IncRNA LINC00473 promotes proliferation, migration, invasion and inhibition of apoptosis of non-small cell lung cancer cells by acting as a sponge of miR-497-5p

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Abstract. Lung cancer is the leading cause of cancer-associated death worldwide and exhibits a poor prognosis. The present study aimed to determine the effect of long non-coding (lnc)RNA-LINC00473 on the development of non-small cell lung cancer (NSCLC) cells by regulating the expression of microRNA (miR)-497-5p. Reverse transcription-quantitative PCR was conducted to detect the level of LINC00473 and miR-497-5p. An MTT assay, flow cytometry and Transwell tests were performed to evaluate the proliferation, apoptosis, migration and invasion of NSCLC cells. Western blotting was performed to detect the expression of apoptosis- and migration-related proteins. RNA immunoprecipitation and a luciferase reporter assay were performed to verify the regulatory relationship between lncRNA-LINC00473 and miR-497-5p. LINC00473 expression was upregulated in lung cancer tissues and NSCLC cells (A549 and H1299) when compared with adjacent tissues or human bronchial epithelial cell lines and the 5-year survival rate was lower in patients with high LINC00473 expression compared with in patients with low LINC00473 expression. A negative correlation between LINC00473 and miR-497-5p was observed in lung cancer tissues. Proliferation, migration and invasion as well as the related protein levels were increased in A549 and H1299 transfected with pcDNA3.1-LINC00473, while the opposite results were obtained in A549 and H1299 transfected with small interfering (si)-LINC00473. Notably, it was demonstrated that LINC00473 could bind directly with miR-497-5p and inhibit its expression. miR-497-5p inhibitors reversed the effect of si-LINC00473. Furthermore, the present study demonstrated that LINC00473 promoted the malignant behaviour of NSCLC cells via regulating the ERK/p38 and MAPK signalling pathways and the expression of miR-497-5p.

Introduction

Lung cancer is a disease with the fastest increasing morbidity and mortality rates, and is a notable public health issue (1). In 2019, there were ~228,150 new cases and 142,670 deaths of lung cancer in the United States (1). It is categorised into small cell lung cancer and non-small cell lung cancer (NSCLC), with the latter composing ~85% of all lung cancer cases in the United States in 2015 (2). Despite the development of therapeutic technology, the effect of lung cancer treatment remains unsatisfactory, with a low survival rate of only 15% (3,4). Therefore, it is important to elucidate the pathogenesis of lung cancer occurrence and progression.

Long non-coding (lnc)RNAs have been found to be involved in tumour initiation and tumorigenesis (5,6). Furthermore, studies have shown that numerous lncRNAs, such as lncRNA cancer susceptibility 15, forkhead box protein C2 and JHDM1D antisense 1, promote the onset and progression of NSCLC (7‑9). LINC00473 is a novel oncogenic lncRNA and is upregulated in various types of human cancer (10). Reportedly, silencing LINC00473 can block the progression of pancreatic cancer by strengthening miR-195‑5p‑targeted downregulation of PD‑L1 (11). Niu et al (12) also reported that LINC00473 regulates the expression of MAPK1 in breast cancer by interacting with miR-198 (12). However, there have been few reports on the effects of LINC00473 in NSCLC.

microRNAs (miRNAs/miRs) are elements that can inhibit the expression of target genes by hindering mRNA translation, where imbalance can result in tumour suppressor gene dysfunction, leading to the occurrence of tumours (13). It has been reported that IncRNA can act as a sponge of miRNAs, reducing their regulatory effects on target miRNAs (14). The present study aimed to elucidate the mechanism by which LINC00473 affects the progression of NSCLC. Using bioinformatics tools, the miRNAs that bind to LINC00473 were predicted, and the miR-497-5p binding site was revealed. Previously miRNA miR-497-5p has been found to inhibit
NSCLC cell proliferation and invasion as a tumour suppressor gene, therefore it was speculated that LINC00473 may influence the progression of NSCLC by targeting miR-497-5p and affecting its activity.

Materials and methods

**Sample collection.** In total, 58 NSCLC tissues and normal adjacent tissues (~5 cm away from the cancerous tissues; 39 males and 19 females; age range, 29-81 years; median age, 56 years) were obtained from patients who underwent resection surgery at The Second Hospital of Shandong University (Jinan, China) between July 2017 and August 2018. All samples were verified after a histopathological double-blind assessment by two independent pathologists and immediately stored at -80°C. Five-year survival rates were calculated by the percentage of patients who survived >60 months after resection surgery. Each patient signed an informed consent form. The protocol of the research was approved by The Ethics Committee of The Second Hospital of Shandong University (Jinan, China).

**Cell culture.** NSCLC cell lines A549 and H1299 and normal human bronchial epithelial cell line (HBE) were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% bovine fetal serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin sulphate (both HyClone; Cytiva) at 37˚C in a humidified incubator with 5% CO₂ atmosphere.

**Cell transfection.** Once cells reached 70-80% confluence, si-LINC00473 (5 nM; 5’-GCGCCCGGAGAGCAUCGAGAU GAA-3’), pcDNA3.1-LINC00473 (Shanghai GenePharma Co., Ltd.), miR-497-5p mimics (50 nM; 5’-CAGCAGCACACUGUGGUUUU-3’) and miR-497-5p inhibitors (100 nM; 5’-ACAAAA CCACAGUGUGCUGCG-3’) (Guangzhou RiboBio Co., Ltd.), miR-497-5p mimics (50 nM; 5’-CAGCAGCACACUGUGGUUUU-3’), pcDNA3.1-LINC00473 and miR-497-5p inhibitors were transfected into A549 and H1299 cells, respectively, using Lipofectamine 2000 according to the manufacturer's instructions. As a control, pcDNA3.1-NC, mimics NC (50 nM; 5’-UUCUCGGAACGU GUCACGUTT-3’) and inhibitors NC (100 nM; 5’-CAGUAC UUUGUGUGUACAA-3’) were also transfected into A549 and H1299 cells non-targeting. After 48 h, cells were collected for subsequent analyses.

**LUCiferase reporter assay.** LINC00473-wild-type (WT) and LINC00473-mutant (MUT) inserts were ligated into pGL3 reporter vectors (Promega Corporation) and transfected into A549 and H1299 cells (5x10⁴), respectively, in 24-well plates and incubated for 24 h at 37°C. In parallel, miR-497-5p mimics or mimics NC LINC00473-WT or LINC00473-MUT were respectively transfected into A549 and H1299 cells using Lipofectamine 2000 according to the manufacturer's instructions. Luciferase activity was detected using the Dual-Luciferase Reporter Assay system (Promega Corporation). *Renilla* luciferase activity was used for normalization. Moreover, the binding site of LINC00473 and miR-497-5p was predicted using DIANA tools (http://carolina.imis.athena-innovation.gr/diana_tools/web/).

**RNA immunoprecipitation.** A549 and H1299 cells were lysed in complete RIP lysis buffer (EMD Millipore) and then incubated with magnetic beads (EMD Millipore) containing human anti-SNRNP70 antibody or IgG (EMD Millipore; cat. nos. CS203216 and CS200621, respectively) overnight at 4°C. After incubation, tubes were placed on a magnetic separator (EMD Millipore) and supernatants were discarded. After washing with RIP washing buffer, the samples were subsequently incubated with proteinase K buffer in a heating block at 55°C for 30 min. Target RNA was extracted using chloroform, purified using two salt solutions (EMD Millipore), a precipitate enhancer (EMD Millipore) and absolute ethanol, and detected by reverse transcriptase-quantitative (RT-q)PCR analysis.

**RT-qPCR.** Total RNA was isolated from both tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed using PrimeScript™ RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. The relative gene expression of LINC00473 and miR-497-5p was analysed using a SYBR® Premix Ex Taq™ kit (Takara Bio, Inc.) and an ABI PRISM® 7500 Two-step Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. The 2^ΔΔCq method (15) was used for quantification of LINC00473 and miR-497-5p expression. GAPDH and U6 expression levels were used as reference controls for LINC00473 and miR-497-5p levels, respectively. Primers used for amplification are listed in Table I.

**Colony formation assay.** A total of 1,000 transfected A549 and H1299 cells were placed in 6-well plates and cultured in RPMI-1640 medium. After 14 days of continuous incubation at 37°C, cells were stained with crystal violet for 10 min at room temperature (Beyotime Institute of Biotechnology) and observed manually under a light microscope (magnification, x40) for the number of colonies to be counted (>50 cells were considered as a colony).

**MTT assay.** The transfected cells were resuspended in RPMI-1640 with 10% FBS, seeded in a 96-well plate (5x10³/well), and incubated at room temperature for 24-72 h. MTT solution was then added, followed by incubation for 4 h and addition of 150 µl DMSO to each well. Optical density at 490 nm was measured using a microplate reader, and data were expressed as absorbance values.

**Apoptosis analysis.** Apoptosis analysis was performed using an Annexin V FITC Apoptosis kit (BD Biosciences; cat. no. 556420). Cells transfected with si-LINC00473, pcDNA3.1-LINC00473 and miR-497-5p inhibitors were cultured for 48 h, then incubated with FITC-labelled Annexin V and PI in the dark at 25°C for 20 min. Thereafter, the apoptosis rate of the cells was measured by flow cytometry using a flow cytometer (BD FACSCanto II; BD Biosciences). FlowJo version 10 software (FlowJo LLC) was used to analyse apoptosis.

**Transwell migration and invasion assays.** After 48 h of incubation, transfected A549 and H1299 cells were trypsinised and...
Table I. Primer sequences for quantitative expression analysis.

| Gene          | Sequence of oligonucleotides, 5'-3' |
|---------------|-------------------------------------|
| LINC00473     |                                     |
| Forward       | GATGGAAAGGAGGGAAGG                  |
| Reverse       | CACCTGGGTGCCAAGG                   |
| microRNA-497-5p |                                   |
| Forward       | CTTCCAGCAGCACACTGTGG               |
| Reverse       | CAGTGCAGGGTCCAGGTAT                |
| GAPDH         |                                     |
| Forward       | AGAAGGCTGGGGCTACTTT                |
| Reverse       | AGGGGCATACCAAGTCTTC                |
| U6            |                                     |
| Forward       | CTCGCTTCGGCAGCACA                  |
| Reverse       | AAGCGTCACAGAATTTGCGT               |

resuspended. For invasion assays, the upper chamber surface of the Transwell insert was coated with 50 mg/l Matrigel (1:8) and air-dried at 4°C. For both migration and invasion assays, 2x10^5 cells were added to the upper chamber containing serum-free RPMI-1640 medium, and the lower chamber was filled with RPMI-1640 medium supplemented with 5% FBS. After 24 h of incubation, the cells were stained with crystal violet for 20 min at room temperature. Finally, the filters were washed with PBS and observed under a light microscope (magnification, x200). Four visual fields were randomly selected and images were captured, and the visible cells were counted.

Western blotting. After 48 h incubation of transfected cells, samples from each group were collected, and lysed with ice cold RIPA buffer (Sigma-Aldrich; Merck KGaA) supplemented with a protease inhibitor cocktail (Roche Diagnostics). The protein concentration was measured using a BCA kit (Takara Bio, Inc.). Proteins (10 ng/lane) were separated via 10% SDS-PAGE, followed by transfer to a PVDF membrane. After 2.5 h of blocking with a BSA (neoFroxx GmbH)-TBST solution (1x TBS, 1% Tween-20 and 5% w/v BSA) at room temperature, the PVDF membrane was incubated with the following primary antibodies: Bax (1:1,000; cat. no. 14796), Bcl-2 (1:1,000; cat. no. 4223), Matrix metalloproteinase (MMP)-2 (1:1,000; cat. no. 40994), MMP-9 (1:1,000; cat. no. 15561), p44/p42 (ERK1/2; 1:1,000; cat. no. 4695), phosphorylated-p44/p42 (p-ERK1/2; Thr202/Tyr204; 1:1,000; cat. no. 4370), p38 (1:1,000; cat. no. 14451) and phosphorylated-p38 (Thr180/Tyr182; 1:1,000; cat. no. 4511) (all Cell Signalling Technology, Inc.) at 4°C overnight. The following day, the samples were incubated with HRP-conjugated secondary antibodies (1:2,000; cat. no. 7074; Cell Signalling Technology, Inc.) at room temperature for 2 h. The protein blots on the membrane were imaged using a chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.). Data were quantified using ImageJ software v1.41 (National Institutes of Health).

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments and were analysed using SPSS 19.0 (IBM Corp). Paired t-tests were used to determine the statistical differences between two groups. The overall survival rate was analysed by the Kaplan-Meier method and differences were tested using the log-rank test. The data difference among ≥2 groups were determined using ANOVA and Bonferroni’s post-hoc test. The correlation between LINC00473 and miR-497-5p was analysed using Pearson’s correlation coefficient. P<0.05 was considered to indicate a statistically significant difference.

Results

LINC00473 expression is upregulated in NSCLC tissues and cells. RT-qPCR results indicated that LINC00473 was significantly upregulated in NSCLC tissues when compared with adjacent tissues (P<0.05; Fig. 1A). Furthermore, survival analysis indicated that patients with a higher expression of LINC00473 had a significantly lower 5-year survival rate compared with patients with low expression of LINC00473 (log-rank P<0.05; Fig. 1B). Additionally, significantly increased expression of LINC00473 was observed in both A549 and H1299 cells compared with HBE cells (P<0.05; Fig. 1C). The present results indicated that LINC00473 expression was upregulated in both NSCLC tissues and cells.

LINC00473 promotes proliferation and inhibits apoptosis of NSCLC cells. As indicated in Fig. 2A, LINC00473 was significantly upregulated in A549 and H1299 cells transfected with pcDNA3.1-LINC00473, compared with those transfected with pcDNA3.1-NC (both P<0.05). Furthermore, the expression of LINC00473 in A549 and H1299 cells was significantly decreased after transfection with si-LINC00473 when compared with the si-NC group (both P<0.05). The results of the MTT and colony formation assays indicated that overexpression of LINC00473 promoted proliferation, whereas its downregulation inhibited the proliferation of A549 and H1299 cells compared with their NC groups (all P<0.05; Fig. 2B and C). Moreover, the apoptosis rate (Annexin V+PI- cells) significantly decreased when LINC00473 was overexpressed and significantly increased when LINC00473 was knocked down (both P<0.05; Fig. 2D). Meanwhile, it was observed that expression levels of Bax decreased while Bcl-2 increased with LINC00473-overexpression, and the opposite was observed when LINC00473 was knocked down, with an increase in Bax and decrease in Bcl-2 expression (P<0.05; Fig. 2E). These results suggested that LINC00473 served a promoting role in proliferation and an inhibitory role in apoptosis of NSCLC cells in vitro.

LINC00473 promotes the migration and invasion of NSCLC cells. A Transwell migration assay demonstrated that the overexpression of LINC00473 significantly enhanced the migration and invasion activities of NSCLC cells (P<0.05; Fig. 3A and B). Conversely, migration was significantly inhibited by LINC00473-knockdown (P<0.05; Fig. 3A and B). In addition, LINC00473-overexpression significantly increased the expression of the metastasis-associated proteins MMP-2 and MMP-9; however, LINC00473-knockdown significantly decreased their expression (all P<0.05; Fig. 3C). The results demonstrated that LINC00473 promoted the migration and invasion of NSCLC cells in vitro.
Figure 1. LINC00473 is upregulated in lung cancer tissues, HEB, A549 and H1299 cells. (A) RT-qPCR was conducted to detect the transcription level of LINC00473. (B) Overall survival rate of patients with lung cancer with either high or low expression of LINC00473. (C) Expression of LINC00473 in human bronchial epithelial and NSCLC cell lines as quantified using RT-qPCR. RT-q, reverse transcription-quantitative.

Figure 2. LINC00473 promotes the proliferation and inhibits apoptosis of NSCLC cell lines. (A) Expression of LINC00473 in NSCLC cell lines transfected with pc-DNA-LINC00473, si-LINC00473 and negative control. (B) MTT and (C) colony formation assays were used to detect the proliferation of NSCLC cells. (D) Apoptosis rate (%) of A549 and H1299 cells. (E) Bcl-2 and Bax expression levels. *P<0.05 vs. pcDNA3.1-NC and blank group; †P<0.05 vs. si-NC and blank group. NSCLC, non-small cell lung cancer; NC, negative control; si, small interfering.
LINC00473 competitively binds to miR-497-5p. As predicted by DIANA tools (http://carolina.imis.athena-innovation.gr/diana_tools/web/), miR-497-5p was a possible target miRNA of LINC00473. To confirm this, two types of luciferase reporter gene vectors (miR-497-5p-WT and miR-497-5p-MUT) were used to investigate whether LINC00473 acts as a sponge to miR-497-5p through direct binding (Fig. 4A). As shown in Fig. 4B and C, after transfection with WT sequences, the miR-497-5p mimics showed significantly inhibited luciferase activity (both P<0.05). However, when the predicted binding site was mutated, overexpression of miR-497-5p showed no change in luciferase activity. Moreover, the expression of miR-497-5p was significantly reduced after overexpression of LINC00473 in both cell lines, but significantly increased after LINC00473-knockdown in A549 and H1299 cells (all P<0.05; Fig. 4D and E). As shown in Fig. 4F, the expression of miR-497-5p in A549 and H1299 cells was lower compared with that in HBE (both P<0.05). Likewise, the expression of miR-497-5p in 58 pairs of NSCLC and adjacent tissues was compared and it was reported that miR-497-5p was significantly downregulated in the former (P<0.05; Fig. 4G). The expression of miR-497-5p was found to be negatively correlated with LINC00473 (P<0.05; Fig. 4H). The results indicated that LINC00473 could competitively bind to miR-497-5p and negatively regulate its expression.

miR-497-5p inhibition attenuates the effect of LINC00473 silencing. As depicted in Fig. 5A, the cell proliferation rates in the si-LINC00473 group were lower compared with the NC group. However, cell viability was enhanced after treatment with miR-497-5p inhibitors (P<0.05). The clone number of A549 cells decreased after LINC00473-knockdown but increased after the addition of miR-497-5p inhibitors (P<0.05; Fig. 5B). Moreover, miR-497-5p inhibition stabilised the increase in apoptosis and the expression of apoptosis-related proteins induced by LINC00473-knockdown (all P<0.05; Fig. 5C and D). Transwell migration assays indicated that both cell migration and invasion abilities were reduced after silencing LINC00473 but were reversed after co-transfection with miR-497 inhibitors (both P<0.05; Fig. 6A). Similarly, the expression of MMP-2 and MMP-9 was inhibited by silencing LINC00473 but was reversed by miR-497 inhibitors (all P<0.05; Fig. 6B). The current results suggested that LINC00473 served its role by regulating miR-497-5p expression.

LINC00473 activates the MAPK signalling pathway. To study the relationship between LINC00473 and the MAPK signalling pathway, the expression of proteins associated with the pathway was analysed. The data indicated that p-ERK1/2 and p-p38 expression increased when LINC00473 was overexpressed and decreased when LINC00473 was knocked down (Fig. 7A). Moreover, the p-ERK1/2/ERK1/2 ratio (P<0.05; Fig. 7B) and p-p38/p38 ratio showed the similar trends. In A459 cells, the expression levels of p-ERK1/2 and p-p38 were significantly decreased by LINC00473-knockdown and increased after treatment with miR-497-5p inhibitors (and...
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both p-ERK1/2/ERK1/2 ratio and p-p38/p38 ratio showed the same trends (P<0.05; Fig. 7C and D). These results indicated that the regulatory mechanism of LINC00473 may be through the MAPK signalling pathway.

Discussion

Multiple studies have suggested that IncRNAs play crucial roles in tumour progression, making them promising therapeutic targets for treatment of diseases such as NSCLC (16-18). Some studies have reported that the IncRNA LINC00473 promotes cell migration and invasion in numerous types of cancer, including pancreatic, mucoepidermoid and breast cancer (11,19,20). He (21) reported that LINC00473 regulates the progression of oesophageal squamous cell carcinoma by affecting 5'-AMP-activated protein kinase catalytic subunit α-1 expression. Furthermore, Mo et al (22) revealed that LINC00473 promotes the progression of hepatocellular carcinoma by sponging miRNA-195 and increasing high mobility group protein HMGI-C expression. However, whether miR-146-5p is involved in the regulation of lung cancer remains unclear. The present study demonstrated that LINC00473 was significantly upregulated in both patient lung cancer tissues and NSCLC cell lines. Additionally, the inhibitory effects of silencing LINC00473 and the promotional effect of its overexpression on the proliferation, invasion and migration of NSCLC cells was investigated.

Several studies have suggested that IncRNAs act as natural sponges to interact with miRNAs by eliminating the inhibitory activity of these miRNAs (23-25). Chen et al (24) showed that LINC00473 weakens the effect of radiotherapy by targeting miR-374a-5p. Wang et al (25) also revealed that in colorectal cancer LINC00473 can promote Taxol resistance by inhibiting miR-15a. In the present study, to explore the underlying mechanism of LINC00473 in NSCLC development, target miRNAs of LINC00473 were investigated. Using bioinformatics methods, it was demonstrated that miR-497-5p contained a binding site for LINC00473. Luciferase reporter assays confirmed that LINC00473 and miR-497-5p bind with each other. Furthermore, the expression of miR-497-5p was decreased by LINC00473 overexpression and enhanced by LINC00473 silencing. Moreover, the inhibition of miR-497-5p attenuated the effect of LINC00473 silencing on cell proliferation, apoptosis, migration and invasion. These data

Figure 4. LINC00473 targets miR-497-5p. (A) Binding site of LINC00473 and miR-497-5p predicted using DIANA. Relative activity of luciferase activity in the (B) A549 and (C) H1299 cell lines. Expression of miR-497-5p in (D) A549 and (E) H1299 cells transfected with pcDNA-LINC00473 and si-LINC00473. (F) Expression of miR-497-5p in HBE and the NSCLC cell lines A549 and H1299. (G) miR-497-5p expression in 58 pairs of lung cancer-affected and adjacent tissues. (H) Correlation analysis of LINC00473 and miR-497-5p in lung cancer. *P<0.05 vs. pcDNA3.1-NC, blank group or HBE cells; #P<0.05 vs. si-NC and blank group. si, small interfering; miR, microRNA; HBE, human bronchial epithelial; Wt, wild-type.
suggested that LINC00473 promotes NSCLC progression in an miR-497-5p-dependent manner.

It has been reported that the MEK/ERK signalling pathway is involved in cell proliferation, apoptosis and migration (26). Several lncRNAs, such as lncRNA-01126 and lncRNA HOTTIP, have been shown to exert their functions by regulating this pathway (27,28). This pathway is involved in the migration and invasion of NSCLC cells induced by the
LncRNA SDPR-AS (29). In addition, the lncRNA MALAT1 has been shown to play a tumour-enhancing role by regulating the development of NSCLC via the MAPK/ERK pathway (30). The present results indicated that LINC00473-knockdown decreased the p-ERK and p-p38 levels, which were reversed by miR-497-5p inhibition. These data indicated that LINC00473 regulates the activity of the MAPK/ERK signalling pathway to influence the proliferation, migration and invasion of NSCLC cells.

Overall, the present results indicated that LINC00473 is highly expressed in NSCLC tissues, and its downregulation suppresses the malignant activities of NSCLC cells. Structurally, LINC00473 competitively binds with miR-497-5p and regulates the MEK/ERK signalling pathway.

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Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
MJS conceived, interpreted the data and designed the study. MJS and SHX confirmed the authenticity of all the raw data. SHX, YHB, HCM and HNZ performed the experiments and analysed the data. SHX wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by The Ethics Committee of The Second Hospital of Shandong University (Jinan, China) and all patients provided written informed consent.

Patient consent for publication
Not applicable.
Competing interests
The authors declare that they have no competing interests.

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