Hepatic stellate cell mediates transcription of TNFSF14 in hepatocellular carcinoma cells via H₂S/CSE-JNK/JunB signaling pathway

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INTRODUCTION

Nowadays hepatocellular carcinoma (HCC) is the second most lethal cancer with persistently increasing mortality in the worldwide due to its highly complex and heterogeneous genetic aberrations [1, 2]. More and more researches suggest that tumor microenvironment (TME) plays an important role in the processes of hepatocarcinogenesis, epithelial-mesenchymal transition (EMT), invasion, and metastasis [3]. TME comprises various cells, including hepatic stellate cells (HSC), liver stromal endothelial cells (LSEC), immune cells and cytokines like extracellular matrix (ECM) [4].

HSC are usually considered to foster the tumor microenvironment formation and contribute to a malignant tumor phenotype. Activated HSC contribute to hepatocarcinogenesis through the secretion of growth factors, and producing ECM and matrix metalloproteinase (MMPs) [5–7]. But, there are more and more studies implicated that HSC could play an anti-tumor role in HCC progression [8, 9]. Hydrogen sulfide (H₂S) is the most recently-discovered gas signal molecule, participated in regulating cell proliferation, apoptosis, oxidative stress, inflammation and angiogenesis [10]. H₂S is mainly produced and cleared in the liver tissues by three enzymes: cystathionine-gamma-lyase (CSE), cystathionine-beta-synthase (CBS), and 3-mercapto-pyruvate sulfo transferase (MPST) [11]. Increasing evidence shows that H₂S affect HCC through the regulation of cell proliferation [12], migration and invasion [13], angiogenesis and immune responses [14]. Intriguingly, our previous study found that exogenous H₂S inhibit HCC progression through the PI3K/Akt/mTOR signaling pathway in vitro [15]. Our previous data found that activated HSC can produce H₂S mainly regulated by CSE. Nowadays, the mechanisms involved in the capacity of HSC released H₂S to affect the HCC have not been worked out, all evidence up to this point suggests these mechanisms have great potential importance.

In this study, we investigate how activated HSC in the HCC microenvironment influence HCC fate. We found that activated HSC released H₂S to induce HCC cell apoptosis. H₂S induced cancer cell apoptosis through activating JNK/JunB signaling, which upregulated tumor necrosis factor superfamily member 14 (TNFSF14) expression.

MATERIALS AND METHODS

Patients

A total of 45 patients with HCC who received hepatectomy were enrolled from Beijing You’an Hospital, affiliated to Capital Medical
Table 1. Sequences of oligonucleotides used for RT-qPCR, ChIP-qPCR, and RNA interference assays.

| Primers for RT-qPCR | Sequences of oligonucleotides |
|---------------------|-----------------------------|
| TNFSF14             | F: 5′-CGTGAAGCTCTACCTTATAT-3′<br>  R: 5′-CCCTCAAGTGTGTTGATATG-3′ |
| CSE                 | F: 5′-AAGACGGCTTCCTCAAGGTT-3′<br> R: 5′-ATATTCCAAACCCCGGATCTGG-3′ |
| GAPDH               | F: 5′-TGAGATGTCGAAGCACGGA-3′<br> R: 5′-CTGGAGAAGCTGATGGGAT-3′ |

| Primers for ChIP-qPCR | Sequences of oligonucleotides |
|-----------------------|-----------------------------|
| TNFSF14               | F: 5′-GACAGACGGCAACUCCCAUACATT-3′<br> R: 5′-UGAAGGGGAUGUGCGUCUGUTT-3′ |
| Scrambled siRNA       | F: 5′-ACGUGACACUCCGGAATT-3′<br> R: 5′-ACGUGACACUCCGGAATT-3′ |

Table 2. Correlation between TNFSF14 expression with clinicopathological characteristics of HCC.

| Feature                        | Low expression of TNFSF14 (n = 22) | High expression of TNFSF14 (n = 23) | P-value |
|-------------------------------|-----------------------------------|-------------------------------------|---------|
| Gender                        | Male                               | 20                                  | 18                  | 0.4140  |
|                               | Female                             | 2                                   | 5                  |         |
| Age (years)                   | ≤50                                | 14                                  | 10                 | 0.2362  |
|                               | >50                                | 8                                   | 13                 |         |
| HBsAg                         | Positive                           | 20                                  | 18                 | 0.4140  |
|                               | Negative                           | 2                                   | 5                  |         |
| Tumor size (cm)               | ≤3                                 | 7                                   | 10                 | 0.5420  |
|                               | >3                                 | 3                                   | 13                 |         |
| Tumor number                  | Single                             | 17                                  | 20                 | 0.4591  |
|                               | Multiple                            | 5                                   | 3                  |         |
| Portal invasion               | No                                 | 10                                  | 20                 | 0.0045  |
|                               | Yes                                | 12                                  | 3                  |         |
| Serum AFP (ug/L)              | ≤20                                | 10                                  | 11                 | >0.9999 |
|                               | >20                                | 12                                  | 12                 |         |
| BCLC HCC stage                | A                                  | 3                                   | 11                 | 0.0011a |
|                               | B                                  | 5                                   | 9                  | 0.019a  |
|                               | C                                  | 14                                  | 3                  |         |

*Compared with BCLC stage A. C. AFP α-fetoprotein, HBsAg Hepatitis B surface antigen, BCLC Barcelona Clinic Liver Cancer, HCC Hepatocellular carcinoma.

Cells

PLC/PRF/5 (ATCC® Cat. No. CRL-8024TM), HepG2 (ATCC® Cat. No. HB-8065TM) and LX-2 cells were cultured in DMEM medium supplemented with 10% FBS in a 37 °C incubator with 5% CO₂. The Transwell insert system was used for co-culture experiments to analyze the effect of LX-2 cells on HCC cells as previously described [37].

CCK-8

Cells proliferation was examined by a Cell Count Kit-8 assay (CCK8, AbMole, Houston, TX, America) according to the protocol. 2 × 10³ cells with different treatments were pipetted into 96-well microplates for measurements. The absorbance values were measured under a wavelength of 450 nm on a Universal Microplate Reader (ELx800; Bio-Tek Instruments Inc, Winooski, VT, USA). The percentage of cell viability was calculated as: Cell proliferation = [(A450sample-background)/ (A 450control-background)] × 100%.

Flow cytometry

Cell apoptosis was measured by FCM analysis using Annexin V-FITC/PI Apoptosis Detection Kit (40302ES20, Yeasen, Shanghai, China). Briefly, cells were collected and incubated with 5 µL Annexin V-FITC and 10 µL PI for 15 min at room temperature. Adjusted the volume to 500 µL with binding buffer. The apoptosis of HCC cells was analyzed with a FACScan-420 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

ELISA

The concentrations of H₂S were examined by a Hydrogen sulfide Assay Kit (JEB-11780, Jin Yibai Biological Technology, Nanjing, China) according to the instruction.

RNA sequencing

RNA sequencing analysis was conducted by Novogene (Beijing, China). In brief, total RNA was isolated by TRIzol reagent (15596026, Thermo Fisher Scientific, Waltham, MA, USA). The RNA quantification and qualification were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Sequencing of the libraries was carried out using the Illumina NovaSeq 6000 platform (Novogene, Beijing, China). All the raw data were uploaded on the Gene Expression Omnibus (GSE193660).

Quantitative real-time reverse transcription PCR (RT-qPCR)

Total RNA was isolated by TRIzol reagent and subsequently subjected to reverse transcription (4368814, High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher Scientific, Waltham, MA, USA). RT-qPCR was performed using the TB Green (RR420A, Takara) on an ABI ViiA7 (GX-XVI R2, Applied Biosystems Life Tech, USA). The relative mRNA expression levels were calculated with 2^−ΔΔCt method and presented as mean fold-change of samples to control. The primers used in the study were listed in the Table 1.

Western blotting

Cells or tissues were lysed in ice-cold lysis buffer. Total protein from cells or tissues was extracted using RIPA lysis buffer (R0010, Solarbio, Beijing, China). Forty µg of each protein were utilized for Western blotting. Membranes were blocked by TBST with 5% nonfat milk (w/v) for 1 h at room temperature. Incubated the membranes with primary antibodies at 4 °C for overnight. Next day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h following washing with TBST. The immuno-complexes were incubated with the enhanced chemiluminescent system (34580 Thermo Scientific, Waltham, MA, USA) and were subsequently exposed by a BioRad Chemidoc XRS+ imaging system (USA). Primary antibodies against JNK(#9252), phospho-JNK (#4668), JunB (#3753), and HDAC1 (#34589) were purchased from Cell Signaling Technology (CST, USA).
TNFSF14 antibody (#AF0329) was purchased from Affinity Biosciences (USA). Horseradish peroxidase-conjugated goat anti-mouse (#7076) and anti-rabbit (#7074) IgG secondary antibodies were purchased from CST. Full and uncropped western blots were uploaded as ‘Supplemental Material’.

Chromatin immunoprecipitation assay

The Chromatin immunoprecipitations (ChIP) assay was carried out according to a ChIP assay kit (#56383 CST). Cells were lysed with TNE buffer. Lysates were collected by centrifugation (15,000 g × 10 min) at 4 °C. Aliquots were incubated with Protein G-Sepharose beads (GE Healthcare) for 1 h. After centrifugation (1000 g × 5 min), the supernatants were incubated with phospho-JunB (#8053 CST) and IgG(#2729 CST) antibodies overnight at 4 °C. Next day, added Protein G-Sepharose beads for 8 h incubation. Then eluted the precipitate with the sample buffer. Finally, the immunoprecipitated chromatin was analyzed by PCR using primers targeting the human TNFSF14 promoter containing JunB binding sites. The primers used for ChIP-PCR are listed in Table 1.

Immunofluorescence staining

Cells were fixed in ice-cold 4% paraformaldehyde solution. Then incubated the cells with primary antibody (p-JunB) for overnight at 4 °C in the wet box. Next day, the cells were washed and subsequently incubated with the secondary antibody conjugated with Alexa Fluor 488 for 1 h at room temperature. Cell nuclei were counterstained with DAPI. Cells were viewed and captured with a fluorescence microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan).

Transient transfection

The small interfering RNAs (siRNAs) technique and TNFSF14-expressing plasmid were used to evaluate the effect of TNFSF14 on H2S-induced HCC cell apoptosis. The siRNAs sequences were listed in the Table 1. Briefly, HCC cells
were seeded into the 6-well plates and cultured to the 80% confluence.

SiRNAs and the plasmids were transfected to HCC cells according to the instruction of Lipofectamine™ 3000 transfection reagent (L3000-015, Thermo Fisher Scientific, Waltham, MA, USA). After 24 h of transfection, the expression of TNFSF14 was evaluated by RT-qPCR and Western blotting.

Data analysis of public databases

The GSE14520 gene expression dataset was downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo). According to the annotation information in the platform, the probes were converted into the gene symbols. We divided the examined HCC patients into TNFSF14 high and low groups based on the median of the gene expression.

Statistical analysis

Each experiment was repeated at least three times independently. All data were presented as mean ± S.D. performed using the SPSS software (version 23.0). Differences between unpaired groups were compared by the Student’s t-test or Mann–Whitney U test. For paired groups, the Wilcoxon
signed-rank test was used. Parametric or nonparametric multiple comparisons were performed using ANOVA-test or Kruskal–Wallis test. Pearson’s χ² test or Fisher’s exact test was used to analyze categorical data. Correlations were assessed by the Spearman correlation. Survival analyses were performed by the Kaplan–Meier and the log rank test. P < 0.05 was considered significant.

RESULTS
Activated hepatic stellate cells induce apoptosis of cancer cells
To examine how hepatic stellate cells (HSC) impact HCC cell behavior, we performed co-culture with activated HSC cell line LX-2 cells and HCC cell lines, HepG2 or PLC/PRF/5 cells. CCK-8 assays showed that cell viability of HCC cells was significantly reduced over time when the cells were co-cultured with LX-2 cells (Fig. 1A, C). Flow cytometry analyses further demonstrated that co-culture with LX-2 cells induced apoptosis of HepG2 and PLC/PRF/5 cells. The maximal apoptosis of these cancer cells was observed at 72 h following co-culture (Fig. 1B, D). These results suggest that activated HSC not only inhibit HCC cell proliferation, but also induce apoptosis of cancer cells.

LX-2 cells induce apoptosis of HCC cells through releasing H2S
How do activated HSC induce apoptosis of HCC cells? We hypothesized that H2S is a crucial mediator in the process given that activated HSC release H2S [16] and H2S is capable of inducing cancer cell apoptosis [15]. ELISA assay confirmed that LX-2 cells increased H2S release over time (Fig. 2A). LX-2 cells released the same amount of H2S at 72 h as 10⁻³ M NaHS did at 24 h (Fig. 2B).

Subsequently, we observed the effects of NaHS, a donor of H2S, and DL-Propargylglycine (PPG), a CSE inhibitor, on LX-2 cell-dependent HCC cell apoptosis. As shown in Fig. S1, NaHS increased H2S release, whereas PPG dose-dependently inhibited mRNA and protein expression of CSE as well as H2S release in LX-2 cells. Flow cytometry analyses showed that NaHS resulted in HCC cell apoptosis (Fig. 2C, D). LX-2-dependent cancer cell apoptosis was significantly reduced by the administration of PPG (Fig. 2C, D). PPG alone did not affect HCC apoptosis (Fig. 2C, D). These results suggest that activated HSC result in HCC cell apoptosis through releasing H2S.

H2S upregulates pro-apoptotic factor TNFSF14 expression in HepG2 cells
To clarify how H2S results in HCC cell apoptosis, we performed RNA-seq in HepG2 cells, which were co-cultured with LX-2 cells or treated with NaHS. A heat-map shows different transcriptome clusters in cells with different administrations (Fig. 3A). LX-2 co-culture and NaHS treatment respectively led to 1047 and 1183 gene upregulation as well 997 and 1144 gene downregulation in HepG2 cells. Among these altered genes, 659 up-regulated genes and 342 down-regulated genes were presented in HepG2 cells treated with either LX-2 co-culture or NaHS (Fig. 3B). Based on the fold change, 30 genes with maximal fold changes were selected to further analyze (Fig. S2). Among these 30 genes, we identified a pro-apoptotic gene TNFSF14 (Fig. 3C). We examined TNFSF14 mRNA and protein in HepG2 and PLC/PRF/5 cells treated with different concentrations of NaHS. NaHS dose-dependently increased expression of TNFSF14 and reached a maximum at 10⁻³ M treated 24 h in both HCC cells (Fig. 3D–G). Furthermore, we observed that TNFSF14 expression was up-regulated when HCC cells were co-cultured with LX-2 cells (Fig. 3H, I). The effect was inhibited by PPG administration (Fig. 3H, I). These results imply that TNFSF14 might be the key factor mediating H2S-induced HCC apoptosis.

TNFSF14 is indispensable for H2S-induced HCC cell apoptosis
To confirm the role of TNFSF14 in H2S-induced HCC cell apoptosis, we knocked down TNFSF14 expression in both HepG2 and PLC/PRF/5. RT-qPCR and Western blotting analyses confirmed that...
NaHS and LX-2 co-culture-induced TNFSF14 mRNA and protein expression was inhibited by siRNA-TNFSF14 in both HepG2 (Fig. 4A, B) and PLC/PRF/5 cells (Fig. 4D, E). Flow cytometric analyses further showed that NaHS and LX-2 co-culture-induced apoptosis of cancer cells was significantly inhibited when TNFSF14 expression was knocked down (Fig. 4C, F). On the other hand, the effect of TNFSF14 was also confirmed through transfection of the tnf14 gene into HCC cells, which showed that transfection with pcDNA3.1-tnf14 results in apparent increase of apoptosis in HepG2 and PLC/PRF/5 cells with different treatments (Fig. S3).
These results suggest that TNFSF14 expression is required for H₂S-induced HCC cell apoptosis.

**H₂S regulates TNFSF14 transcription through JNK/JunB signal**

To clarify how H₂S regulates the expression of TNFSF14, we analyzed PROMO dataset and adopted JASPAR software to predict potential transcript factors binding to the TNFSF14 promoter. JunB got the highest scored among all the analyzed factors (Fig. 5A upper panel) and possessed multiple binding sites on the TNFSF14 promoter (Fig. 5A lower panel). CHIP assays confirmed the binding of p-JunB to the TNFSF14 promoter in both HepG2 and PLC/PRF/5 cells (Fig. 5B). The binding was increased by NaHS treatment (Fig. 5B). Immunofluorescence staining and Western blotting showed that administration of NaHS remarkably induced p-JunB expression and nuclear translocation in both HCC cell lines (Fig. 5C–D). Besides NaHS, LX-2 cell co-culture also increased p-JunB expression and nuclear translocation (Fig. 5E). JunB phosphorylation and nuclear translocation required upstream p-JNK. Administration of SP600125, a p-JNK inhibitor, completely inhibited NaHS- or LX-2 cell co-culture-induced p-JunB expression and nuclear translocation in both cancer cells (Fig. 5F). CSE gene mRNA expressions in tumor tissues compared with surrounding non-tumor tissues (Fig. 6A). Western blottings analyses performed in 6 patients confirmed the results (Fig. 6B). CSE mRNA expressions in tumor tissues were positively correlated with that of TNFSF14 (r = 0.438, P < 0.05, Fig. 6C). We further investigated the association between TNFSF14 expression and tumor characteristics. Patients with high TNFSF14 expression in HCC showed less portal invasion, lower Barcelona Clinic Liver Cancer (BCLC) HCC stages and longer survival and disease-free time than those with low TNFSF14 expression (Table 2 and Fig. 6D, E). These results were similar as the data in the GSE 14520: TNFSF14 and CSE gene expression were significantly downregulated in tumor tissues compared with surrounding non-tumor tissues (Fig. 6A). Western blotting analyses performed in 6 patients confirmed the results (Fig. 6B). These results were similar as the data in the GSE 14520: TNFSF14 and CSE gene expression were significantly downregulated in tumor tissues compared with surrounding non-tumor tissues (Fig. 5A). CSE gene expressions in HCC tissues were positively associated with TNFSF14 expression (r = 0.168, P < 0.05, Fig. 6A). In addition, patients with high TNFSF14 expression showed less alpha-fetoprotein (AFP) levels, lower ALT, lower BCLC HCC stages, and lower metastasis risk than those with low TNFSF14 expression (P < 0.05, Fig. S4). The patients with high TNFSF14 expression had longer survival and disease-free time (Fig. S4).

**DISCUSSION**

HSC, as the central modulators of the tumor microenvironment, have long been considered as a tumor promoter during hepatocarcinogenesis by the secretion of growth factors such as hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF) and connective tissue growth factor (CTGF), altering components of the ECM, regulating angiogenesis and immune surveillance [17–21]. In stark contrast, HSC are a source of transforming growth factor beta (TGF-β), which exert tumor-suppressing activity by inducing cytostasis and apoptosis of hepatocytes in early tumor phases [8]. Recently, HSC were reported to be negative regulators of HCC progression through upregulating endothelin which is capable of inhibiting tumor-promoting cytokines, including IFG2, RBP4, DKK1, and CCL5 [9]. The findings of our study shed further insight into the increasing appreciation that HSC do not just act as pro-tumorigenic role, but may inhibit tumor progression. In our study, we found that HSC cells co-cultured with HepG2 and PLC/PRF/5 cells could promote cell apoptosis which was time-dependently manner. Further research found that HSC cells through releasing H₂S induced HCC cells apoptosis, which was relieved by adding PPG (CSE inhibitor) to reduce the amount of H₂S.

As a gas signaling molecule, the role of H₂S in HCC is still unclear. The previous reports shown that in the HepG2 and PLC/PRF/5 cells, endogenous H₂S/CSE pathway regulated cell growth mainly through EGFR pathway [12]. Another similar study found that in the HepG2 cells, H₂S/CSE pathway was activated after irradiated, which led to the long-term cell invasion and tumor metastasis [22]. The function of exogenous H₂S has also been studied in HCC cells. In the PLC/PRF/5 cells, 500 μM NaHS could induce cell proliferation, migration, and anti-apoptotic via activating NF-kB pathway [13]. Other researchers found that exogenous H₂S effectively restricted the tumor development in H22 HCC-bearing mice and HCC cell lines proliferation by blocking STAT3 and NF-kB pathways [14, 23]. Similar with our previous study, 10⁻³ M NaHS treated HepG2 and HLE cells could inhibit cells proliferation, migration and anti-apoptotic via mTOR signaling pathway [15]. In this study, our results showed that both endogenous and exogenous H₂S promoted HCC cells apoptosis, and the apoptosis was reduced when endogenous H₂S was inhibited by adding PPG.

How do H₂S regulate HCC cells apoptosis? Based on the RNA-sequencing results, we found that both endogenous and exogenous H₂S could up-regulate TNFSF14 in HepG2 cell. TNFSF14, mainly expressed on activated T cells, is activated in Natural Killer (NK) cells, and immature dendritic cells (DC) [24]. Previous studies reported that TNFSF14 act as an immunomodulatory factor to induce tumor regressions and apoptosis through enhancing T cells proliferation, differentiation, and cytokines secretion [25–27]. In human epithelial cancer cell lines, TNFSF14 promotes apoptosis and inhibits proliferation, which effect is independent of T cells [28–31]. However, there are few reports about the effect of TNFSF14 in HCC. In our study, we found that H₂S induced HCC apoptosis partly due to the TNFSF14 gene. TNFSF14 mRNA and protein expression levels were upregulated in HepG2 and PLC/PRF/5 cells under H₂S treatment. Knockdown of TNFSF14 reduced H₂S-induced HCC apoptosis. To figure out the precise molecular mechanism of H₂S regulating the transcription of TNFSF14 gene, the transcription...
The regulatory region of TNFSF14 gene was analyzed on the JASPAR website. We found transcription factor JunB binds to the TNFSF14 gene. The transcriptional activity of JunB is enhanced through phosphorylation-JNK [32]. In HCC cells, JNK/JunB exerts anti-tumor effects by promoting tumor cell apoptosis and inhibiting cell growth [33]. In this study, we found that H₂S regulated TNFSF14 transcription mainly through activating JNK/JunB singal in both HepG2 and PLC/PRF/5 cells.

All these in vitro results demonstrated that H₂S, released by LX-2 cells, participated in the JNK/JunB-TNFSF14 pathway to promote HCC cell apoptosis. Further validation was performed in the GEO dataset and our cohort, the results showed that patients with high CSE and TNFSF14 expression in HCC showed less portal invasion, dataset and our cohort, the results showed that patients with high expression levels in tumorous tissues (HCC; N = 45) and nontumorous tissues (NT; N = 45). B Western blotting analysis for the expression of CSE and TNFSF14 in the tumorous and nontumorous tissues in 6 patients with HCC. C Correlation between CSE mRNA expression levels and TNFSF14 mRNA expression levels in tumorous tissues (N = 45). D, E Overall survival and disease-free survival curves showed that patients with high expression levels of TNFSF14 have better prognosis. All Bars, ± SD; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Fig. 6 TNFSF14 expression is associated with the clinical outcome of HCC. A TNFSF14 mRNA and CSE mRNA expression levels in tumorous tissues (HCC; N = 45) and nontumorous tissues (NT; N = 45). B Western blotting analysis for the expression of CSE and TNFSF14 in the tumorous and nontumorous tissues in 6 patients with HCC. C Correlation between CSE mRNA expression levels and TNFSF14 mRNA expression levels in tumorous tissues (N = 45). D, E Overall survival and disease-free survival curves showed that patients with high expression levels of TNFSF14 have better prognosis. All Bars, ± SD; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

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ACKNOWLEDGEMENTS
This work was supported by the National Natural Science Foundation (81672725, 81970525), Beijing Natural Science Foundation(7212052), Beijing Hospitals Authority Youth Programme (QML20211701), Sino-German Cooperation Group (GZ1517), and Sino-German Mobility program (M-0200). The authors express their gratitude to the National Natural Science Foundation (81672725, 81970525), Beijing Natural Science Foundation(7212052), Beijing Hospitals Authority Youth Programme (QML20211701), Sino-German Cooperation Group (GZ1517), and Sino-German Mobility program (M-0200) for financial support.

AUTHOR CONTRIBUTIONS
The authors declare no competing interests.

ETHICS STATEMENT
The study protocol was approved by the Ethics Committee of Beijing You‘an Hospital affiliated to Capital Medical University. The informed consent was obtained from all enrolled patients.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41419-022-04678-z.

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