (5'-AGGAUUCE UGGGAAACCUGGC-3') and negative control (inhibitor NC, 5'-UCUACUCCUUCUAGGGUGUGA-3) were all purchased from Shanghai GenePharma Co., Ltd. The transfection of above siRNAs or miRNA mimics (final concentration: 50 nM) into A549/DDP cells (4x10⁵/per well of 6-well plate) was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were collected for further experiments 48 h after transfection.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA (1 µg) was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit (cat. no. 4368814, Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT-qPCR was performed using the ABI 7500 RT-PCR system (Applied Biosystems; Thermo Fisher Scientific). The primers were synthesized by Takara Biotechnology Co., Ltd. The primers sequences are provided in Table I. 18s rRNA was used as internal references for IncRNA, mRNA and miRNA. U6 small nuclear RNA was used as internal references for miRNA. The relative expression levels were quantified using the 2-∆∆Cq method (16). RT-qPCR reactions were performed in triplicate with the following conditions: 95°C for 2 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Cell proliferation assay. The sensitivity of cells to DDP treatment was determined using a CellTiter 96® Non-Radioactive Cell Proliferation Assay kit (Promega Corporation). In brief, transfected cells were seeded in triplicate into 96-well plates at a density of 4x10³ cells/well in 100 µl RPMI-1640 medium (HyClone; GE Healthcare Life Sciences). After 12 h, A549 and A549/DDP cells were treated with different concentrations of DDP (5, 10, 20, 40 and 80 µM) for 1, 2 and 3 days. Subsequently, MTT (10 µl; 5 mg/ml) was added into each well and incubated for 4 h at 37°C. Then 100 µl of dimethyl sulfoxide was added to dissolve the solution and solubilize the crystals. The optical density was detected at 570 nm using a microplate reader (Bio-Rad Laboratories, Inc.). The in vitro DDP activity was expressed in terms of concentrations capable of suppressing cell proliferation by 50% (IC₅₀). This assay was performed in triplicate.

Flow cytometric analysis of apoptosis. The Annexin V-FITC Apoptosis Detection Kit (Nanjing KeyGen Biotech Co., Ltd.) was used to evaluate cell apoptosis. Briefly, A549/DDP cells (10⁶ cells/ml) were harvested 48 h after transfection and washed twice with ice-cold PBS. The cells were then resuspended in 500 µl of binding buffer. Next, the cells were stained with 5 µl of Annexin V-FITC and 5 µl of propidium iodide, and incubated at 25°C for 15 min in the dark according to the manufacturer's protocol. Cell apoptosis was measured via FACS Calibur flow cytometry (BD Biosciences). Results were analyzed using BD FACSDiva software (version 8.0; BD Biosciences). This assay was performed in triplicate. Apoptotic rate was calculated using the sum of early apoptotic and late apoptotic cells.

Western blotting. Total protein was extracted from the cells using RIPA solution containing phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology). Protein concentration was measured using BCA reagent (Beyotime Institute of Biotechnology). Total protein (30 µg) was separated via SDS-PAGE (10% gel) and then transferred to PVDF membranes (EMD Millipore). After blocking with 5% non-fat milk at 25°C for 2 h, the membranes were subsequently incubated overnight at 4°C with the following primary antibodies: Anti-Bcl-2 (1:1,000; cat. no. 4223); anti-Bax (1:1,000; cat. no. 5023); anti-MRP1 (1:1,000; cat. no. 14685); anti-P-glycoprotein (P-gp; 1:1,000; cat. no. 12683) and anti-GAPDH (1:5,000; cat. no. 5174) (all purchased from Cell Signaling Technology, Inc.). Next, membranes were washed with TBS containing 1% Tween 20 (TBST), followed by incubation with horseradish peroxidase-linked secondary antibodies (1:5,000; cat. no. 4050-05; Southern Biotech) at 25°C for 2 h. The membranes were then washed with TBST. Protein bands were visualized via chemiluminescence using an ECL kit (Thermo Fisher Scientific, Inc.). Parallel blotting of GAPDH served as the internal reference.

Luciferase reporter assay. The putative binding sites between LINC00707 and miR-145 were predicted using Starbase v2.0 (http://starbase.sysu.edu.cn/agoClipRNA.php?source=IncRNA). Wild-type (WT) and mutant (MUT) LINC00707 containing putative miR-145 binding sequences were generated and cloned downstream of the luciferase reporter vector, psi-CHECK-2 (Promega Corporation). The mutants were subsequently co-transfected with luciferase plasmids (0.5 µg/per well), and miR-145 mimics (final concentration, 50 nM) or miRNA negative controls (final concentration, 50 nM) using the Lipofectamine 2000 reagent. Luciferase activities were detected 48 h post-transfection using a Dual-Luciferase Reporter Assay System (Promega Corporation). Renilla
luciferase activity was normalized to Firefly luciferase activity. This assay was performed in triplicate.

Statistical analysis. Statistical analyses were performed using SPSS software (version 19.0; IBM Corporation). All data are expressed as the mean ± standard deviation from three independent experiments. Differences between two groups were identified using Student’s t-test, and differences between more than two groups were identified using ANOVA followed by a least significant difference post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

LINC00707 expression is upregulated in A549/DDP cells. To confirm the DDP-resistant phenotype of purchased A549/DDP cells, the half-maximal inhibitory concentration (IC\textsubscript{50}) value of DDP, and the protein expression levels of multidrug-resistance-related proteins MRP1 and P-gp were examined in A549/DDP and parental A549 cells. The results showed that the IC\textsubscript{50} value of DDP in A549/DDP cells was 4.5-fold, 4.6-fold or 5.6-fold higher at 1, 2 or 3 days, respectively, when compared with A549 cells (Fig. 1A). In addition, the protein expression levels of MRP1 and P-gp were markedly increased in A549/DDP cells when compared with those in A549 cells (Fig. 1B). These results verified the DDP-resistant phenotype of A549/DDP cells. The RT-qPCR results showed that LINC00707 expression was significantly upregulated in A549/DDP cells when compared with A549 cells (Fig. 1C). Therefore, this finding suggested that LINC00707 may contribute to DDP resistance in A549/DDP cells.

LINC00707 knockdown reduces the IC\textsubscript{50} value of DDP in A549/DDP cells. LINC00707 expression levels were observed to be significantly decreased in A549/DDP cells transfected with si-LINC00707 when compared with cells transfected with si-NC in the control group (Fig. 2A). An MTI assay revealed that si-LINC00707 significantly reduced the IC\textsubscript{50} value of DDP in A549/DDP cells compared with the si-NC group (Fig. 2B). Thus, these results indicated that LINC00707 knockdown induces DDP sensitivity in A549/DDP cells.

LINC00707 knockdown enhances apoptosis and influences the expression of multidrug-resistance-related proteins in A549/DDP cells. Flow cytometric analysis demonstrated that LINC00707 knockdown significantly accelerated

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5' → 3')</th>
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<tr>
<td>LINC00707</td>
<td>F: GCTGCACATTTGAGACAGATA</td>
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<tr>
<td></td>
<td>R: ATGTTCGACGTCCATCTCAT</td>
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<tr>
<td>Bcl-2</td>
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<td></td>
<td>R: CCATCGATGTATCTCTCC-3</td>
</tr>
<tr>
<td>MRP1</td>
<td>F: GCCTCAAGGAGTATTCAGAG</td>
</tr>
<tr>
<td></td>
<td>R: CCATCGATGTATCTCTCC-3</td>
</tr>
<tr>
<td>P-gp</td>
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<tr>
<td></td>
<td>R: GCGGCGAATACGAATGCCCC</td>
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<td></td>
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<tr>
<td>U6</td>
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<tr>
<td></td>
<td>R: GCGGCGAATACGAATGCCCC</td>
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F, forward primer; R, reverse primer; miR, microRNA; MRP1, multidrug resistance protein 1; P-gp, P-glycoprotein; LINC00707, long intergenic non-coding RNA 00707.
Figure 2. LINC00707 knockdown decreases the IC_{50} values of DDP in A549/DDP cells. (A) LINC00707 expression levels were measured in A549/DDP cells transfected with either si-LINC00707 or si-NC using reverse transcription-quantitative PCR. (B) IC_{50} values of DDP in either si-LINC00707- or si-NC-transfected A549/DDP cells were measured using an MTT assay. **P<0.01; ***P<0.001 vs. si-NC. IC_{50}, half-maximal inhibitory concentration; LINC00707, long intergenic non-coding RNA 00707; A549/DDP cells, DDP-resistant A549 cells; si-LINC00707, LINC00707 siRNA; si-NC, siRNA negative control.

Figure 3. LINC00707 knockdown enhances apoptosis and DDP sensitivity in A549/DDP cells. (A and B) Apoptotic cell rates of A549/DDP cells transfected with either si-LINC00707 or si-NC were measured using flow cytometry. (C) Relative mRNA expression levels of Bcl-2 in A549/DDP cells transfected with either si-LINC00707 or si-NC were measured using RT-qPCR. (D) Relative mRNA expression levels of Bax in A549/DDP cells transfected with either si-LINC00707 or si-NC were measured using RT-qPCR. (E) Relative protein expression levels of Bcl-2 and Bax in A549/DDP cells transfected with either si-LINC00707 or si-NC were measured using western blotting. (F) Relative mRNA expression levels of MRP1 in A549/DDP cells transfected with either si-LINC00707 or si-NC were measured using RT-qPCR. (G) Relative mRNA expression levels of P-gp in A549/DDP cells transfected with either si-LINC00707 or si-NC were measured using RT-qPCR. (H) Relative protein expression levels of MRP1 and P-gp in A549/DDP cells transfected with either si-LINC00707 or si-NC were measured using western blotting. ***P<0.001 vs. si-NC. MRP1, multidrug resistance protein 1; P-gp, P-glycoprotein; LINC00707, long intergenic non-coding RNA 00707; RT-qPCR, reverse transcription-quantitative PCR; A549/DDP cells, DDP-resistant A549 cells; PI, propidium iodide; si-LINC00707, LINC00707 siRNA; si-NC, siRNA negative control.
A549/DDP cell apoptosis when compared with the si-NC group (Fig. 3A and B). Furthermore, the expression levels of the apoptosis-associated proteins Bcl-2 and Bax were analyzed in A549/DDP cells transfected with si-LINC00707 or si-NC using RT-qPCR and western blotting. Results showed that LINC00707 knockdown significantly inhibited Bcl-2 mRNA and protein expression, and promoted Bax mRNA and protein expression in A549/DDP cells when compared with the si-NC group (Fig. 3C-E). Moreover, multidrug-resistance-related proteins, MRP1 and P-gp, were selected as detection indexes to analyze the effect of LINC00707 knockdown on DDP resistance in A549/DDP cells. The results showed that LINC00707 knockdown significantly decreased MRP1 and P-gp mRNA and protein levels in A549/DDP cells when compared with the si-NC group (Fig. 3F-H). Thus, these results indicated that LINC00707 knockdown enhances apoptosis and DDP sensitivity in A549/DDP cells.

**LINC00707 functions as a molecular miR-145 sponge in A549/DDP cells.** Bioinformatics analysis was used to determine the putative binding sites between LINC00707 and miR-145, and the results revealed a miR-145 binding site in LINC00707 (Fig. 4A). RT-qPCR analysis revealed that LINC00707 knockdown markedly promoted miR-145 expression in A549/DDP cells (Fig. 4E). Overall, these results suggest that LINC00707 may function as a molecular sponge by competitively binding to miR-145.

**miR-145 downregulation weakens the effect of LINC00707 knockdown in A549/DDP cells.** To gain insight into the mechanism via which LINC00707 knockdown enhanced DDP sensitivity in A549/DDP cells, an miR-145 inhibitor or inhibitor NC was further transfected into A549-DDP cells transfected with si-LINC00707. RT-qPCR analysis revealed that miR-145 inhibitor significantly downregulated miR-145 expression in A549/DDP cells compared with inhibitor NC (Fig. 5A). Additionally, it was demonstrated that the miR-145 inhibitor significantly downregulated miR-145 expression in A549/DDP cells transfected with si-LINC00707 when compared with the control group (Fig. 5B). It was further explored whether miR-145 downregulation reversed the effect of LINC00707 knockdown in A549/DDP cells. An MTT assay showed that miR-145 downregulation significantly increased the IC_{50} value of DDP in A549/DDP cells.
ZHANG et al: SILENCING LINC00707 ENHANCES CISPLATIN SENSITIVITY

Transfected with si-LINC00707 (Fig. 5C). Flow cytometric analysis also demonstrated that miR-145 downregulation inhibited apoptosis in A549/DDP cells transfected with si-LINC00707 and miR-145 inhibitor (Fig. 5D and E). Furthermore, western blotting results indicated that miR-145 downregulation promoted Bcl-2, MRP1 and P-gp expression, while reducing Bax expression in A549/DDP cells transfected with si-LINC00707 and miR-145 inhibitor (Fig. 5F and G). Thus, these results indicated that miR-145 downregulation may reverse the effect of LINC00707 knockdown in A549/DDP cells.

Discussion

DDP is considered to be a classical chemotherapeutic drug used for treating patients with NSCLC. However, DDP resistance among patients with NSCLC presents a significant
barrier towards successful chemotherapy (17). Therefore, there is an urgent need to elucidate the molecular and biological mechanisms underlying the development of DDP resistance. In the present study, LINC00707 expression was found to be significantly upregulated in A549/DDP cells. Correspondingly, LINC00707 knockdown enhanced A549/DDP cell sensitivity towards DDP. Moreover, it was demonstrated that miR-145 was a target of LINC00707 and that miR-145 downregulation was capable of reversing the effect of LINC00707 knockdown in A549/DDP cells.

A number of studies have shown that IncRNA dysregulation fuels drug resistance in human cancers, including NSCLC (18,19). For example, IncRNA maternally expressed 3 was shown to be downregulated in DDP-resistant NSCLC cells, and its overexpression enhanced DDP sensitivity in NSCLC cells in vitro (18). Therefore, comprehensive elucidation of IncRNA regulatory mechanisms in drug resistance may provide a promising therapeutic strategy for the treatment of NSCLC. LINC00707 was previously identified to be an oncogene in various cancers. It was shown to be upregulated in hepatocellular carcinoma cells, thereby promoting hepatocellular carcinoma progression (8). Its expression was also shown to be highly upregulated in gastric cancer tissues and cells, thus promoting their proliferation and metastasis by interacting with human antigen R (9). In lung cancer, Ma et al (10) found that LINC00707 expression was clearly upregulated in lung adenocarcinoma tissues and cells; notably, LINC00707 promoted lung adenocarcinoma progression by regulating cell division control protein 42. However, the role of LINC00707 in the progression of DDP resistance in NSCLC still remains unclear. Herein, it was revealed that LINC00707 expression was highly upregulated in A549/DDP cells. LINC00707 knockdown reduced the IC_{50} value of DDP in A549/DDP cells. In addition, LINC00707 knockdown enhanced the percentage of apoptotic A549/DDP cells, inhibited the expression of anti-apoptotic protein Bcl-2 and promoted the expression of pro-apoptotic protein Bax in A549/DDP cells.

These results indicated that LINC00707 knockdown enhances the DDP sensitivity of A549/DDP cells. By investigating the underlying mechanism, it was demonstrated that LINC00707 knockdown inhibited the expression of MRPI and P-gp. The official full name of MRPI is ATP binding cassette subfamily C member 1. The official full name of P-gp is ATP binding cassette subfamily B member 1. Both MRPI and P-gp are members of the superfamily of ATP-binding cassette transporters, which is involved in multidrug resistance. The increased expression of MRPI and P-gp usually represents the enhancement of multidrug resistance (20). Therefore, the results of the present study suggest that LINC00707 knockdown enhances DDP sensitivity by weakening multidrug resistance.

Recent studies have proposed that IncRNAs, including LINC00707, may serve as molecular miRNA sponges, thus affecting their target gene expression indirectly. For example, Jia et al (21) reported that LINC00707 sponged miR-370-3p to promote the osteogenesis of human bone marrow-derived mesenchymal stem cells by increasing WNT2B. In addition, Tu et al (22) found that LINC00707 contributed to hepatocellular carcinoma progression by sponging miR-206, which led to the upregulation of CDK14. In the present study, miR-145 was identified as a target of LINC00707. Changes in LINC00707 expression resulted in corresponding changes in miR-145 expression, thereby indicating that miR-145 expression was negatively regulated by LINC00707. miR-145, a known tumor suppressor, has been commonly reported to be downregulated in various types of human cancers, including colorectal cancer (23), breast cancer (24) and NSCLC (25), and has been shown to suppress tumor cell proliferation, apoptosis, migration and invasion (26). In the present study, it was revealed that miR-145 downregulation markedly reversed LINC00707 knockdown-induced cell dysfunction in A549-DDP cells. This was determined by the increased IC_{50} value of DDP, reduced apoptosis, increased Bcl-2, MRPI and P-gp protein expression levels, and attenuated Bax protein expression levels in A549/DDP cells. Interestingly, Zhan et al (15) reported that miR-145 promoted MRPI mRNA degradation by directly targeting its 3'-UTR, thereby sensitizing gallbladder cancer cells to DDP. Similarly, in the present study, it was demonstrated that miR-145 downregulation led to upregulated MRPI protein expression, thus suggesting that miR-145 downregulation may enhance DDP activity in A549/DDP cells by regulating the expression of MRPI. These results indicated that silencing of LINC00707 enhances DDP sensitivity in NSCLC cells by sponging miR-145.

There are certain limitations in this study. Firstly, the effect of LINC00707 knockdown-induced DDP resistance was analyzed only in A549/DDP cells; additional NSCLC cells were not included. Furthermore, although miR-145 was identified as a target of LINC00707, its target genes were not identified. Finally, the effect of LINC00707 knockdown on A549/DDP cells was not verified in vivo. Despite these limitations, the present study indicated that LINC00707 contributed to the progression of DDP resistance in NSCLC cells.

In conclusion, LINC00707 was identified to be highly expressed in A549/DDP cells, and its knockdown was in turn found to significantly enhance DDP sensitivity in A549/DDP cells by sponging miR-145. Therefore, these findings suggest that LINC00707 may be a potential target in the treatment of DDP-resistant NSCLC patients.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author.

Authors' contributions

HZ was involved in study design, conducting all experiments and preparing the manuscript. YL was involved in data collection and literature analysis. WX was responsible for performing the cell culture. KL and CL were responsible for performing the western blot analysis. All of the authors read and approved the final manuscript.
Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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