Effects of mdig on proliferation and apoptosis of lung cancer cells

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Abstract. Expression of mineral dust-induced gene (mdig) in lung cancer NCI-H1650 cells was detected to investigate the effects of mdig on proliferation and apoptosis of NCI-H1650 cells. NCI-H1650 lung cancer cells were cultured *in vitro*. The expression of mdig in NCI-H1650 cells was lowered using ribonucleic acid interference (RNAi) technique. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis were used to detect the effects of mdig small interfering RNA (siRNA) on messenger RNA (mRNA) and the protein expression of mdig in lung cancer NCI-H1650 cells, respectively. The effect of mdig on the proliferation of NCI-H1650 cells was observed through 3-(4,5)-dimethylthiazol (-z-yl)-3,5-di-phenyl tetrazolium bromide (MTT) assay, and flow cytometry was used to detect the impact of mdig on cell cycle and apoptosis of NCI-H1650 cells. The influence of mdig on caspase-3 and poly (ADP-ribose) polymerase 1 (PARP1) proteins in NCI-H1650 cells were investigated via western blot analysis. The results of RT-qPCR and western blot analysis showed that mdig siRNA obviously inhibited the expression of mRNA and protein of mdig in NCI-H1650 cells. Results of the MTT assay showed mdig siRNA could significantly reduce the proliferation ability of NCI-H1650 cells. In addition cell cycle detection showed that mdig siRNA caused NCI-H1650 cell arrest at G1 phase. Apoptosis detection results indicated that mdig siRNA promoted apoptosis of NCI-H1650 cells. Western-blot analysis revealed that mdig siRNA upregulated the expression of cleaved caspase-3 and cleaved PARP1 proteins in NCI-H1650 cells. Mdig is highly expressed in lung cancer NCI-H1650 cells while mdig siRNA can inhibit proliferation of NCI-H1650 cells and accelerate apoptosis. The underlying mechanism may be related to inhibited cell cycle progression and upregulated expression of cleavage proteins (cleaved caspase-3 and cleaved PARP1).

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Introduction

Lung cancer is one of the most common malignant tumors, with morbidity and mortality ranking the first among those of malignancies, and has become Top 1 among malignant tumors in mankind (1). Studies of World Health Organization have shown that incidence and mortality rates of lung cancer are increasing annually worldwide from the middle of last century to now, and current death roll of patients due to lung cancer has far exceeded the total number of patients who die of breast cancer, prostate cancer and colorectal cancer (2,3).

Survival time of patients has been extended using surgical treatment, chemotherapy, radiotherapy, targeted therapy and other methods, but the 5-year survival rate in lung cancer patients is only 15% (4). Studies have found that main causes of the high incidence and mortality rates of lung cancer are changes in related genes and signal transduction pathways, such as increase of proto-oncogene expression, decrease of tumor suppressor gene expression and imbalance of intracellular signal transduction pathway, thereby improving tumor cell proliferation, invasion and metastasis abilities, so that tumor cells easily metastasize (5-8). Therefore, studies of tumor-associated genes and their signal transduction pathways in lung cancer cells are useful to identify the mechanisms of occurrence and development of lung cancer, and are of very important clinical significance for finding new drug targets and methods of treatment.

Mineral dust-induced gene (mdig) is a lung cancer-related gene that was first discovered in miners' alveolar macrophages in 2005 (9). Mdig, located on human chromosome 3q11.2, contains 1510 bases in full length. It is able to encode a protein consisting of 465 amino acids and has a molecular weight of 53 kDa (10). A slight expression of mdig was found in normal human tissue cells, but its expression is high in various tumor tissues and cell lines (11-14). A further study showed that mdig can promote tumor cell proliferation and block cell cycle progression (15).

There are few detailed reports on the effects of mdig on proliferation and apoptosis of lung cancer cells and mechanisms of action. Therefore, this study used ribonucleic acid interference (RNAi) technology to silence mdig expression in lung cancer NCI-H1650 cells and then adopted reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis to detect the impact of mdig small interfering RNA (siRNA) on the expression of messenger RNA (mRNA) and protein of mdig in NCI-H1650 cells. The effects of mdig silencing on proliferation, cycle distribution,

apoptosis and apoptosis-related proteins of NCI-H1650 cells were further studied.

Materials and methods

Materials. Materials used in the study were: NCI-H1650 human lung cancer cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China); Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (Hyclone, Logan, UT, USA); TRIzol kits and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA); bicinchoninic acid (BCA) protein quantification kits and cell lysis buffer (Beyotime Biotechnology, Nantong, China); reverse transcription kits, RT-qPCR kits, primer syntheses, mdig siRNA, and negative control siRNA (N-siRNA) (Takara, Dalian, China); mdig, cleaved caspase-3, cleaved poly (ADP-ribose) polymerase 1 (PARP1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibodies, and horseradish peroxidase (HRP)-labeled secondary antibodies (Proteintech Group, Inc., Wuhan, China); cycle detection kits and Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits (Beyotime Biotechnology, Nantong, China). The study was approved by the Ethics Committee of Peking Union Medical College Hospital (Beijing, China).

Cell culture and siRNA transfection. NCI-H1650 cells were cultured in DMEM containing 10% fetal bovine serum at 37°C in a 5% CO_2 incubator, the medium was changed every two days, and digestion and passage were performed after cells spread to 80% of the bottom of the culture bottle. Before transfection, NCI-H1650 cells in logarithmic phase were inoculated into a 6-well plate at a density of 2×10^5/well. After 24 h, siRNA transfection was conducted according to instructions of Lipofectamine 2000 for 48 h, with the siRNA sequence shown in Table I. This study included normal control group (control), negative control group (N-siRNA) and experimental group (mdig siRNA).

Effect of mdig siRNA on mRNA expression of mdig in NCI-H1650 cells detected by RT-qPCR. Cells in each group were transfected for 48 h and then collected to extract total RNA according to recommended methods of TRIzol kits. When the absorbance ratio [the absorbance at 260 and 280 nm (A260/280)] of samples was between 1.8 and 2.0, the next reverse transcription reaction was carried out. Then, PCR amplification was performed using the obtained complementary deoxyribonucleic acid (cDNA) as a template according to primer sequence shown in Table II. Specific reaction conditions were as follows: pre-denaturation at 95°C for 3 min, then 95°C for 15 sec, 62°C for 30 sec, 72°C for 30 sec, with 40 cycles in total. GADPH was used as the internal reference. The cycle threshold (Cq) value was output from the instrument, and experimental results were analyzed using the $2^{-\Delta\Delta Cq}$ method (16).

Impact of mdig siRNA on protein expression of mdig in NCI-H1650 cells via western blot analysis detection. Cells were collected from each group after 48 h of transfection, added with cell lysis buffer, and centrifuged at 3,000 x g for 10 min at 4°C to extract proteins. The concentration of the extracted protein was

Table I. siRNA sequences.

Name	Sequence name	siRNA sequence
mdig	Sense	5'-UUGUCCGAACGUGUCACGUTT-3'
siRNA	Antisense	5'-ACGUGACACGUUCGGAGAATT-3'
N-siRNA	Sense	5'-GGGCAACGAUUCAGUUUCATT-3'
	Antisense	5'-UGAAACUGAAUCGUUGCCCTT-3'

Table II. RT-qPCR primer sequences.

Gene	Primer name	Primer sequence
mdig	Forward	5'-GGCAACGATTCAGTTTCACCAA-3'
	Reverse	5'-TGTACACATTCGAGCCAACCAAG-3'
GADPH	Forward	5'-CCTGGTATGACAACGAATTTG-3'
	Reverse	5'-CAGTGAGGGTCTCTCTCTTCC-3'

measured by BCA protein concentration kits. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out, 40 μg of protein were loaded on each well, subjected to wet membrane transfer, blocked in 5% bovine serum albumin (BSA) solution, and incubated at 4°C with mdig and GAPDH antibodies (diluted at 1:1,000). After washing the membrane, proteins were added with HRP-labeled secondary antibodies dropwise and incubated at room temperature for 2 h, followed by membrane washing. Then, electrochemical luminescence (ECL) scotography was performed. A gel imager was used for scanning or photographing. Gray value was measured using Gel Pro 4.0 image analysis software (Media Cybernetics, Inc., Rockville, MD, USA).

Role of silenced mdig in proliferation of NCI-H1650 cells disclosed through 3-(4,5)-dimethylthiazol (-z-yl)-3,5-diphenyl tetrazolium bromide (MTT) assay. A single cell suspension was inoculated into a 96-well plate, with 5×10^3 cells in each well. Then, siRNA experiment was performed. After 48 h, 20 μl (5 μg/μl) MTT solution was added into each well and the plate was incubated for 4 h in the dark. After that, 100 μl dimethyl sulfoxide (DMSO) solution was added to each well and the plate was vibrated to dissolve purple crystals. A microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) was applied to measure absorbance [optical density (OD) value] at 490 nm, and the cell proliferation rate was calculated as: proliferation rate = (OD in mdig siRNA group/ OD in control group) x100%.

Influence of silenced mdig on NCI-H1650 cell cycle discovered via flow cytometry. Cells transfected for 48 h were collected from each group and fixed overnight at 4°C in pre-cooled 70% ethanol. The cells were re-suspended in 500 μl staining buffer, added with 25 μl propidium iodide (PI) (50 μg/ml) and 10 μl ribonuclease A (RNase A) (10 mg/ml), mixed evenly and incubated at 37°C for 30 min. Subsequently

Table III. Effect of mdig siRNA on cell cycle of NCI-H1650 cells.

| Group	Proportion of cells in different phases (%)		
	G1 phase	S phase	G2/M phase
Control	64.53±3.24	24.20±3.31	11.27±2.17
N-siRNA	66.38±2.95	22.07±3.52	11.55±1.98
mdig siRNA	81.45±4.33[a]	9.66±1.93[a]	8.89±1.13

In comparison with control group, [a]$p<0.01$.

Figure 1. Expression of mdig mRNA in NCI-H1650 cells detected via RT-qPCR. In comparison with control group, the mdig siRNA group has an obviously reduced expression of mdig mRNA in NCI-H1650 cells, [**]$p<0.01$.

the cells were washed with phosphate-buffered saline once. Cell cycle analysis was performed using a flow cytometer (Becton Dickinson and Company, Franklin Lakes, NJ, USA), and the proportion of cells in different phases was expressed as a percentage (%).

Effect of silenced mdig on NCI-H1650 cell apoptosis through flow cytometry and western blot analysis. Cells were collected from each group after 48 h of transfection, and then added with 0.3 ml binding buffer suspension cells. Each test sample was added with 5 μl Annexin V and 5 μl PI, incubated in the dark at room temperature for 15 min, and added with additional 0.2 ml binding buffer. A flow cytometer (Becton Dickinson and Company) was used to detect the apoptotic rate of cells in each group.

Cells were transfected for 48 h and then collected. Expression of cleaved caspase-3 and cleaved PARP1 proteins in cells in each group was detected as described earlier.

Statistical analysis. Statistical Product and Service Solutions (SPSS) 17.0 software (International Business Machines Corporation, Armonk, NY, USA) was used for data analysis. Data are expressed as mean ± standard deviation (SD). The t-test was employed for comparison among groups. P<0.05 suggested that the difference was statistically significant.

Results

Impact of mdig siRNA on mRNA expression of mdig in NCI-H1650 cells. After transfection for 48 h, the expression level of mdig mRNA in the mdig siRNA group was significantly lower than that in control group (p<0.01) (Fig. 1). However, there was no significant difference in the expression level of mdig mRNA between control group and N-siRNA group, suggesting that mdig siRNA can specifically interfere with the expression of mdig mRNA.

Effect of mdig siRNA on protein expression of mdig in NCI-H1650 cells. After 48 h of transfection, changes in mdig protein level were detected through western blotting. The results revealed that compared with that in control group, expression level of mdig protein in cells in mdig siRNA group was distinctly decreased (p<0.01) (Fig. 2). Changes in the expression levels of mdig protein in both control group and N-siRNA group were not significant, indicating that mdig
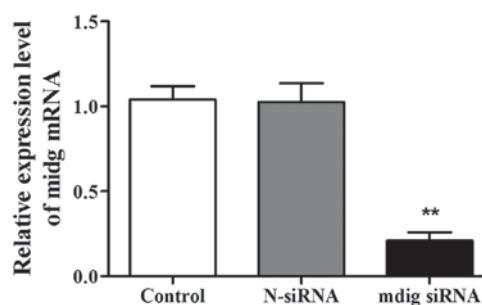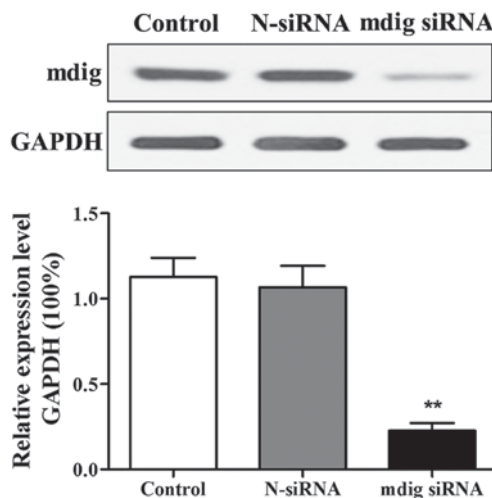

Figure 2. Expression of mdig protein in NCI-H1650 cells detected by western blot analysis. Expression level of mdig protein in NCI-H1650 cells is clearly decreased in mdig siRNA group vs. the control group, [**]$p<0.01$.
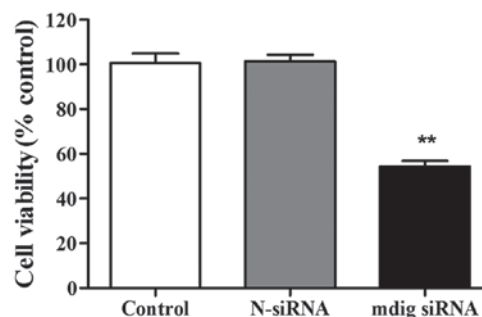

Figure 3. Viability of NCI-H1650 cells detected by MTT assay. Viability of NCI-H1650 cells in mdig siRNA group is overtly lower than that in control group, [**]$p<0.01$.

siRNA is able to specifically interfere with expression of mdig protein.

Role of mdig siRNA in of NCI-H1650 cell proliferation. The role of mdig siRNA in the proliferation ability of NCI-H1650 cells was detected through MTT assay. There was no evident difference in cell viability between control group and N-siRNA group. Cell viability was obviously inhibited in mdig siRNA group compared to control group (p<0.01) (Fig. 3).
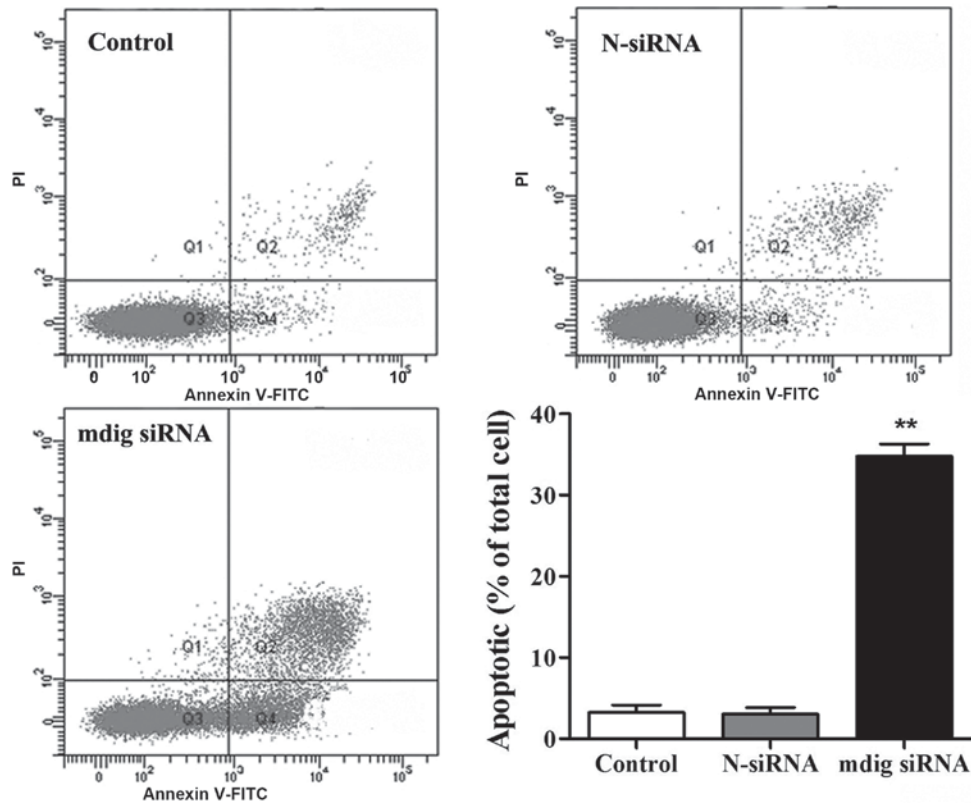
Figure 4. Detection of NCI-H1650 cell apoptosis via Annexin V-FITC flow cytometry. In comparison with control group, mdig siRNA group has a distinctly upward proportion of apoptotic cells, **p<0.01.
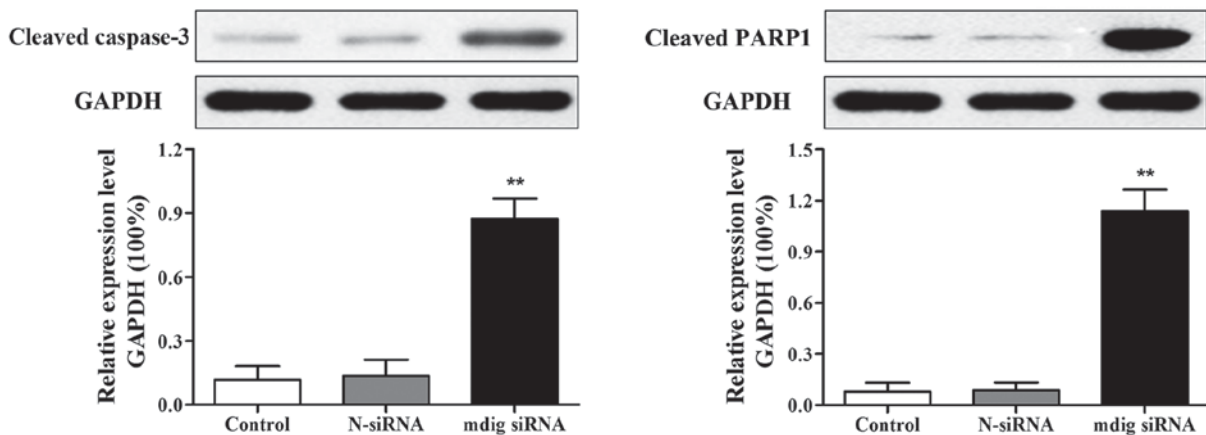

Figure 5. Measurement of expression of cleaved caspase-3 and cleaved PARP1 in NCI-H1650 cells using western blot analysis. Expression levels of proteins (cleaved caspase-3 and cleaved PARP1) in mdig siRNA group is significantly enhanced compared with those in control group, **p<0.01.

Effect of mdig siRNA on cell cycle of NCI-H1650 cells. Effect of mdig siRNA on cell cycle of NCI-H1650 cells was observed via flow cytometry. Compared to control group, the mdig siRNA group had significantly increased proportion of cells in G1 phase (p<0.01), and clearly decreased proportion of cells in S phase (p<0.01) (Table III). However, in G2 phase there were no obvious changes: proportion of cells in different phases was not changed evidently in either the control group or N-siRNA.

Influence of mdig siRNA on of NCI-H1650 cell apoptosis. Annexin V-FITC apoptosis detection kits were used for the detection of impact of mdig siRNA on apoptosis of NCI-H1650 cells. No obvious difference was found in apoptosis between the control and N-siRNA groups (Fig. 4). Compared to that in control group, the proportion of apoptotic cells in mdig siRNA group was overtly increased (p<0.01).

Effects of mdig siRNA on the expression of apoptotic proteins in NCI-H1650 cells. After transfection for 48 h, the expression of apoptotic proteins (cleaved caspase-3 and cleaved PARP1) was measured via western blot analysis, and the results revealed that expression levels of cleaved caspase-3 and cleaved PARP1 were obviously increased in mdig siRNA group than those in control group (p<0.01). No visible changes in expression levels

of cleaved caspase-3 and cleaved PARP1 were observed in control group or N-siRNA group (Fig. 5).

Discussion

Lung cancer is a malignancy with the highest morbidity and mortality worldwide (17,18). Research data have shown that the incidence of lung cancer is on the increase in China, and China may become the Top 1 in lung cancer morbidity and mortality worldwide if no effective preventive and control measures are taken (19). At present, deaths caused by lung cancer are more than that due to liver cancer and account for approximately 23% of the total deaths caused by malignancies making lung cancer the leading cause of death caused by malignant tumors (20). Therefore, lung cancer seriously affects life and health, and new breakthroughs in its clinical treatments and drugs must be made.

The mdig-encoded protein has a molecular weight of 53 kDa, and is mainly located in the nucleus. The protein contains a conserved Jumonji C (JmjC) domain that determines the function of mdig protein (21). A study found that mdig is highly expressed in lung cancer, esophageal cancer, gastric cancer and other tumors, and that mdig is a proto-oncogene (22). A study by Zhang *et al* revealed that mdig is a mineral dust-induced gene, and that the expression level of mdig mRNA is significantly enhanced when NCI-H1650 cells are treated with silica (23). In addition, it was found in the study by Komiya *et al* that mdig is a target gene of proto-oncogene c-myc that can induce overexpression of mdig (24). A study by Lu *et al* revealed that various lung cancer cell lines have high mdig expression, and in patients with lung cancer, expression of mdig mRNA is increased by more than 6 times in lung cancer tissues than that in normal tumor-adjacent tissues (25). Caspase-3 plays a key role in the process of apoptosis; when caspase-3 is cleaved and activated, cells enter an irreversible process of apoptosis (26). PARP1 is a kind of DNA repair enzyme in the nucleus, while cleaved caspase-3 can cleave and activate PARP1, leading to unrepaired cell DNA damage, thereby resulting in apoptosis (27).

In this study, mdig siRNA was used to transfect NCI-H1650 cells and silence mdig expression in the cell line. RT-qPCR and western blot analysis were used to detect mRNA and protein expression of mdig in NCI-H1650 cells. The results showed that mdig siRNA was able to significantly decrease expression of mRNA and protein of mdig in NCI-H1650 cells and the roles of mdig in proliferation, cell cycle and apoptosis of NCI-H1650 cells. Furthermore, we found that silenced mdig clearly reduced proliferation capabilities of NCI-H1650 cells and blocked NCI-H1650 cells in G1 phase. At the same time, this study employed flow cytometry and western blotting to observe the impact of silenced mdig on apoptosis of NCI-H1650 cells, and the results revealed that the apoptotic rate of NCI-H1650 cells in mdig siRNA group was obviously increased. Western blot analysis, revealed that, silenced mdig upregulated the expression of cleaved caspase-3 and cleaved PARP1 proteins in NCI-H1650 cells, thereby inducing apoptosis. It was found that in pancreatic cancer PANC-1 cells, apoptosis rate of cells was overtly enhanced when PANC-1 cells were transfected with mdig siRNA, suggesting that mdig is capable of inhibiting apoptosis in PANC-1 cells (28).

In conclusion, mdig has a high expression in lung cancer NCI-H1650 cells. In addition, mdig siRNA is able to inhibit proliferation and promote apoptosis of NCI-H1650 cells. The underlying mechanism may be linked to inhibited cell cycle progression and upward expression of apoptosis proteins (cleaved caspase-3 and cleaved PARP1).

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

XX and HL were responsible for PCR and western blot analysis. LC and YZ helped with cell culture and siRNA transfection. HL, ZS and YC contributed to MTT assay and flow cytometry. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Peking Union Medical College Hospital (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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