MicroRNA-103a-3p potentiates chemoresistance to cisplatin in non-small cell lung carcinoma by targeting neurofibromatosis 1

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Abstract. Lung cancer remains the leading cause of cancer-associated mortality worldwide, and non-small-cell lung cancer (NSCLC) contributes to ~80% of these deaths. However, both primary and acquired cisplatin resistance frequently occurs within the disease and represents a huge clinical treatment problem. The underlying molecular mechanisms are not yet completely understood, but in recent years, microRNAs (miR) have been reported to play vital roles in the development of lung cancer and chemoresistance. In the present study, it was revealed that there were increased expression levels of miR-103a-3p in both NSCLC cell lines and human NSCLC samples that exhibited resistance to cisplatin. The results also revealed that the inhibition of miR-103a-3p in A549/cisplatin cells significantly sensitized these cells to cisplatin, while inhibition of miR-103a-3p expression inhibited tumor growth and enhanced the function of cisplatin in a xenograft animal model. Furthermore, the present study demonstrated that miR-103a-3p regulates cisplatin resistance by targeting neurofibromatosis 1 (NF1) via activating ERK signaling in vitro and in vivo. In conclusion, NF1 was identified as a special miR-103a-3p target in the present study, and it was revealed that targeting NF1 via miR-103a-3p may help reverse chemoresistance and provide a biomarker to cisplatin responsiveness in NSCLC.

Introduction

Non-small cell lung cancer (NSCLC) accounts for ~85% of all primary lung cancer cases worldwide in 2018 (1). Chemoresistance is responsible for the high prevalence and mortality of NSCLC, as this phenomenon enhances NSCLC progression and makes NSCLC difficult to treat (2). The underlying molecular mechanisms of chemoresistance are not yet understood (3). The aim of the present study was to elucidate these mechanisms in order to identify a method to decrease chemotherapy resistance in NSCLC.

MicroRNAs (miRNAs or miRs) are an important component of epigenetic mechanisms that decrease gene expression at the post-transcriptional level by binding to 3’-untranslated regions (UTRs) of target mRNAs (4). miRNA is an important regulator in a number of biological processes, such as cell proliferation, invasion, metastasis and apoptosis (5,6). Abnormal expression of miRNAs has been demonstrated to be associated with tumor chemotherapy resistance, including resistance to cisplatin (7). However, how miRNAs regulate cisplatin resistance is not yet clear. Previous studies have demonstrated the suppressive role of miR-103a-3p in chemotherapy-resistance in numerous different types of cancer such as bladder carcinoma, malignant mesothelioma and glioma (8-11). However, whether miR-103a-3p regulates cisplatin resistance in NSCLC remains unknown.

Neurofibromin 1 (NF1) plays a role as a key negative regulator of the Ras signaling pathway, negatively regulating mitogen-activated protein kinase-extracellular signal-regulated kinase (ERK) signaling (12). Recent studies revealed that primary and acquired chemotherapy-resistance of lung adenocarcinomas in patients was significantly correlated with lower NF1 expression (12,13). However, the underlying molecular mechanism of how NF1 downregulates cisplatin resistance in NSCLC remains unknown.

In the present study, it was demonstrated that expression levels of miR-103a-3p were significantly increased in patients with cisplatin-resistant NSCLC and can induce cisplatin resistance in NSCLC cells by directly targeting NF1 to activate ERK signaling. Furthermore, the results revealed that overexpression of miR-103a-3p can overcome cisplatin resistance of NSCLC cells.

Materials and methods

Human samples and cell lines. A total of 20 patients (age, 38-69 years; male 12, female 8) with primary NSCLC who underwent surgical resections from January 2016 to December 2017 at The Affiliated Tumor Hospital of Xinjiang
Medical University were included in the present study, and their adjacent normal lung tissues (5 cm away from the tumor tissue) were obtained. The aforementioned 20 patients' serum was also included in the present study. All samples were properly preserved for future use. The tissues were stored in liquid nitrogen. Venous blood samples of all participants were collected in EDTA K2 anticoagulation tubes from an antecubital vein, which were stored in -80°C fridge. The present study was approved by the Research Ethics Board of the Xinjiang Medical University. All patients who provided tissues and serum provided written informed consent and all of them agreed to the use of their samples in scientific research.

Human lung adenocarcinoma A549 and PC-9 cells, which were purchased from the American Type Culture Collection, were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) with 10 U/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO2. The cisplatin-resistant A549/PC-9 cell lines were established in the center laboratory of The Affiliated Tumor Hospital of Xinjiang Medical University and were preserved in 1 µmol/l cisplatin (cat. no. BP809; Sigma-Aldrich; Merck KGaA).

Cell transfection. miR-103a-3p inhibitors (100 nM), miR-103a-3p mimics (50 nM) and negative control (NC), ERK siRNAs, NFI siRNAs and their negative controls were synthesized by RiboBio. The final concentration of siRNAs was 50 nM and the sequences are presented in Table I. Transfection was performed using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 10 µl lentivirus (1x10⁵ TU/ml; Shanghai GeneChem Co., Ltd.) carries a puromycin resistance gene, which can be used to reject untransfected cells following transfection with lentivirus. The pre-experimental results revealed that when 2 mg/ml puromycin was added to the culture medium then cultured at 37°C for 48 h, A549 cell viability was 0% without any transfection. Total RNA and protein were prepared 72 h after transfection and were used for reverse transcription-quantitative PCR (RT-qPCR) or western blot analysis.

Cell viability assay. A549 and PC9 cells were seeded into 96-well plates (5x10³ cells/well) either directly or 24 h after transfection and allowed to attach overnight. Freshly prepared cisplatin was then added at 5, 10, 20, 50 and 100 µM at 37°C. After 24-h, cell viability was assessed using a Cell Counting Kit-8 (cat. no. HY-K0301; MedChemExpress) according to the manufacturer's protocol.

Protein extraction and western blotting. Total protein was collected using radioimmunoprecipitation assay buffer (cat. no. R0278; Sigma Aldrich; Merck KGaA) containing protease inhibitors (Merck KGaA). The supernatant protein concentration was measured using a bicinchoninic acid kit (Beijing Dingguo Changsheng Biotechnology Co., Ltd.). A total of 30 µg of protein per lane was separated via 10% SDS-PAGE electrophoresis and transferred to PVDF membranes (Bio-Rad Laboratories, Inc.). After the membrane was transferred, PVDF was blocked with 5% skim milk powder at room temperature for 1 h. Membranes were then incubated with primary antibodies against ERK (1:1,000; cat. no. 9102), p-ERK (Thr202/Tyr204) (1:2,000; cat. no. 4370), NFI (1:100; cat. no. 14623), (all from Cell Signaling Technology, Inc.) and GAPDH using the 2-ΔΔCq method (14). Thermocycling conditions were as follows: 95°C for 10 sec; 58°C for 20 sec; 72°C for 10 sec and 40 cycles. For the analysis of NFI expression, RT and qPCR were performed using a High-Capacity cDNA Reverse Transcription kit and QuantiTect Sybr Green PCR kit (Thermo Fisher Scientific, Inc., respectively). The thermocycling conditions were as follows: 95°C for 10 sec; 60°C for 20 sec; 72°C for 10 sec and 40 cycles. The expression levels of NFI were quantified in relation to the expression levels of GAPDH using the 2-ΔΔCq method (14). The primer sequences were described in Table II. All primers were obtained from Guangzhou RiboBio Co., Ltd.

RNA preparation and RT-qPCR. Total RNA was isolated from NSCLC tissues or cell lines using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA (All-in-One™ miRNA First-Strand cDNA Synthesis Kit for miRNA; cat. no. AMRT-0020 and All-in-One™ First-Strand cDNA Synthesis Kit for mRNA; cat. no. AORT-0020; GeneCopoeia, Inc.) under the following protocol: 37°C for 60 min then 85°C for 5 min. miRs from serum were isolated using the miRNeasy Serum/Plasma kit (Qiagen) according to the manufacturer's protocol.

Immunochemistry. NSCLC xenograft specimens. Specimens were formalin (10%) fixed for 24 h in 4°C and paraffin-embedded tumor tissues (3 µM) were examined to ensure a tumor content of >75% by a pathologist. IHC was performed using Antigen Retrieval Dako Target Retrieval solution (pH 6.0) and Histostain-Plus 3rd Gen IHC Detection kit (Invitrogen; Thermo Fisher Scientific, Inc.; 1:10,000 and 40 cycles. For the analysis of NF1 expression, RT and qPCR were performed using a High-Capacity cDNA Reverse Transcription kit and QuantiTect SYBR Green PCR kit (Thermo Fisher Scientific, Inc., respectively). The thermocycling conditions were as follows: 95°C for 10 sec; 60°C for 20 sec; 72°C for 10 sec and 40 cycles. The expression levels of NFI were quantified in relation to the expression levels of GAPDH using the 2-ΔΔCq method (14). The primer sequences were described in Table II. All primers were obtained from Guangzhou RiboBio Co., Ltd.

Target gene prediction. National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov) and TargetScan
These results demonstrated that the miR-103a-3p expression levels increased following treatment with inhibitors (Fig. 1A and solid tumor (Fig. 1B)). A549/cisplatin cells were transfected with control lentivirus or miR-103a-3p inhibitors expression lentivirus as previously described. After drug (puromycin, 2 mg/ml) screening for transfection, 1x10^6 cells in 100 μl of phosphate-buffered saline were subcutaneously injected into left side of each mouse. When the tumors reached ~100 mm^3, mice were treated with or without cisplatin (3 mg/kg body weight; 6 mice per group) by intraperitoneal injection every 3 days. After 4 weeks of treatment, the mice, average weight 20 g, were sacrificed by cervical dislocation (maximum tumor volume was 1,300 mm^3), and the tumor weight was measured. The methods of the animal models used in the present study were approved by the Research Ethics Board of The Affiliated Tumor Hospital of Xinjiang Medical University.

### Results

Cisplatin resistance is closely associated with miR-103a-3p overexpression in NSCLC cells. The miR-103a-3p expression levels in 20 human NSCLC samples (10 cisplatin-resistant samples and 10 cisplatin-sensitive samples) from different patients were analyzed in the present study, in order to investigate the association between miR-103a-3p levels and cisplatin resistance. It was revealed that miR-103a-3p was significantly increased in the samples from patients with cisplatin-resistant NSCLC in both serum (Fig. 1A) and solid tumor (Fig. 1B). A549/cisplatin had increased remarkably compared to parental cell A549 (Fig. 1C) in vitro, and PC-9/cisplatin demonstrated also changed (Fig. 1D). These results demonstrated that miR-103a-3p exhibits high expression levels in NSCLC cells and could affect the development of cisplatin resistance.

In order to investigate this hypothesis, miR-103a-3p overexpressed A549 cells were treated with cisplatin and cell viability assays were performed. The results revealed that the miR-103a-3p expression levels increased following treatment with miR-103a-3p mimics or decreased following treatment with inhibitors (Fig. 2A). High expression levels of miR-103a-3p caused A549 cells to exhibit significantly greater levels of resistance to cisplatin treatment compared to control A549 cells, indicating the potential of miR-103a-3p as a therapeutic target for cisplatin resistance.

### Statistical analysis

All data are presented as the mean ± standard deviation. One-way analysis of variance followed by Tukey’s post hoc test was used to evaluate the comparisons of multiple groups. The SAS statistical software package (version 6.12; SAS Institute, Inc.) was used to perform all statistical analyses. A P<0.05 in the two-tailed test was considered to indicate a statistically significant difference.

### Table I. Transfection reagent sequences.

| Name          | Sequence                                      |
|---------------|-----------------------------------------------|
| miR-103a-3p   | 5'-AGCAGCAUGUACAGGCCUAUGA-3'                  |
| miR-103a-3p   | 5'-AUAGCCCUAGAUGCUCUU-3'                      |
| inhibitor     | 5'-TCATAGCCCTGTACAATGCTCT-3'                  |
| Inhibitor     | 5'-CAGTACTTTTGTGTAACAA-3'                     |
| si NF1, F     | 5'-AGATGAAACGATGGTCAAAC-3'                    |
| si NF1, R     | 5'-CGTGAACCTGTTAGAATGCGA-3'                   |
| si ERK, F     | 5'-GGACACCAGUACCACAUU-3'                      |
| si ERK, R     | 5'-AAUGUGUUGAGCUUGUCC-3'                     |

miR, microRNA; siRNA, small interfering; ERK, extracellular signal-regulated protein kinase; NF1, neurofibromin 1; F, forward; R, reverse.

### Table II. Primer sequences.

| Name          | Sequence                                      |
|---------------|-----------------------------------------------|
| U6, F         | 5'-GCTTCGGCACACATATAACTAAAT-3'                |
| U6, R         | 5'-CGCTTCAGAATTGTGCTCAT-3'                    |
| GAPDH, F      | 5'-TGAAAGTGGAGTCAACGAGAT-3'                   |
| GAPDH, R      | 5'-CATGTGGCGACTGAGTCCACAC-3'                  |
| NF1, F        | 5'-CGAAATGGCAGAGACCTTAC-3'                    |
| NF1, R        | 5'-GACCACTTGAGCACGCC-3'                       |

miR, microRNA; F, forward; R, reverse; si, small interfering; NF1, neurofibromin 1.
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Figure 1. miR-103a-3p expression levels are increased in patients with cisplatin-resistant NSCLC. The level of miR-103a-3p was significantly increased in (A) the serum and (B) the tumors of patients with NSCLC that acquired resistance to cisplatin treatment compared with cisplatin-sensitive patients. (C) The level of miR-103a-3p was significantly increased in the cisplatin resistant cell line, A549/cisplatin, compared with the cisplatin-sensitive cell line, A549. (D) The level of miR-103a-3p was significantly increased in cisplatin resistant cell line, PC-9/cisplatin, compared with the cisplatin-sensitive cell line, PC-9. The levels of miR-103a-3p were assessed using reverse transcription-quantitative PCR. *P<0.05 and **P<0.01 with comparisons indicated by lines. miR, microRNA; NSCLC, non-small cell lung carcinoma; cDDP, cisplatin.

to the control and miR-103a-3p inhibitors group (Fig. 2B). miR-103a-3p mimics reversed the inhibitory effect of cisplatin on A549 cells (Fig. 2C). Overall, these results demonstrate that high expression levels of miR-103a-3p significantly contribute to the development of cisplatin resistance in NSCLC cells.

miR-103a-3p induces ERK signaling in NSCLC cells by targeting NF1 expression. In order to investigate the role of miR-103a-3p in the regulation of ERK signaling, the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov) and TargetScan databases (http://www.targetscan.org/vert_72; http://starbase.sysu.edu.cn/) to comprehensively to screen miR-103a-3p target genes and identified NF1 as a tentative target of miR-103a-3 A different 3'-UTR of the NF1 gene was constructed in the present study, which contained three sites that interacted with miR-103a-3p (Fig. 4A). In order to confirm whether miR-103a-3p decreases NF1 expression in A549 cells, NF1 expression was measured following overexpression or inhibition of miR-103a-3p in A549 cells. At the protein (Fig. 4B) or mRNA (Fig. 4C) levels, miR-103a-3p negatively regulated NF1 expression in NSCLC cells. In addition, the present study assessed the binding of miR-103a-3p to NF1 3'-UTR by synthesizing each site of the NF1 3'-UTR that could interact with miR-103a-3p into the firefly luciferase reporter plasmid. These sites were then transfected into A549 cells with miR-103a-3p mimics or control oligonucleotides. The luciferase assay results revealed that the signal was significantly decreased following transfection with the third site of the NF1 3'-UTR (Fig. 4D). These results indicate that miR-103a-3p directly interacts with the third site of the NF1 3'-UTR. A three-nucleotide mutation
The site of miR-103a-3p was inserted into the 3'-UTR to confirm whether the expression of NF1-luciferase is dependent on the 3'-UTR sequence (third binding site) complementary to the miR-103a-3p seed sequence, as presented in Fig. 4A. The data from the present study indicate that the 3'-UTR mutation had no effect on miR-103a-3p overexpression or luciferase activity, but wild-type 3'-UTR significantly repressed the luciferase activity following miR-103a-3p overexpression in A549 cells. Inhibition of miR-103a-3p significantly enhanced the luciferase activity associated with the wild-type 3'-UTR (Fig. 4E). Furthermore, it was demonstrated that miR-103a-3p regulates ERK signaling by targeting NF1.

Overexpression of NF1 reversed miR-103a-3p-induced upregulation of phosphor-ERK in A549 cells (Fig. 5A). In contrast, silencing NF1 reverses the inhibition of miR-103a-3p on ERK phosphorylation (Fig. 5B). Consistent with these results, the cell viability assay indicated that miR-103a-3p overexpression and cisplatin-induced resistance were overcome by NF1 overexpression in A549 cells (Fig. 5C). In summary, these data indicate that miR-103a-3p regulates cisplatin resistance of NSCLC cells via NF1, which activates ERK signaling.

**Inhibition of miR-103a-3p can reverse cisplatin resistance of NSCLC cells in vivo.** The results of the present study indicate that overexpression of miR-103a-3p induces cisplatin resistance, but whether miR-103a-3p interference can overcome cisplatin resistance in NSCLC cells remains unclear. To demonstrate this hypothesis, cell viability changes in two...
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Figure 4. NF1 is a target gene of miR-103a-3. (A) Sequence alignment of miR-103a-3p with the 3'-UTR of NF1 gene. The red labeled nucleotides represent point mutations in the 3'UTR sequence. miR-103a-3p negatively regulates NF1 expression at both the (B) protein and (C) mRNA levels. A549 cells were transfected with miR-103a-3p mimic or inhibitor. After 72 h of transfection, cells were subjected to reverse transcription-quantitative PCR and western blot analysis. (D) 3'-UTR luciferase reporter assay for NF1. Each miR-103a-3p binding site of NF1 3'-UTR was cloned into luciferase reporter constructs and transfected into A549 cells with miR-103a-3p mimic or control oligonucleotides. The luciferase assay revealed that the signal was significantly decreased following transfection with the third site of the NF1 3'-UTR. (E) The third NF1 3'-UTR binding site inhibits the expression of firefly luciferase. The indicated luciferin plasmid was transfected with miR-103a-3p mimic or control oligonucleotides. *P<0.05 and **P<0.01 with comparisons indicated by lines. NF1, neurofibromin 1; miR, microRNA; 3'-UTR, 3'-untranslated region; NC, negative control; mut, mutant.

Figure 5. miR-103a-3p activates ERK signaling by inhibiting NF1 expression in NSCLC cells. (A) Overexpression of NF1 inhibits miR-103a-3p-induced ERK phosphorylation. A549 cells were transfected with miR-103a-3p mimic or/and NF1 expression plasmid 72 h. (B) Silencing of NF1 suppressed miR-103a-3p inhibitor-induced inhibition of ERK phosphorylation. A549 cells were transfected with miR-103a-3p inhibitor and/or NF1 siRNA 72 h. (C) Overexpression of NF1 inhibits miR-103a-3p-induced cisplatin resistance in A549 cells. Cells were transfected with the indicated nucleotides and/or plasmid, then treated with indicated concentration of cisplatin for 72 h. *P<0.05 and **P<0.01 with comparisons indicated by lines. NF1, neurofibromin 1; miR, microRNA; NC, negative control; ERK, extracellular signal-regulated protein kinase; siRNA, small interfering RNA; p, phosphorylated.
different cisplatin-resistant A549 cell clones treated with miR-103a-3p inhibitor and cisplatin were then examined. The results revealed that the combination of miR-103a-3p inhibitor and cisplatin was more effective than cisplatin only treatment (Fig. 6A). Furthermore, a xenograft model generated by the lentiviral transfected cisplatin-resistant cell line A549/cisplatin was used to observe whether this theory is correct in vivo. When the average volume of the xenograft tumor reached 100 mm$^3$, the mice received 4 weeks of cisplatin treatment. The results revealed that in the A549/cisplatin xenograft model, the combination of miR-103a-3p inhibitor and cisplatin was superior to cisplatin or miR-103a-3p inhibitor alone in inhibition of A549/cisplatin xenograft growth (Fig. 6B). The combination of the two can significantly slowed tumor growth in volume and in weight (Fig. 6C and D). The expression levels of Ki-67 were markedly lower in the miR-103a-3p inhibitor combined with cisplatin treatment group compared to the control (Fig. 6E).

Discussion

Over the past 10 years, NSCLC has become a prevalent malignancy worldwide, but cisplatin-based chemotherapy resistance is one obstacle standing in the way of treatment (15). A number of studies have demonstrated that dysregulation of specific miRs results in the development of chemoresistance in a number of different types of cancer (16,17). In the present study, NF1 was identified as a target of miR-103a-3. It was revealed that miR-103a-3p overexpression could decrease NF1, improve cell viability and desensitize NSCLC to cisplatin both in vivo and in vitro. Chen et al (12) reported that miR-641 can contribute to erlotinib resistance in NSCLC cells by targeting NF1. MiR and NF1 play an important role in NSCLC treatment resistance. Furthermore, the present study demonstrates the association between miR-103a-3p and the development of cisplatin chemoresistance in NSCLC.

There are numerous reasons underlying drug resistance, which include factors such as increases in drug efflux, alternations in drug targets, DNA repair, cell cycle regulation and evasion of apoptosis (12,18). It has previously been demonstrated that selective regulation of miR activity can improve responsiveness to chemotherapy (18) miR-103a-3p expression has been demonstrated in several different cancer cell lines such as bladder carcinoma cell and glioma cell line (8-10), and miR-103a-3p has been indicated to be important in proliferation and metastasis (8,10). In the present study, it was revealed that miR-103a-3p was significantly increased in patients with NSCLC who acquired resistance to cisplatin treatment, as well as increased cisplatin resistance in NSCLC cell lines. It was also demonstrated that overexpression of miR-103a-3p can decrease NF1 levels, desensitize A549/cisplatin cells to cisplatin, and promote tumor growth in a nude mice model. In addition, it was revealed that miR-103a-3p is partially complementary to the 3'-UTR of the NF1 mRNAs using bioinformatics (TargetScan) and that miR-103a-3p can affect luciferase activity due to canonical binding to the NF1 3'-UTR. Thus, the present study clearly established an inverse association between miR-103a-3p and NF1. Furthermore, overexpression of NF1 can reverse high ERK phosphorylation levels, which had been induced by overexpression of miR-103a-3. On the other hand, low phosphorylation levels, which had been caused by inhibition of miR-103a-3p, were increased via inhibition of NF1.
miR-103a-3p levels are highly expressed in breast cancer, pancreatic cancer and ovarian cancer, and are closely associated with tumor invasion and metastasis (19-22). There are some inconsistent results, however; Fasihi et al (23) assessed whether hsa-miR-103a-3p plays an important role in colorectal carcinoma via regulation of the Wnt signaling pathway. They hypothesized that miR-103a-3p overexpression resulted in cell cycle progression and decreased apoptotic rate in SW480 cells. A single miR may disrupt multiple pathways involved in regulating cancer cell survival or drug response. Thus, the effect of miR-103a-3p was determined by the function of target genes in the present study. However, the molecular mechanism underlying the differing functions of miR-103a-3p in different cancers remains unclear; in addition, whether miR-103a-3p affect the therapeutic effect of tyrosine kinase inhibitors treatment in NSCLC requires further examination.

In the present study, the study combined clinical and experimental studies to establish a role for miR-103a-3p in regulating cisplatin chemoresistance in NSCLC. Upregulated expression of miR-103a-3p dramatically enhances the sensitivity of NSCLC cells to cisplatin chemotherapy. These results are also helpful for developing potential therapeutics for the treatment of NSCLC chemoresistance.

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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

SY conceived and designed this study. HZ was responsible for doing the main experimental. HZ and JY were jointly involving in extracting data and writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Board of The Affiliated Tumor Hospital of Xinjiang Medical University (Urumqi, China). All patients who provided tissues and serum provided written informed consent and all of them agreed to the use of their samples in scientific research. The methods of the animal models used in the present study were approved by the Research Ethics Board of The Xinjiang Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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