KDM6A suppresses hepatocellular carcinoma cell proliferation by negatively regulating the TGF-β/SMAD signaling pathway

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Abstract. Lysine demethylase 6A (KDM6A) is a Jumonji-C domain-containing histone demethylase that specifically catalyzes the removal of histone H3 lysine-27 trimethylation. KDM6A is a member of the KDM6 family, the biological role of which has been reported in various types of cancer, including bladder and lung cancer, as well as pancreatic ductal adenocarcinoma. However, the role of KDM6A in hepatocellular carcinoma (HCC) is not completely understood. Therefore, the present study aimed to determine the biological function of KDM6A in HCC progression. The expression profile of KDM6A was examined in HCC surgical specimens using reverse transcription-quantitative PCR. In addition, the role of KDM6A in the proliferation capacities of HCC cell lines was examined in vitro and in vivo using crystal violet and MTT assays. The underlying mechanism by which KDM6A exerts its function was explored by western blotting. The present study indicated that KDM6A was significantly downregulated in HCC tissues compared with normal control tissues. The role of KDM6A in HCC cell proliferation was also determined. KDM6A overexpression significantly inhibited HCC cell proliferation, whereas KDM6A knockdown significantly promoted HCC cell proliferation compared with the corresponding control groups. Consistently, KDM6A overexpression suppressed HCC cell tumorigenesis in vivo. The western blotting results indicated that KDM6A overexpression decreased the phosphorylation levels of smad2, whereas KDM6A knockdown increased the phosphorylation levels of smad2 compared with the corresponding control groups. Therefore, the present study suggested that KDM6A may inhibit HCC cell proliferation by negatively regulating the TGF-β/SMAD signaling pathway, suggesting that KDM6A may serve as a potential target for the diagnosis and treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, resulting in high morbidity and mortality, with 854,000 new cases and 810,000 cases of mortality each year (1). HCC is also the fifth most commonly occurring cancer, as well as the third leading cause of cancer-related death worldwide (2). Multiple therapeutic strategies, such as liver resection, transarterial chemoembolization, radiotherapy and sorafenib administration, have been used for the treatment of patients with HCC. However, the prognosis of patients with HCC remains poor due to its complexity, high recurrence and early vascular invasion (3,4). Increasing evidence has revealed that HCC is associated with multi-gene mutations (5). Molecular targeting has been suggested as a novel therapeutic approach for patients with advanced HCC, as it significantly increases the survival time of patients and has favorable curative effects (6). Therefore, understanding the development of HCC and identifying novel molecular therapeutic targets has received increasing attention.

Lysine demethylase 6A (KDM6A; also known as ubiquitously transcribed X chromosome tetratricopeptide repeat protein) is a Jumonji-C domain-containing histone demethylase that catalyzes the removal of histone H3 lysine-27 trimethylation (7,8). As a member of the KDM6 family, KDM6A is also a key molecule that regulates cell fate decisions and cell identity during normal development by controlling the expression of pluripotency and lineage-specific genes (9,10). It has also been reported that KDM6A is frequently targeted by somatic inactivating mutations in various types of cancer, such as esophageal and urethral cancer, as well as pancreatic ductal adenocarcinoma and multiple myeloma (11). Mechanistically, KDM6A mutations suppress cell proliferation by activating retinoblastoma protein-related genes, which suggests that KDM6A serves as a putative tumor suppressor (12). Furthermore, the biological function of KDM6A in a number of malignancies, including bladder and lung cancer, as well as pancreatic ductal adenocarcinoma, has been reported (13-16). However, the cellular and molecular mechanisms underlying KDM6A in HCC are not completely understood.

The present study investigated the importance of KDM6A during HCC progression. The results indicated that KDM6A expression was downregulated in human HCC tissues compared with corresponding normal control tissues.
In addition, KDM6A overexpression inhibited HCC cell proliferation in vitro and in vivo. Therefore, the present study identified a potential molecular mechanism underlying the role of KDM6A during HCC development.

Materials and methods

HCC tissue samples. A total of 30 paired primary HCC tissues and adjacent normal tissues (distance, <2 cm) were obtained from patients (age, 32-68 years; mean age, 46±3.2 years; 22 male patients and 8 female patients) who had undergone a hepatectomy between February and July 2019 at Gansu Provincial Hospital (Lanzhou, China). The inclusion criteria were as follows: i) Patients clinically diagnosed with liver cancer following surgery; ii) R0 resection based on histological examinations; and iii) paired normal tissue was adjacent to tumor tissue (distance, <2 cm). The exclusion criteria were as follows: i) Patients with distant metastasis; and ii) patients who had received radiotherapy or chemotherapy before surgery. Paired samples were subjected to RNA extraction for reverse transcription-quantitative PCR (RT-qPCR) and immunohistochemistry (IHC) analyses. The present study was approved by the Institutional Review Board of The Institute for Gansu Provincial Hospital (Lanzhou, China). All patients provided written informed consent.

IHC. Tumor tissues and paired normal tissues were fixed in 4% formalin at room temperature for 24 h, embedded in paraffin and cut into 5-µm thick consecutive sections. Paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated using a descending ethanol gradient before being subjected to antigen retrieval in a sodium citrate solution (pH 6.0) for 20 min at 98°C and endogenous peroxidase activity blocking with 3% hydrogen peroxide. The sections were washed three times with 0.01 M PBS for 5 min each and blocked in 0.01 M PBS containing 0.3% Triton X-100 and 5% BSA (Gibco; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. Subsequently, sections were incubated with an anti-KDM6A antibody (cat. no. orb333886; 1:100; Biorbyt Ltd.) at 4°C overnight. After washing with 0.01 M PBS, sections were incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (cat. no. 7074; 1:5,000; Cell Signaling Technology, Inc.) at room temperature for 1 h. Subsequently, DAB substrate was used to visualize KDM6A protein expression levels. Stained sections were observed in five randomly selected, independent high-power microscopic field of view using an inverted light microscope (magnification, ×400).

RNA extraction and RT-qPCR. Tissues were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Total RNA was extracted from tumor tissues and adjacent noncancerous tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA (2 µg) was reverse transcribed into cDNA using the PrimeScript™ RT Reagent kit (Takara Bio, Inc.), according to the manufacturer's instructions, with the following thermocycling conditions: 37°C for 15 min and 85°C for 5 sec. Subsequently, qPCR was performed using SYBR® Premix Ex Taq (Takara Bio, Inc.). The sequences of the primers used for qPCR are listed in Table I. The following thermocycling conditions were used for qPCR: Initial denaturation for 1 min at 95°C; 40 cycles of denaturation for 20 sec at 95°C, annealing at 56°C for 60 sec and extension at 72°C for 2 min; followed by final extension at 72°C for 6 min. mRNA expression levels were quantified using the 2^(-ΔΔCt) method (17) and normalized to the internal reference gene GAPDH.

Cell culture. Human HCC cell lines (YY-8103, SNU-398, MHCC97-L, Hep3B, LM3 and Huh7) and 293T cells were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO2. The Huh7, LM3 and Hep3B cell lines have high invasive characteristics, whereas the YY-8103, SNU-398 and MHCC-97L cell lines have less invasive characteristics (18).

Cell transfection. A KDM6A coding sequence was constructed and inserted into the p23-3xflag-GFP vector (Invitrogen; Thermo Fisher Scientific, Inc.) to generate a KDM6A overexpression vector. The empty p23 vector was used as the negative control. Lentiviral short hairpin (sh)RNAs targeting KDM6A were designed and constructed into pLKO.1-TRC vectors (Shanghai GenePharma Co., Ltd.). A non-targeting scrambled (SCR) oligonucleotide (Shanghai GenePharma Co., Ltd.) was constructed into pLKO.1-TRC vectors served as the negative control. The sequences of the shRNAs are provided in Table I.

To produce lentiviral particles for KDM6A overexpression and knockdown, the core plasmid (1.5 µg) was co-transfected with the packaging plasmids 3.0 µg pMD2.G and 3.0 µg psPAX2 (Shanghai GenePharma Co., Ltd.) into 293 T cells (5x10^6 cells per well) using a calcium phosphate co-precipitation method in six-well plates (19). At 12 h post-transfection, the medium was refreshed. At 24 and 48 h post-transfection, the cell culture supernatants containing the virus were collected and filtered through a 0.45 µm membrane. Subsequently, the virus was concentrated by centrifugation at 50,000 x g for 140 min at 4°C. The pellet was resuspended in DMEM solution, aliquoted and stored at -80°C until further use. For the transduction process, Huh7 and LM3 cells (1x10^6 per well) were grown to 60% confluence in 6-well plates and transduced with 40 µl viral supernatant (1x10^8 units per well) and 5 µg/ml polybrene (Hanheng Biological Technology Co., Ltd.) for 24 h at 37°C. The medium was refreshed and the cells were transferred to 10-cm dishes. After 2 days at 37°C, KDM6A-overexpression Huh7 and LM3 cells were screened by green fluorescence via flow cytometry using an IX-71 flow cytometer (Olympus Corporation). KDM6A-knockdown YY-8103 and SNU-398 cells were cultured and screened in medium containing 3 µg/ml puromycin (Hanheng Biological Technology Co., Ltd.) for 4 days at 37°C. Subsequently, individual puromycin-resistant colonies were isolated and used for subsequent experiments. Transfection efficiency was verified by western blotting.

Western blotting. Total protein was extracted from cells using RIPA Lysis Buffer (Thermo Fisher Scientific, Inc.) supplemented with phenylmethylsulfonyl fluoride (Thermo Fisher
Total cell lysates were centrifuged at 10,000 x g for 15 min at 4˚C, following which the supernatant was collected. Protein concentration of the supernatant was determined using Bradford reagent (Sigma-Aldrich; Merck KGaA). Protein (20 µg per lane) was separated via 10% SDS-PAGE and transferred onto nitrocellulose membranes (EMD Millipore). The membranes were blocked with 5% fat-free milk for 1 h at room temperature. Subsequently, the membranes were incubated at 4˚C overnight with the following primary antibodies: Anti-KDM6A (1:1,000; cat. no. orb333886; Biorbyt Ltd.), anti-transforming growth factor (TGF)-β (1:1,000; cat. no. 3711; Cell Signaling Technology, Inc.), anti-phosphorylated (p)-smad2 (1:1,000; cat. no. 18338; Cell Signaling Technology, Inc.), anti-Smad2 (1:1,000; cat. no. 12570-1-AP; ProteinTech Group), anti-GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.). The membranes were then incubated with secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology, Inc.). Signals were visualized using chemiluminescence reagents (BioRad Laboratories). The grey values of the bands were analyzed using Quantity One software (BioRad Laboratories). The expression levels of KDM6A and GAPDH were quantified and normalized to the corresponding control. The expression levels were calculated using the formula: % protein expression = (I / I0) x 100%, where I is the integrated density of the protein band and I0 is the integrated density of the control band.

**Table I. Primer and shRNA sequences.**

| Gene     | Sequence (5'→3')                           |
|----------|--------------------------------------------|
| KDM6A    | F: TTCCTCGGAAAGGTGCTATTCA                  |
|          | R: GAGGCTGGTTGCAGATTTCA                    |
| GAPDH    | F: ATGACCCTTATGGACCTCA                     |
|          | R: GAGATGATCACCCCTTTTGGCT                  |
| KDM6A-SCR| F: TTCCTGGAACTTCGAGGT                    |
|          | R: ACGTGACAGTTGGAGA                       |
| KDM6A-sh1| F: CCGGGGACATAGACTAAGGAATAAACTCAGTTATTTCTAGTATGTGCTT |
|          | R: AAATCAAAAAAGCACATAGACTAAGGAATAAACTCAGGTTATTATCCCTAGTCATGTC               |
| KDM6A-sh2| F: CCGGGGACACGAATTAAATGTTATTTACTCAGGTAATACCTAAATTCGAGGTCTTCTTTTGT         |
|          | R: AAATCAAAAAAGCACACGAATTAAATGTTATTTACTCAGGTAATACCTAAATTCGAGGTCTTCTTTTGT |

sh, short hairpin RNA; KDM6A, lysine demethylase 6A; SCR, scrambled.
Inc.), anti-p-smad4 (1:1,000; cat. no. 10231-8-AP; ProteinTech Group, Inc.), anti-smad4 (1:1,000; cat. no. 10231-1-AP; ProteinTech Group, Inc.), anti-proliferating cell nuclear antigen (PCNA; 1:1,000; cat. no. 10205-2-AP; ProteinTech Group, Inc.), anti-Ki67 (1:1,000; cat. no. 27309-1-AP; ProteinTech Group, Inc.), anti-GAPDH (1:1,000; cat. no. 10494-1-AP; ProteinTech Group, Inc.) and anti-Flag (1:4,000; cat. no. F7425; Sigma-Aldrich; Merck KGaA). Following primary incubation, the membranes were incubated with an anti-rabbit (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.) or anti-mouse (1:3,000; cat. no. 7076; Cell Signaling Technology, Inc.) horse-radish peroxidase-conjugated secondary antibody for 1 h at room temperature. The immunoreactive protein bands were visualized using an enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and the medium was changed every 3 days. After 2 weeks, the medium was removed and cells were fixed with 20% methanol at room temperature for 10 min. Subsequently, cells were stained with 0.5% crystal violet at room temperature for 10 min. Stained cells were washed to PBS and observed using a light microscope (magnification, x4). Subsequently, 1 ml glacial acetic acid was added to each well and the optical density of each well was measured at a wavelength of 600 nm using a microplate reader.

Crystal violet assay. Wild-type and transfected cells were seeded (1x10^3 cells/well) into 6-well plates. Cells were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and the medium was changed every 3 days. After 2 weeks, the medium was removed and cells were fixed with 20% methanol at room temperature for 10 min. Subsequently, cells were stained with 0.5% crystal violet at room temperature for 10 min. Stained cells were washed to PBS and observed using a light microscope (magnification, x4). Subsequently, 1 ml glacial acetic acid was added to each well and the optical density of each well was measured at a wavelength of 600 nm using a microplate reader.

MTT assay. Cells were seeded (1x10^3 cells/well) into 96-well plates in triplicate. After incubation for 1-7 days at 37°C, 20 µl MTT solution (5 mg/ml) was added to each well and incubated at 37°C for 4 h. Subsequently, the culture medium was removed, 200 µl DMSO was added and the plates were gently
agitated to dissolve the formazan crystals. The absorbance of each well was measured at a wavelength of 570 nm using an automatic microplate reader.

**In vivo tumorigenesis analysis.** A total of 8 male BALB/c nude mice (age, ~5 weeks; median weight, 20 g; weight range, 18-21 g) were obtained from Beijing Huafukang Bioscience Co., Ltd. Mice were housed with free access to regular chow diet and water under specific pathogen-free conditions in laboratory cages at 23±3˚C with 35±5% humidity and 12-h light/dark cycles. The mice divided into were divided into two groups (n=4 per group): i) Huh7 p23 negative control Vector and ii) Huh7 KDM6A. KDM6A overexpression vector- or p23 negative control vector-transfected Huh7 cells (1x10^6 cells in a total volume of 100 µl PBS) were then subcutaneously injected using a 1 ml injection syringe into the right flank of each mouse in their corresponding groups. Tumor size was measured on a weekly basis. Tumor volume was calculated according to the following formula: Tumor volume (mm$^3$)=0.5xlengthxwidth$^2$. At the end of the experiment (week 4), mice were sacrificed by cervical dislocation. The tumors were excised, photographed and weighed. The maximum tumor volume observed was 609 mm$^3$. All animal experiments were performed according to guidelines of the Institutional Animal Care and Use Committee of Gansu Provincial Hospital (20) and were approved by Ethics Committee of the Gansu Provincial Hospital.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism (version 5; GraphPad Software, Inc.). Data are presented as the mean ± SD. Comparisons between two groups were determined using the unpaired Student's t-test. Comparisons between multiple groups were determined using one-way ANOVA followed Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

**Results**

**KDM6A is downregulated in HCC tissues.** To explore the potential roles of KDM6A in HCC, the mRNA expression
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levels of KDM6A in 30 paired HCC and corresponding normal tissues were examined. KDM6A mRNA levels were significantly decreased in 25/30 (83%) HCC samples compared with the corresponding normal samples (Fig. 1A). IHC analysis indicated that KDM6A was primarily expressed in the cytoplasm of both normal and tumor tissues. Consistent with the RT-qPCR results, HCC tissues displayed a decreased staining intensity compared with the corresponding normal tissues in two patients (Fig. 1B), which indicated that KDM6A expression was decreased in HCC tissues compared with corresponding normal tissues. In addition, the expression of KDM6A in several HCC cell lines was assessed. KDM6A expression was notably higher in YY‑8103 and SNU‑398 cells compared with Huh7 and LM3 cells (Fig. 1C and D).

KDM6A overexpression inhibits HCC cell proliferation in vitro. Based on the aforementioned clinical data, it was hypothesized that KDM6A may influence HCC cell proliferation in vitro. To evaluate the biological function of KDM6A in HCC cells, Huh7 and LM3 cell lines were transfected with empty (control) or KDM6A overexpression vectors. Western blotting was performed to confirm the transfection efficiency of KDM6A overexpression in HCC cells (Fig. 2A).

The MTT assay results indicated that the absorbance values of KDM6A-overexpression Huh7 and LM3 cells were significantly lower compared with empty vector-transfected cells on day 7 (Fig. 2B and C). In addition, the results of the crystal violet assay indicated that KDM6A overexpression markedly inhibited Huh7 and LM3 cell proliferation compared with empty vector-transfected cells (Fig. 2D). Similarly, the absorbance values of KDM6A-overexpression Huh7 and LM3 cells were significantly lower compared with empty vector-transfected cells (Fig. 2E).

KDM6A knockdown promotes HCC cell proliferation in vitro. Using gene-specific shRNAs, KDM6A expression was successfully knocked down in YY‑8103 and SNU‑398 cells (Fig. 3A). Similar to the overexpression experiments, the effect of KDM6A knockdown on HCC cell proliferation was assessed. The results of the MTT assay indicated that the absorbance values of KDM6A-knockdown YY‑8103 and SNU‑398 cells were significantly higher compared with SCR-transfected cells on day 7 (Fig. 3B and C). Consistently, the crystal violet assay results indicated that KDM6A knockdown markedly promoted YY‑8103 and SNU‑398 cell proliferation compared with SCR-transfected cells (Fig. 3D). Furthermore, the absorbance values of crystal violet in KDM6A-knockdown YY‑8103 and SNU‑398 cells were significantly higher compared with SCR-transfected cells (Fig. 3E).

KDM6A overexpression suppresses HCC cell tumorigenesis in vivo. Based on the in vitro results, empty vector- and KDM6A overexpression vector-transfected Huh7 cells were subcutaneously injected into the right flank of nude mice. Subsequently, the tumors were excised and photographed. Consistent with the in vitro results, KDM6A overexpression notably suppressed tumor growth compared with the empty vector control group in the xenograft mouse model (Fig. 4A).
In particular, KDM6A overexpression tumors were lighter and grew at a reduced rate compared with empty vector control tumors (P<0.001; Fig. 4B and C). The results suggested that KDM6A may serve as a tumor suppressor during HCC cell tumorigenesis in vivo. In addition, the western blotting results indicated that the protein expression levels of TGF-β, p-smad2, Ki67 and PCNA were significantly decreased in KDM6A overexpression tumors compared with empty vector control tumors (Fig. 4D).

**KDM6A inhibits TGF-β/SMAD signaling in HCC cells.** To explore the molecular mechanism underlying the effects of KDM6A on HCC, p-smad2, total smad2, p-smad4, total smad4 and TGF-β protein expression levels were measured in KDM6A-overexpression and KDM6A-knockdown cells. The results indicated that the expression levels of TGF-β and p-Smad2 were significantly decreased in KDM6A-overexpression cells compared with empty vector control cells (Fig. 5A and B). By contrast, KDM6A-knockdown cells displayed significantly increased TGF-β and p-smad2 expression levels compared with SCR-transfected cells (Fig. 5C and D). However, there was no significant difference in the expression levels of p-smad4 in KDM6A-overexpression or KDM6A-knockdown cells compared with the corresponding control cells.
addition, KDM6A overexpression significantly decreased the expression levels of proliferation markers, such as Ki67 and PCNA, compared with empty vector control cells. By contrast, KDM6A knockdown significantly increased the expression levels of Ki67 and PCNA compared with SCR-transfected cells (Fig. 5).

Discussion

A number of inactivating somatic mutations and copy number losses related to KDM6A have been identified in numerous malignancies, including pancreatic ductal adenocarcinoma, multiple myeloma, and esophageal and urethral cancer (11). KDM6A gene loss induces squamous-like metastatic pancreatic cancer, specifically in women, by deregulating the COMPASS-like complex (15). Furthermore, loss of KDM6A has been correlated with a poor prognostic subtype of human pancreatic cancer, where it serves a functional role as a tumor suppressor (13). KDM6A can also inhibit the invasion of HCT-116 colon cancer cells by upregulating E-cadherin expression, whereas KDM6A knockdown enhances epithelial-mesenchymal transition (EMT)-mediated stem cell properties in breast cancer cells (21,22). Moreover, Terashima et al (16) reported that KDM6A served an antagonistic role in TGF-β-induced lung cancer cell EMT and migration.

The present study indicated that KDM6A mRNA levels were significantly downregulated in HCC tissues compared with corresponding control tissues. Western blotting was performed to verify the transfection efficiency of KDM6A overexpression and knockdown in HCC cell lines. HCC cell proliferation was significantly inhibited by KDM6A overexpression compared with empty vector-transfected cells. Conversely, KDM6A knockdown promoted HCC cell proliferation compared with SCR-transfected cells. Furthermore, KDM6A overexpression also suppressed HCC cell tumorigenesis in vivo. Further mechanistic studies indicated that the expression levels of TGF-β and p-smad2 were significantly decreased by KDM6A overexpression compared with empty vector-transfected cells. By contrast, KDM6A knockdown increased TGF-β and p-smad2 expression levels, which are related to tumor progression (23), compared with SCR-transfected cells. Therefore, the present study indicated that KDM6A may inhibit HCC cell proliferation by negatively regulating TGF-β/SMAD signaling. TGF-β can influence gene expression by interacting with SMAD protein transcription factors, such as smad2, smad3 and smad4, to induce heterodimer formation (24,25). TGF-β signaling serves a complex role during the development and progression of various diseases (26). Specifically, activation of the TGF-β signaling pathway has been associated with increased growth and invasion of malignant cells during the late stages of tumor progression (27-29). To the best of our knowledge, the present study was the first study to indicate that KDM6A may be involved in HCC cell proliferation.

In summary, the present study demonstrated that KDM6A may serve a critical role in HCC cell proliferation. The results provided a mechanistic understanding of the role of KDM6A during tumor development, suggesting that KDM6A may serve as a potential target for the diagnosis or treatment of HCC.

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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

YL and JY conducted the experiments. XZ and HL provided the clinical samples. XZ, HL and JG analyzed the data. YL, XZ, HL and JG drafted and revised the manuscript. JG designed and supervised the study. 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 Gansu Provincial Hospital (approval no. KY-20190089; Lanzhou, China). Written informed consent was obtained from all subjects and the study was conducted in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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