miR-4732-5p promotes ovarian cancer mobility by targeting MCUR1

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Abstract. MicroRNAs (miRNAs/miRs) play critical roles in tumor progression. However, the role of miR-4732 and its underlying molecular mechanism in ovarian cancer (OC) remain unclear. In the present study, the high expression of miR-4732 was confirmed to be associated with the mortality of patients with OC following surgery, according to The Cancer Genome Atlas Ovarian Cancer database (TCGA-OV). Additionally, the expression of miR-4732 was positively associated with an increased tendency to exhibit an early TNM stage (IIA, IIB and IIC) of OC, indicating its promotional role in the early stages of tumorigenesis. By performing *in vitro* gain-of-function experiments, the transient transfection of IGROV1 cells with miR-4732-5p mimics enhanced cell viability according to Cell Counting Kit-8 assay, and cell migration and invasion in Transwell assays. However, though the application of loss-of-function experiments, the transient transfection of IGROV1 cells with miR-4732-5p inhibitors hindered cell viability, cell migration and invasion *in vitro*. Mitochondrial calcium uniporter regulator 1 (MCUR1) was validated as a downstream direct target of miR-4732-5p through bioinformatics analysis, western blotting and luciferase assays. Therefore, the results of the present study provide evidence that miR-4732-5p may promote OC cell mobility through the direct targeting of the tumor suppressor, MCUR1.

Introduction

Ovarian cancer (OC) is the fifth leading cause of cancer-associated mortality among women worldwide (1). Among the gynecological malignancies, OC is the leading cause of mortality due to its late diagnosis and recurrence (2). In general, ~75% of patients with OC are initially diagnosed with intra-abdominal disease and a 5-year survival is encountered in <40% of patients with stage III disease (3,4). Ovarian tumors are prone to becoming diffuse, resulting in peritoneal and intraperitoneal metastasis; these tumors also relatively resistant to conventional chemotherapeutics (4). All of the aforementioned factors contribute to a strong frequency of therapeutic recurrence and resistance.

MicroRNAs (miRNAs/miRs) are defined as short non-coding RNA molecules of 19 to 22 nucleotides in length. They function as tumor suppressors or oncogenes (oncomiRs) in the initiation and progression of human cancer. miRNA mimics or molecules targeted at miRNAs (antimiRs) have been reported to be promising as therapeutic drugs in pre-clinical development (5), including a mimic of the tumor suppressor miR-34 (6), which reached phase I clinical trials for hepatocellular carcinoma, and anti-miRs targeting miR-122, which reached phase II trials for hepatitis (7). Several miRNAs have been reported to be involved in OC tumorigenesis, invasion or metastasis, the function of extracellular matrix and angiogenesis (8). For example, circulating levels of different panels of miRNAs may be used as promising classifiers for OC (8-10), greatly enhancing the feasibility of circulating miRNAs as non-invasive prognostic markers (11). Therefore, examining the expression profile and underlying mechanisms of miRNAs in OC may be crucial.

Recently, miR-4732-5p expression in plasma-derived exosomes was identified as a potential diagnosis biomarker for distinguishing patients with epithelial OC (EOC) from healthy subjects with 85.7% sensitivity and 82.4% specificity, and as a monitor for EOC progression from the early to the late stage (12). However, the role of miR-4732-5p and its underlying molecular mechanisms in OC remain unclear. In the present study, the expression of the miR-4732 was analyzed using The Cancer Genome Atlas (TCGA) database and the functional effects of miR-4732-5p on OC cell proliferation, migration and invasion were defined by gain-of-function and loss-of-function experiments *in vitro*. Finally, mitochondrial calcium uniporter regulator 1 (MCUR1) was validated as one of the direct targets of miR-4732-5p, potentially involved in the promotion role of miR-4732-5p in OC, at least partially.

Materials and methods

TCGA database. OC RNA expression, miRNA expression and clinical data were downloaded from TCGA database (https://xenabrowser.net/datapages/). Firstly, the link for

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'GDC TCGA Ovarian Cancer (TCGA-OV) (15 datasets)' was selected using the following web link: https://xenabrowser.net/datapages/. Subsequently, the links 'gene expression RNAseq/HTSeq-FPKM (n=379) GDC Hub' for RNA expression (https://xenabrowser.net/datapages/?dataset=TCGA-OV.htseq_fpkm.tsv&host=https%3A%2F%2Fgdc.xenahubs.net&removeHub=https%3A%2F%2Fxena.treehouse.gi.ucsc.edu%3A443), 'stem loop expression/miRNA Expression Quantification (n=498) GDC Hub' for miRNA expression (https://xenabrowser.net/datapages/?dataset=TCGA.OV.sampleMap%2FmiRNA_HiSeq_gene&host=https%3A%2F%2Ftcga.xenahubs.net&removeHub=https%3A%2F%2Fxena.treehouse.gi.ucsc.edu%3A443), 'phenotype/Phenotype (n=758) GDC Hub (https://xenabrowser.net/datapages/?dataset=TCGA-OV.GDC_phenotype.tsv&host=https%3A%2F%2Fgdc.xenahubs.net&removeHub=https%3A%2F%2Fxena.treehouse.gi.ucsc.edu%3A443) and survival data (n=731) GDC Hub' (https://xenabrowser.net/datapages/?dataset=TCGA-OV.survival.tsv&host=https%3A%2F%2Fgdc.xenahubs.net&removeHub=https%3A%2F%2Fxena.treehouse.gi.ucsc.edu%3A443) were selected, in order to acquire expression level information for RNA, miRNAs and clinical information. The clinical phenotype and miR-4732 expression were matched according to the sample ID. In addition, the normalized expression data (level 3) of miR-4732 were analyzed according to TNM stage, lymph node metastasis and survival status following surgery. Finally, receiver operating characteristic (ROC) curve was performed to predict the mortality of patients. The expression value of miR-4732 at the maximum Youden's Index (Sensitivity + Specificity-1) was used as the cut-off value to divide the high and low miR-4732 expression groups. Kaplan-Meier (K-M) survival curve analysis was used to analyze the overall survival (OS) percentage of patients with a high or low miR-4732 expression.

Cells and cell culture. The IGROV1 (cat. no. CTCC-009-0048) human OC cells were obtained from the Cell Center of Meisen CTCC (https://www.ctcc.online/). The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C with 5% CO_2. 293FT cells were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (cat. no. R70007) and cultured according to manufacturer's manuals.

Cell transfection with miR-4732-5p mimics or inhibitors. miRNA mimics or inhibitors for miRabse accession no. MIMAT0019855 were synthesized by Guangzhou RiboBio Co., Ltd., and transiently transfected into $1x10^5$ IGROV1 cells at a 50 nmol/l concentration using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol cultured in an incubator at 37˚C with 5% CO_2 for 24 h to subsequent experimentation. The synthesis of mimic sequences (micrON™ miRNA mimics) was designed as double-strand 5'-UGUAGAGCAGGG AGCAGGAAGCU-3'; the inhibitor sequence (micrOFF™ miRNA inhibitor) was chemically modified as single-strand 5'-AGCUUCCUGCUCCCUGCUCUACA-3'; the control sequences were: Mimics control [micrON™ mimic negative control (NC) #24; cat. no. miR1N0000002-1; https://www.ribobio.com/product_detail/?sku=miR1N0000002-1-1] and inhibitor control (micrOFF inhibitor NC #24; cat. no. miR2N0000002-1; https://www.ribobio.com/product_detail/?sku=miR2N0000002-1-1); all sequences were designed and purchased from RiboBio Co. Ltd.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA extraction and cDNA synthesis for miRNAs by adding polyA tail followed by onligodT-adaptor primer revers transcription were performed according to a previous study (13). Briefly, total RNA was isolated from the cells using miRNeasy Mini kits (Qiagen, Inc.) according to the manufacturer's protocols. Poly(A) Polymerase (New England BioLabs, Inc.) was used to add Poly(A) tails to 100 ng of total RNA 3'-ends according to the manufacturer's protocols. Subsequently, RNA was reverse transcribed using an oligodT-adaptor primer (5'-GCGAGCACAGAATTAATACGACTCACTATA GGTTTTTTTTTTTTVN-3') and Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). The following primers were used to amplify the miRNA or U6: miR-4732-5p forward, 5'-TGTAGAGCAGGG AGCAGGAAG-3' and reverse, 5'-GCGAGCACAGAATTA ATACGAC-3'; and U6 forward, 5'-CTCGCTTCGGCAGCA CA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The primer pairs were designed using Primer-blast. (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The expression levels of miRNAs were evaluated using SYBR™ Select Master Mix (Thermo Fisher Scientific, Inc.) and an ABI7500 fast System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling conditions were as follows: 10 min at 95˚C followed by 40 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 42 sec. U6 was used as the reference gene. The expression level of miR-4732-5p (fold change relative to the control group) was calculated using the $2^{-\Delta\Delta Cq}$ method, whereby ΔCq=Cq (miR-4732-5p)-Cq (U6) (14).

Cell viability assay. A total of $1x10^3$ cells/well were seeded into 96-well plates in triplicate and cultured for 1, 2, 3 and 4 days. Cell viability was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.), according to the manufacturer instructions. Briefly, a total of 10 μl CCK-8 solution was added to each well at the 0, 24, 48, 72 and 96 h time points. Following incubation at 37˚C with 5% CO_2 for 1 h, the absorbance at a wavelength of 450 nm was then measured using a microplate reader (iMark; Bio-Rad Laboratories, Inc.). The experiments were performed in three independent experiments in triplicate.

Transwell migration and invasion assays. The cells ($1x10^4$) were seeded into the upper chambers of a Transwell plate (cat. no. 3422, 8.0 μM; Corning, Inc.) pre-coated in triplicate without or with Matrigel (1:30 dilution with RPMI-1640 medium at 37˚C for 30 min), in 100 μl RPMI-1640 medium containing 1% FBS. A total of 500 μl RPMI-1640 medium containing 10% FBS as a chemoattractant was placed in each lower chamber. Following a 24-h incubation at 37˚C in a cell incubator (Heracell VIOS 160i; Thermo Fisher Scientific, Inc.), the migrated or invaded cells were fixed with 4% formaldehyde for 5 min at room temperature and stained with 1% crystal violet (cat. no. G1062; Beijing Solarbio Science

& Technology Co., Ltd.) for 5 min at room temperature. The number of migrated or invasive cells was counted using a light microscope (Leica Dim8; Leica Microsystems GmbH; magnification, x20) in four randomly selected microscopic fields. The experiments were performed in three independent experiments in triplicate.

Western blotting. The 10^6 cells were harvested and protein extracts were obtained with RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). The concentration of protein was determined by BCA BCA assay kit (Beijing Solarbio Science & Technology Co., Ltd.). Equal amounts (30 μg) of protein were electrophoresed on 10% SDS-PAGE gels, and then transferred to PVDF membrane (MilliporeSigma). After blocking with 5% non-fat milk solution at room temperature for 1 h, the membranes were incubated with anti-MCUR1 (1:5,000; cat. no. 13706; Cell Signaling Technology, Inc.) and anti-actin (1:10,000; cat. no. 3700; Cell Signaling Technology, Inc.) antibodies at 4˚C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:50,000; cat. no. 111-035-003; Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 1 h. ECL solution (Millipore) was used to visualize specific bands and the immunoblots were scanned on an Amersham Imager 680 (AI680; GE Healthcare).

Prediction of the candidate targets of miR-4732-5p using bioinformatics analysis. Firstly, TargetScan version 7.1 (http://www.targetscan.org/vert_71/) was used to predict the direct candidate targets. After selecting 'Human' in the species option inputting miR-4732-5p as the microRNA name, candidate genes for hsa-miR-4732-5p were predicted. Secondly, Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/) was used to identify the OS-related genes (15). The most differential survival genes were acquired by selecting 'Survival/Most Differential Survival Genes' function, 'OC' for dataset selection, 'overall survival' for method and 'Median' value of the gene expression in the cancer tissues for group cut-off. Finally, the predicted targets using TargetScan and survival-related genes ($P<0.05$) were intersected using a Venn plot and the potential candidate targets of miR-4732-5p were obtained. In addition, a literature search using PubMed (https://pubmed.ncbi.nlm.nih.gov/) for these candidate targets was performed to define the oncogene or suppressor role of these genes in the cancer.

Dual luciferase report assay. The wild-type (WT) MCUR1 3'-UTR fragments or the mutant (mutation at the predicted miR-4732-5p binding sites), were inserted downstream of the Firefly luciferase gene in the pGL3-control (cat. no. E1741, Promega Corporation) plasmid. A total of 0.5 μg of the Firefly reporter plasmid pGL3-MCUR1 WT/Mutation (containing position 612-618 of MCUR1 3'UTR at sequences of "CUCUACA" and corresponding mutation with sequences of "AGAGCAG", 26 ng of pRL-TK plasmids (cat. no. E2241, Promega Corporation) containing *Renilla* luciferase, along with 10 pmol/ml miR-4732-5p mimics or negative control, were transfected into 293FT cells seeded into 24-well plate in triplicate. Following a 48-h transfection using Lipofectamine 2000® (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.), cell lysates were collected, and luciferase activities were measured using the Dual Luciferase Reporter System (Promega Corporation) according to manufacturer's protocol with a Tecan Infinite M200 Pro instrument (Tecan Group, Ltd.). The luminescence intensity of Firefly luciferase was normalized to that of *Renilla* luciferase.

Statistical analysis. Data were analyzed using SPSS 13.0 software (SPSS, Inc.). The continuous variables with normal distribution (Shapiro-Wilk test) are expressed as the mean ± standard deviation from three independent experiments. Statistical significance was determined using an unpaired two-tailed Student's t-test for two groups, and using one-way ANOVA with a Bonferroni post hoc test for multiple comparisons. OS was analyzed using the K-M method (log-rank test). The cut-off value for miR-4732-5p expression was based on the ROC corresponding to the maximum the Youden's index (Sensitivity + Specificity-1). Pearson's correlation analysis was used to investigate the expression correlation between miR-4732 and MCUR1 expression. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miR-4732 in OC tissues. The miR-4732 expression levels in TCGA-OV database were downloaded and analyzed. Although there was no significant difference among the TNM subgroups according to the results of one-way ANOVA, there was a trend towards an increased miR-4732 expression in the early TNM stage (IIA, IIB and IIC; Fig. 1A). In addition, in the late TNM stage (IIIA, IIIB, IIIC and IV), the expression levels of miR-4732 appeared to reach a specific threshold, being similar to those in the IIC stage (Fig. 1A), indicating that the miR-4732 may play a role in the early stages of tumorigenesis. No statistically significant differences were observed when comparing the expression levels of miR-4732 in tissue groups with and without lymph node metastasis (Fig. 1B). However, the expression levels of miR-4732 in the patients with the 'death' OS status option (OS=1 in the survival analysis acquired from TCGA-OV) following surgery were significantly higher than those with the 'alive' OS status option (OS=0 in the survival analysis acquired from TCGA-OV; Fig. 1C; unpaired student's t-test, one-tailed; $P=0.035$). Herein, a one-tailed test was used, since the present study was only concerned about one direction, not both, indicating that the increased level of miR-4732 expression predicted a poor prognosis. According to the ROC curve analysis (Fig. 1D), the cut-off value for miR-4732 expression was determined by the point corresponding to the maximum the Youden's index (Sensitivity + Specificity-1). According to this cut-off value, the patients were classified into the high or low miR-4732 expression groups. The K-M survival curve suggested that the OS of patients with a high miR-4732 expression exhibited no significant difference as compared with that of those with a low miR-4732 expression (Fig, 1E; log-rank test; $P=0.6138$). These results indicate that miR-4732 expression may be used for defining a poor prognosis or mortality, although not for defining the OS time using the K-M survival curve for deceased patients after surgery.

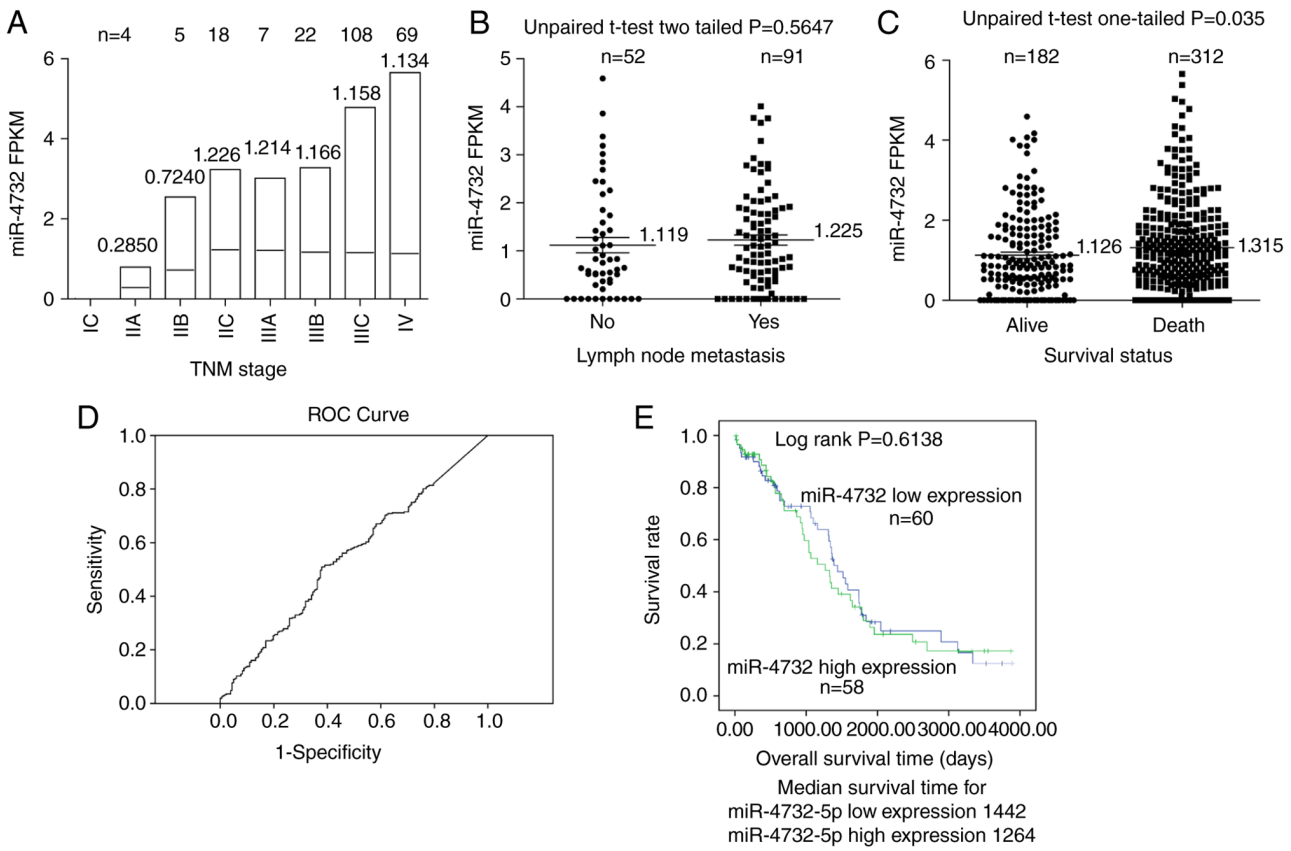

Figure 1. Expression levels of miR-4732 in patients with OC. miRNA expression data were downloaded from TCGA database. Expression levels of miR-4732 in patients with OC grouped according to (A) TNM stage, (B) lymph node metastasis, and (C) survival status. (D) ROC curve analysis for miR-4732 expression in the tumor tissues. (E) Overall survival analysis of miR-4732 expression in patients with OC using the Kaplan-Meier curve method. OC, ovarian cancer; TCGA, The Cancer Genome Atlas; ROC, Receiver operating characteristic.

Ectopic overexpression of miR-4732 promotes the viability, migration and invasion of IGROV1 cells in vitro. miR-4732 includes miR-4732-5p and miR-4732-3p. Recently, miR-4732-5p expression in plasma-derived exosomes from patients with EOC was confirmed to be increased, as compared with that of healthy subjects, and that it could be used as a diagnostic marker or as a monitor for EOC progression from the early to the late stage (12). Herein, since it was hypothesized that miR-4732-5p may function as an oncogene in OC, the phenotypic alterations were evaluated following the induced overexpression of miR-4732 using miRNA mimics in IGROV1 cells. RT-qPCR analysis demonstrated miR-4732-5p expression was increased 166-fold, as compared with the cells transfected with miRNA NC (Fig. 2A; P<0.0001). Concurrently, IGROV1 cell viability significantly increased following transfection with miRNA mimics in comparison with the cells transfected with mimics NC, as evaluated using CCK-8 assay (Fig. 2B; P<0.0001). Furthermore, the migratory or invasive ability of the IGROV1 cells transfected with miRNA mimics was markedly enhanced, as compared with the cells transfected with NC (Fig. 3C; P<0.001), as shown by Transwell assay. These results thus suggested that miR-4732-5p overexpression promoted IGROV1 cell proliferation, migration and invasion *in vitro*.

Inhibition of miR-4732-5p suppresses cell viability, migration and invasion in vitro. To further confirm the aforementioned results on the viability and the migratory and invasive ability of OC cells, the IGROV1 cells were then transfected with miR-4732-5p inhibitors. RT-qPCR analysis demonstrated that miR-125a-5p expression was inhibited by 68% in the cells transfected with miR-4732-5p inhibitors (Fig. 3A; P<0.001). Consistent with the aforementioned results, the viability (Fig. 3B; P<0.0001), as well as the migratory or invasive ability (Fig. 3C; P<0.001) of the IGROV1 cells transfected with miR-4732-5p inhibitors was significantly decreased compared to cells transfected with inhibitor NC. Therefore, miR-4732-5p silencing exerted an inhibitory effect on IGROV1 cell proliferation, migration and invasion *in vitro*.

Tumor suppressor MCUR1 may be a direct target of miR-4732-5p. TargetScan version 7.1 predicted a total of 99 direct targets, and GEPIA identified 2,607 OS-related genes. In total, 11 candidate targets were obtained through the intersection genes of above 2 tables according to the presented Venn plot (Fig. 4A). According to the hypothesis of miR-4732-5p being an oncogene in OC, it was suggested that targets should act as tumor suppressor in OC. Thus, a literature search using PubMed (https://pubmed.ncbi.nlm.nih.gov/) was performed. Two out of the 11 genes [ribosomal protein S6 kinase A2 (RPS6KA2) (16) and MCUR1 (17)] had been reported as tumor suppressors, five genes [CACNA1C (18), FCGBP (19), MAVS (20), CKAP2 (21) and BTLA (22)] as oncogenes, ESCO1 was reported as a drug-related gene (23) and three genes

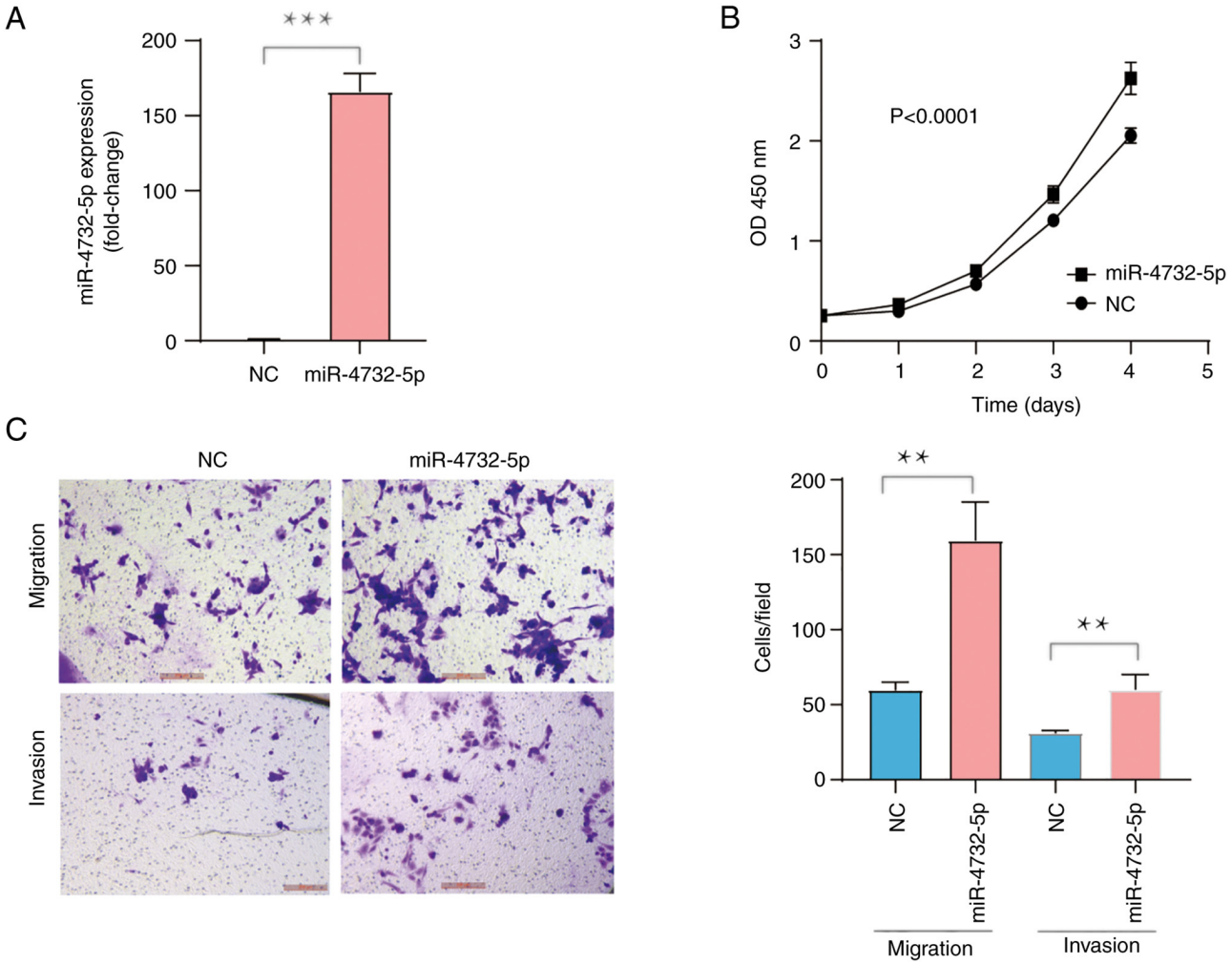

Figure 2. Transfection of IGROV1 cells using miR-4732-5p mimics promotes cell viability, cell migration and invasion *in vitro*. (A) Analysis of miR-4732-5p expression in cells transfected with miR-4732-5p mimics or NC. (B) Analysis of cell viability using Cell Counting Kit-8 assay. (C) Analysis of cell migration and invasion using Transwell assay. Scale bars, 200 μm. Data are presented as the mean ± standard deviation. Three individual experiments with at least three replicates were performed. **P<0.001 and ***P<0.0001, miR-4732-5p mimics group vs. the NC group. NC, negative control.

(SLC51A, PFN4 and CTXN2) had no reference reported in OC. According to the OS of patients analyzed using GEPIA K-M survival curve analysis, the increased expression of RPS6KA2 predicted a poor OS (Fig. 4B), which was contrary to the suppressive role of RPS6KA2 reported in OC (16), while the increased levels of MCUR1 expression predicted an improved OS (Fig. 4C), which was consistent with the tumor suppressor role of MCUR1 reported in OC (17). Thus, MCUR1 was selected to be validated in further experiments. The expression of MCUR1 in the cells was then validated after altering the miR-4732-5p expression levels. Consistent with the prediction, MCUR1 expression was decreased in the cells following transfection with miR-4732-5p mimics, whereas it was increased in the cells following transfection with miR-4732-5p inhibitors (Fig. 4D). Usually, miRNA inhibited downstream targets by complementary binding to the seed region of mRNA 3'UTR. There was one binding region at the position 612-618 WT fragment of MCUR1 3'UTR for miR-4732-5p predicted by TargetScan (Fig. 4E, binding site). The WT MCUR1 3'UTR fragments or the mutant type at the predicted miR-4732-5p binding sites were inserted pGL3-control plasmid downstream of the Firefly luciferase gene. Dual-luciferase reporter assay suggested that the relative luciferase activity was significantly suppressed in miR-4732-5p mimics group for the WT MCUR1 3'UTR (Fig. 4E bar graph; P<0.001); however, the contrary was observed for the mutant type of MCUR1 3'UTR (Fig. 4E), as compared with the negative control, indicating that miR-4732-5p may inhibit MCUR1 by binding to the predicted binding region. These results indicated that miR-4732-5p may function as an oncogene by directly targeting a tumor suppressor, MCUR1. However, it was not possible to identify a negative correlation between the miR-4732-5p and MCUR1 expression in the GEPIA correlation analysis (Fig. 4F).

Discussion

The hsa-miR-4732-5p has been reported either as an oncogene or tumor suppressor in various types of cancer. Firstly, miR-4732-5p has been reported to promote breast cancer progression by targeting tetraspanin 13 (TSPAN13) (24). In addition, a high level of miR-4732-5p has also been reported as a predictor for relapse following S-1 adjuvant chemotherapy in gastric cancer (25). By contrast, miR-4732-5p has been shown to inhibit tumor cell proliferation, migration and

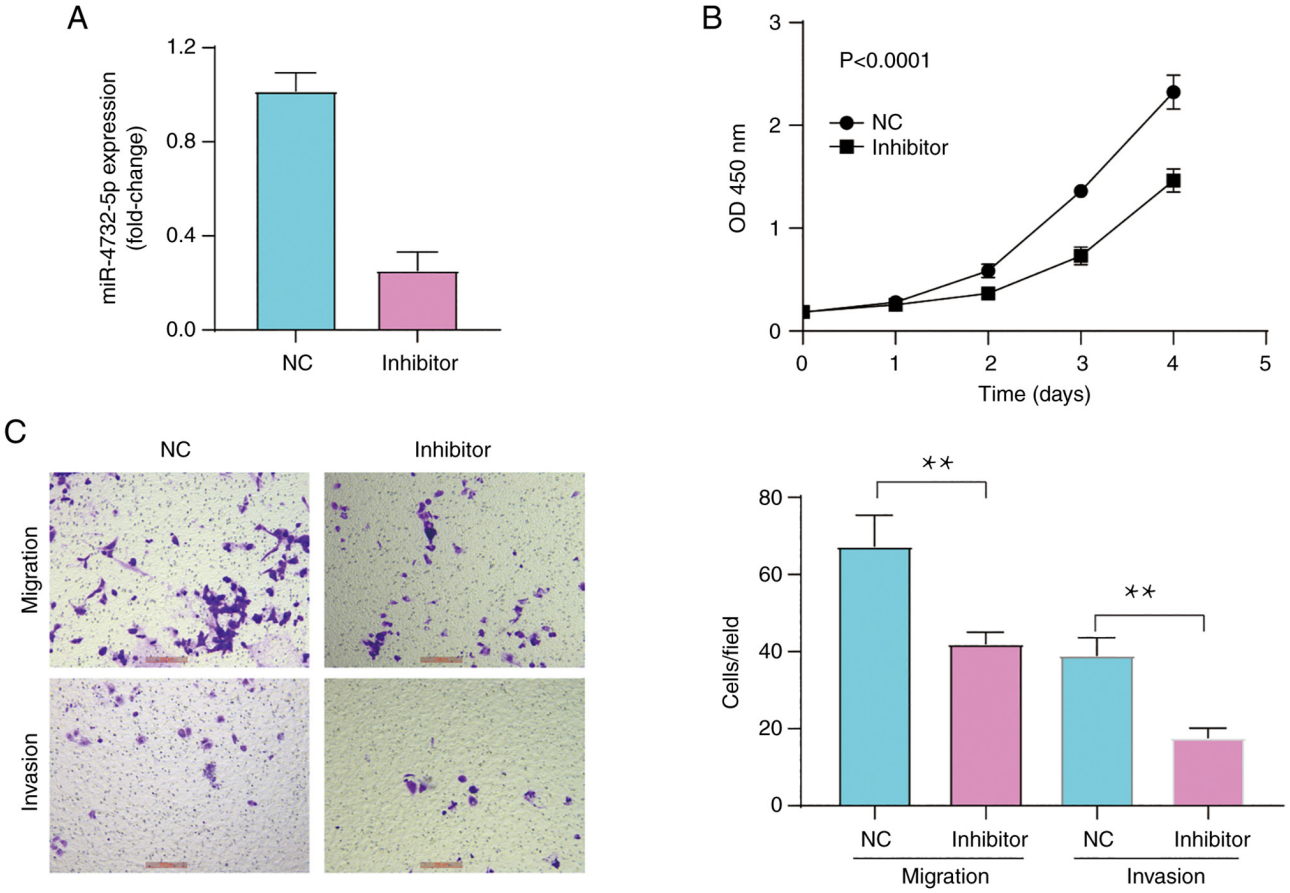

Figure 3. Inhibition of miR-4732-5p expression in IGROV1 cells suppresses cell viability, cell migration and invasion *in vitro*. (A) miR-4732-5p expression in IGROV1 cells transfected with inhibitors or NC. (B) Analysis of cell proliferation using Cell Counting Kit-8 assay. (C) Analysis of cell migration and invasion using Transwell assay. Scale bars, 200 μm. Data are presented as the mean ± standard deviation. Three individual experiments with at least three replicates were performed. **P<0.001, inhibitor group vs. the NC group. NC, negative control.

invasion in non-small cell lung cancer through the modulation of TSPAN13 (26) and in lung adenocarcinoma via the PI3K/Akt/GSK3β/Snail pathway (27). In OC, the increased expression of miR-4732-5p in plasma-derived exosomes is a promising non-invasive diagnostic biomarker, being able to distinguish cancer patients from healthy individuals and also monitor cancer progression from the early to the late stage (12). These findings may suggest the critical role of miR-4732-5p and its diverse functions in cancer progression. However, the effect of miR-4732-5p on the malignant phenotype of OC cells remains undefined. In the present study, the oncogenic role of miR-4732-5p in OC was determined by gain-function and loss-function experiments in order to elucidate an underlying potential mechanism.

In accordance with the aforementioned studies (12,25), the results of the present study demonstrated that the expression of miR-4732 in the patients with the 'death' OS status following surgery in the survival analysis acquired from TCGA-OV was significantly higher than in those with the 'alive' OS status, suggesting that it may be a poor prognostic predictor for patients with OC.

The expression levels of miR-4732 reported in TGCA include both miR-4732-3p and miR-4732-5p expression. Since the high expression of miR-4732-5p in plasma-derived exosomes is a promising non-invasive diagnostic biomarker in OC (12), only the effects of miR-4732-5p on OC cells were analyzed in the present study. The role of miR-4732-3p in OC needs to be investigated in future studies. Previously, it was reported that miR-4732-5p can function as either a tumor suppressor or oncogene (oncomiRs) in various type of cancer. Consistent with the findings of previous studies on breast and gastric cancer supporting the oncomiR role of miR-4732-5p (24,25), the results from present study demonstrated that miR-4732-5p also functioned as an oncogene in OC. The results from previous research (26,27) mentioning miR-4732-5p as a tumor suppressor gene, which are inconsistent with the results of the present study may be attributed to the differential background of various cancer types.

In total, 11 candidate direct target genes were obtained by intersecting 99 genes predicted using TargetScan and 2,607 OS-related genes from GEPIA. Through further reference analysis, the analysis was limited to two possible target genes of miR-4732-5p, including RPS6KA2 and MCUR1. Previously, it was reported that RPS6KA2 reduced the proliferation, induced G1 arrest, increased apoptosis, reduced the levels of phosphorylated extracellular signal-regulated kinase and altered the levels of other cell cycle proteins in UCI101 cells (16), and small interfering RNA against RPS6KA2 demonstrated the opposite effect in 41M cells (16). In a previous study on the MCUR1 gene in OC (17), the expression of MCUR1 was acquired from TCGA database and it was revealed that the decreased expression of MCUR1 was

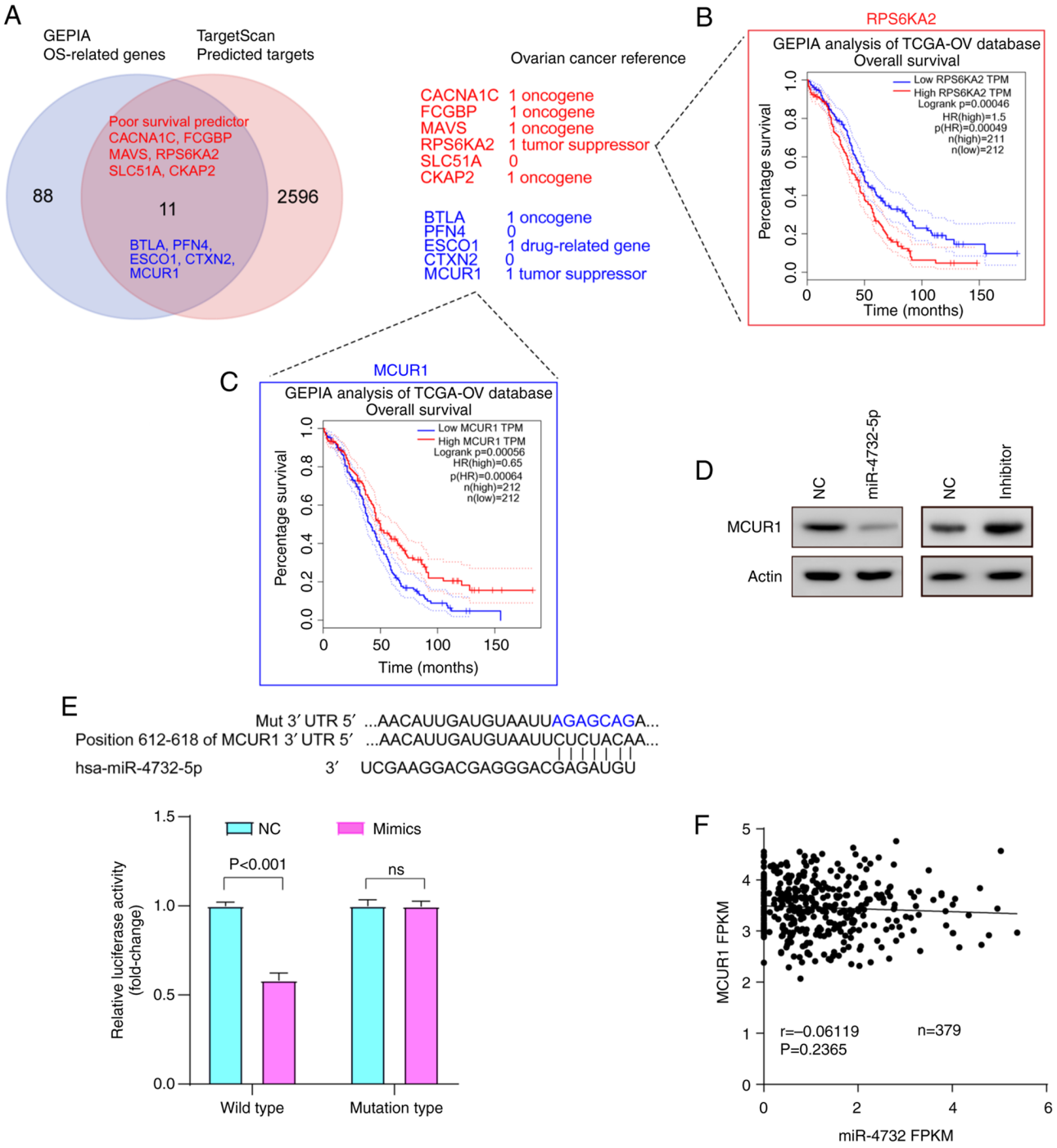

Figure 4. MUCR1 is a target gene of miR-4732-5p. (A) Venn diagram illustrating the intersection genes of possible candidate target genes predicted using TargetScan and OS-related genes identified using GEPIA. (B) K-M survival curve of RPS6KA2 in OC using GEPIA. (C) K-M survival curve of MUCR1 in OC using GEPIA. (D) Western blotting was used to determine MUCR1 protein levels in the cells with an altered miR-4732-5p expression achieved using either mimics or inhibitors. (E) The binding region between wild-type MCUR1 3'UTR and miR-4732-5p, as predicted using TargetScan and its corresponding mutant type. Dual-luciferase reporter assay suggested that relative luciferase activity was significantly suppressed in miR-4732-5p mimics group for the wild-type MCUR1 3'UTR, not for the mutated form of MCUR1 3'UTR. (F) The correlation between MUCR1 and miR-4732 expression in OC by GEPIA. MUCR1, mitochondrial calcium uniporter regulator 1; OS, overall survival; GEPIA, Gene Expression Profiling Interactive Analysis; K-M, Kaplan-Meier; OC, ovarian cancer; RPS6KA2, ribosomal protein S6 kinase A2; NC, negative control.

associated with a poor prognosis of patients with OC. In the present study, the K-M survival curves in relation to two candidate genes, RPS6KA2 and MCUR1, were investigated using GEPIA analysis. The results indicated that the increased expression of the tumor suppressor RPS6KA2 was associated with a poorer prognosis, which was inconsistent with the tumor suppressor role of RPS6KA2 in OC. This may be explained by the fact that one gene can play exactly the opposite role in two different contexts, similarly to the roles of the well-known tumor suppressor 'wild-type' version of p53 (WTp53), which has been reported to promote tumors by stimulating the cancer metabolic switch of the oxidative phosphorylation in glycolysis by promoting the PUMA-mediated disruption of mitochondrial pyruvate uptake in hepatocellular carcinoma, rather

than exerting a suppressive effect (28). MCUR1 was selected for further study due to the available consistent results from the GEPIA analysis. Additionally, MCUR1 was validated as the direct target of miR-4732-5p, which is consistent with a previous study on OC (17). According to OS of patients examined using the GIPIA K-M survival curve analysis, the high level expression of RPS6KA2 predicted a poor OS (Fig. 4B), which was contrary to the suppressive role of RPS6KA2 reported in OC (16), while a high level of MCUR1 expression predicted a good OS (Fig. 4C), which was consistent with the tumor suppressive role of MCUR1 reported in OC (17). In the present study, it was hypothesized based on previously available data (12) that miR-4732-5p, as an oncogene, targeted tumor suppressors and the target genes were narrowed down into two genes, RPS6KA2 and MCUR1, based on previous reference (16,17). However, there is only one study available in OC which suggests RPS6KA2 (16) and MCUR1 (17), respectively, as tumor suppressors. Thus, additional further experimental data are required in order to validate any possible suppressive role. In a study on the MCUR1 gene in OC (17), the expression of MCUR1 was acquired from TCGA database and it was found that the decreased expression of MCUR1 was associated with a poor prognosis of patients with OC.

Of note, a limitation of the present study is the lack of MCUR1 and RPS6KA2 overexpression experiments. Thus, the gene function of MCUR1 in OC warrants further confirmation. Furthermore, the expression correlations between MCUR1 and miR-4732-5p in both OC and non-cancerous cell lines require further investigation. Biologically, MCUR1 protein plays a crucial role in mitochondrial calcium uptake (29). Mitochondrial Ca^{2+} uptake occurs via the mitochondrial Ca^{2+} uniporter (MCU) complex and MCUR1 acting as a scaffold factor for MCU channel function is an essential MCU regulators. Thus, it was suggested that MCUR1 may regulate cancer progression by affecting the cellular Ca^{2+} concentration and Ca^{2+}-related signaling. It was determined that miR-4732-5p may bind to the MCUR1-3'UTR and inhibit its expression though the UTR binding site, as presented in Fig. 4E. However, it was not possible to identify the negative correlation between miR-4732-5p and MCUR1 expression in the GEPIA correlation analysis (Fig. 4F). As regards RPS6KA2, it has been reported that RPS6KA2 suppresses proliferation, induces G1 arrest, increases apoptosis, reduces the levels of phosphorylated extracellular signal-regulated kinase and alters the levels of other cell cycle proteins in OC cell lines (16). Thus, it can be hypothesized that RPS6KA2 may reduce cancer progression through a similar mechanism.

In conclusion, the results of the present study supported the conclusions of a previous study (12) that miR-4732-5p may function as an oncogene in OC by possibly targeting MCUR1.

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Availability of data and materials

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

Authors' contributions

XL performed the majority of the experiments. XW and JW participated in the *in vitro* study and acquired cell viability data. XL designed the experiments, coordinated the study and wrote the manuscript. XL, XW and JW confirm the authenticity of all the raw data. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Ningbo Women and Children's Hospital (Approval No.:EC2022-024).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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