

## c-Met-targeted RNA interference inhibits growth and metastasis of glioma U251 cells in vitro

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**Abstract** Angiogenesis plays an essential role in tumor growth and metastasis and is a promising target for cancer therapy. c-Met, a receptor tyrosine kinase, and its ligand, hepatocyte growth factor (HGF), are critical in cellular proliferation, motility, invasion, and angiogenesis. The present study was designed to determine the role of c-Met in growth and metastasis of glioma U251 cells using RNA interference (RNAi) technology in vitro. We constructed three kinds of shRNA expression vectors aiming at the c-Met gene, then transfected them into glioma U251 cells by lipofectamine<sup>TM</sup> 2000. The level of c-Met mRNA was investigated by real-time polymerase chain reaction (RT-PCR). The protein expression of c-Met was observed by immunofluorescence staining and western blotting. U251 cell growth and adherence was detected by methyl thiazole tetrazolium assay. The apoptosis of U251 cells was examined with a flow cytometer. The adherence, invasion, and in vitro angiogenesis assays of U251 cells were done. We got three kinds of c-Met specific shRNA expression vectors which could efficiently inhibit the growth and metastasis of U251 cells and the expression of c-Met in U251 cells. RT-PCR, immunofluorescence staining and western blotting showed that inhibition rate for c-Met expression was up to 90%, 79% and 85%, respectively. The expression of c-Met can be inhibited by RNA interference in U251 cells, which can inhibit the growth and metastasis of U251 cell and induce cell apoptosis. These results

indicate that RNAi of c-Met can be an effective antiangiogenic strategy for glioma.

**Keywords** c-Met · Apoptosis · Adherence · Gene therapy · Glioma · RNA interference

### Abbreviations

HGF	Hepatocyte growth factor
c-Met	Hepatocyte growth factor receptor
RNAi	RNA interference
RPMI	Roswell park memorial institute
PBS	Phosphate-buffered saline
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
HRP	Horseshoe peroxidase
NC	Nitrocellulose
MTT	Methyl thiazole tetrazolium

### Introduction

A variety of growth factors such as vascular endothelial growth factor, epidermal growth factor, and transforming growth factor  $\alpha$  appear to play a crucial role in human carcinogenesis and angiogenesis. Recently, attention has been focused on the role of the hepatocyte growth factor (HGF)/receptor system because of its multifunctional properties such as cell proliferation, cell movement, and morphogenesis [1, 2]. The receptor for HGF is a protein product of a proto-oncogene, c-Met [3], which encodes a transmembrane tyrosine kinase (P<sup>190 c-Met</sup>) with structural and functional features of a growth factor receptor [4]. Autophosphorylation of this receptor by ligand binding stimulates its intrinsic tyrosine kinase activity with

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resultant changes in cellular morphology, motility, growth, invasion, and angiogenesis. Overexpression of this oncogene was shown in different human solid tumors [5, 6]. We have previously found HGF and its receptor c-Met played an important role in the formation, progression and angiogenesis of glioma and could promote tumor proliferation and intratumoral microvascular formation, and was closely related to the prognosis of the patients [7–11].

RNA interference (RNAi) is the sequence-specific, posttranscriptional gene silencing method initiated by double-stranded RNAs, which are homologous to the suppressed gene [12, 13]. Therefore, the present study was designed to determine the role of c-Met in growth and metastasis of glioma U251 cells using RNA interference (RNAi) technology in vitro.

## Materials and methods

### Cell culture, RNA interference and transfection

ECV304 cells and human glioma cells U251 (Wuhan University of China) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 µg/ml streptomycin, and 100-units/ml ampicillin. The cells were plated in 24- or 6-well plates at 50%–70% confluence 24 h prior to transfection. For experiments with U251 cells, c-Met short hairpin RNA (shRNA) sequences [14] were isolated from the parental pSuper vector by *EcoRI* and *XhoI* digestion and cloned into p(si)<sup>2</sup>-puro [15]. Cells were infected with retrovirus produced as described [15] and selected with puromycin (2 µg/ml). The sequence of met1, met2 and met3 is 5'-AGAATGTCATTCTACATGAGC-3' and 5'-ATGTGAACGCTACTTATGTGC-3', and 5'-ATCAGAACCAGAGGCTTGGTC-3', respectively. An irrelevant RNAi control plasmid was constructed for green fluorescent protein (GFP) gene, pShRNA-GFP. The sequence (5'-AGCTGACCCTGAAGTTCATCT-3') was designed to target the nucleotides 126–144 of the GFP coding region. Transfection of cells was carried out with Lipofectamine<sup>TM</sup> 2000 reagent (Invitrogen, Carlsbad, CA).

### Real-time polymerase chain reaction for c-Met

Total RNA was isolated from cultured cells and real-time polymerase chain reaction (RT-PCR) was performed using the RNeasy and one step RT-PCR kit from Qiagen Corp. RT-PCR of G3PDH, a housekeeping gene served as a control. The sequences used for primers are 5'-ACAGTGGCATGTCAACATCGCT-3' (sense) and 5'-GCTCGGTA GTCTACAGATTC-3' (antisense) for c-Met (656 bp) [16], 5'-ACCACAGTCCATGCCATCAC-3'(sense) and 5'-TCC

ACCACCCTGTTGCTG TA-3' (antisense) for G3PDH (1,000 bp) [17]. For RT-PCR, two pairs of primers were added into a reaction tube, the program consisted of an initial reverse transcription at 50°C for 30 min, denaturation at 95°C for 10 min, followed by 24 cycles of amplification (denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 68°C for 1 min) and a final extension at 68°C for 10 min. The products were then separated by electrophoresis on 1.5% agarose gel, the bands were visualized using UV light and analyzed by Genetools software.

### Immunofluorescence staining

Cells were harvested on d 2 post-transfection for analysis, washed once with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at 4°C. After blocked with goat serum, the cells were incubated with monoclonal mouse anti-c-Met for 2 h at 37°C. After three washes, the cells were incubated with Cy3-conjugated rabbit anti-mouse secondary antibodies for 1 h at 37°C and washed three times with PBS. The stained cells were mounted and analyzed under fluorescence microscope.

### Western blotting

Cells were harvested on d 3 post-transfection, washed twice with 10 ml of PBS, SDS buffer, boiled for 5 min, separated by 10% SDS-PAGE gel electrophoresis, transferred onto a nitrocellulose membrane, incubated with c-Met antibodies at a dilution of 1/400 and HRP-conjugated rabbit anti-mouse antibody at a dilution of 1/4000. The HRP substrate was observed on the NC membrane. After three washes, the NC membrane was incubated with actin antibody and HRP-conjugated second antibody. The HRP substrate was observed again.

### Measurement of cell growth

Cell proliferation was measured by the methyl thiazole tetrazolium (MTT) assay [18]. Cells were seeded in 24-well plates at a density of  $1 \times 10^4$  cells/well. After a 24-h incubation, 200 µl of 5 mg/µl solution of MTT (Sigma, Guangzhou, China) in PBS was added to each well. The plates were then incubated for 4 h at 37°C. The precipitate was then solubilized in 100% dimethylsulfoxide (Sigma), 100 µl/well, and shaken for 15 min. Absorbance was determined with an enzyme-linked immunosorbent assay reader (model318; Shanghai, China) at 540 nm. Each assay was performed nine times. The results were expressed as mean  $\pm$  SE of controls.

### Measurement of apoptosis by flow cytometry

In preparation of flow cytometry (FCM), U251 cells were centrifuged 72 h after transfection. The cells were washed with PBS and fixed in 70 ml/l cold ethanol. Samples were treated with RNase (10 g/l), resuspended, and stained with 10 g/l propidium iodide. After 30 min at room temperature in the dark, the cells were analyzed using a FCM scan flow cytometer. Apoptotic cells appeared in the cell cycle distribution as cells with DNA contents less than  $G_1$  cells, and the percentage of apoptotic cells was calculated.

### Adhesion assay

Cells were seeded in quadruplicate at a density of  $1 \times 10^4$  cells/well in 96-well plates coated with BSA (10 g/l), Matrigel (50 mg/l), or fibronectin (Fn) (10 mg/l). The cells were cultured at 37°C for 60 min, and the MTT assay was performed as above [18–21].

### Tumor cell adherence to ECV304

ECV304 cells were plated onto 96-well plates at a density of  $5 \times 10^4$  cells/well. After 48 h, the supernatant was aspirated and cells were plated at a density of  $5 \times 10^4$  cells/well. After 30 min, the wells were gently washed twice with PBS to remove unattached cells, and 100  $\mu$ l of rose bengal (25%) was added for 5 min. The supernatant was aspirated, the wells were gently washed twice with PBS, and finally 200  $\mu$ l 95% ethanol/PBS (1:1) was added. After 20 min, the absorbance at 540 nm was recorded.

### Invasion assay

The invasion assays with cells were performed using Transwell polycarbonate membrane inserts in 24-well plates (Corning, Lowell, MA) following the manufacturer's instructions. Briefly, the underside of each polycarbonate microporous membrane was coated with Matrigel (1:100) at 37°C for 5 min and allowed to sit overnight. Then, 50  $\mu$ l Matrigel (1:30) and 200  $\mu$ l sterile water were added to the upper compartment at 37°C. After 2 days, 200  $\mu$ l of the invasion buffer [2 ml BSA (2%) + 38 ml RPMI 1640] was added into the upper compartment and, 1 h later, the upper compartment fluid was aspirated. Cells at a density of  $5 \times 10^4$  cells/well were added into the upper compartment, and 800  $\mu$ l of the Fn solution (10  $\mu$ g/ml) was added into the lower compartment. The cells were allowed to migrate for 48 h. The inserts were then fixed in 10% formalin, stained with hematoxylin and eosin, and rinsed by dipping in water. The cells on the upper surface of the membrane were removed with a cotton bud. The membranes were air-dried overnight, excised from the insert, and mounted onto

glass slides for microscopic analysis. The migrated cells were counted at high-power magnification ( $\times 40$ ) from four randomly selected fields. Each experiment was repeated three times.

### In vitro angiogenesis assay

The test was performed using the In vitro Angiogenesis Assay Kit (Chemicon International, Temecula, CA) following the manufacturer's instructions. Briefly, 96-well plates were coated with cold solution (50  $\mu$ l/well of a solution containing 900  $\mu$ l of ECMatrix per 100  $\mu$ l of  $10 \times$  diluent buffer), which was allowed to polymerize at room temperature for about 60 min. Then, wells were seeded with 100  $\mu$ l of a  $5 \times 10^4$  cells/ml suspension of ECV304, ECV304 transiently transfected with pShRNA-GFP, or ECV304 transiently transfected with pShRNA-met1. Tube formation was assessed after 12 h.

## Results

### c-Met mRNA inhibition in U251 cells by RT-PCR

The inhibition rate of pShRNA-met1, pShRNA-met2 and pShRNA-met3 was 90%, 76% and 85% respectively in U251 cells compared with the control plasmid pShRNA-GFP (Fig. 1a, b).

### c-Met protein inhibition in U251 cells by immunofluorescence staining

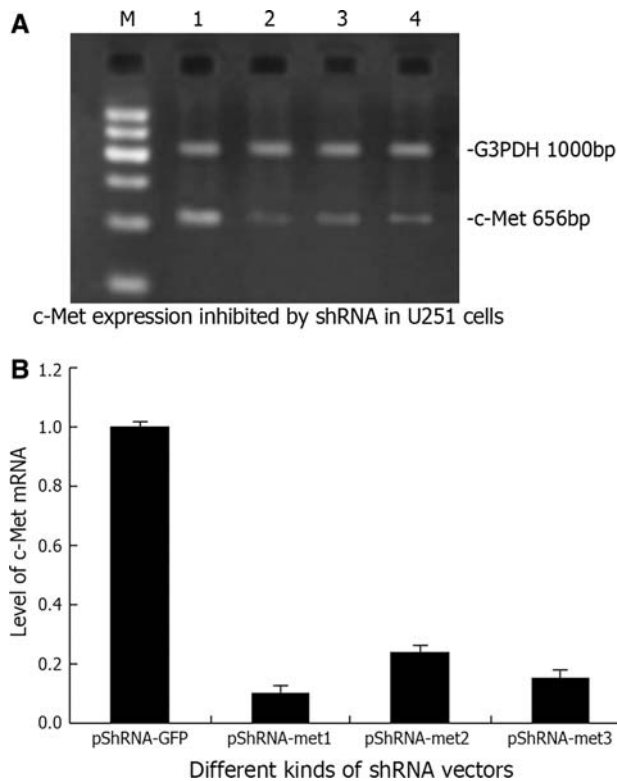
The inhibition rate of pShRNA-met1, pShRNA-met2 and pShRNA-met3 was 79%, 58% and 70% respectively in U251 cells compared with the control plasmid pShRNA-GFP (Fig. 2a, b). c-Met was stained red and located in plasma of cells.

### c-Met protein inhibition in U251 cells by western blotting

The inhibition rate of pShRNA-met1, pShRNA-met2 and pShRNA-met3 was 85%, 61% and 78% respectively in U251 cells compared with the control plasmid pShRNA-GFP (Fig. 3a, b).

### Cell proliferation assay

As shown in Fig. 4, pShRNA-met1, pShRNA-met2 and pShRNA-met3 caused a statistically significant reduction of cell viability to 16.5%, 38.4% and 28.6% ( $P < 0.05$ ), respectively, whereas pShRNA-GFP had not such change ( $P > 0.05$ ).



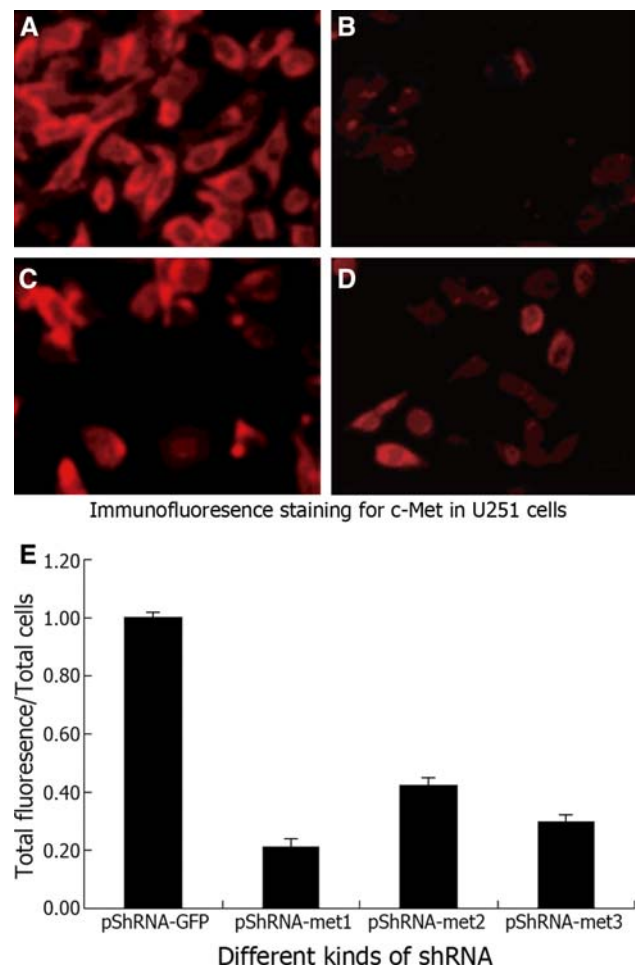
**Fig. 1** c-Met gene expression inhibited by shRNAs (a) and the inhibition rate of pShRNA-met1, pShRNA-met2 and pShRNA-met3 (b) in U251 cells. Lane 1 pShRNA-GFP; lane 2 pShRNA-met1; lane 3 pShRNA-met2; lane 4 pShRNA-met3

#### Induction of apoptosis by the c-Met shRNA

Cells exposed to the oligonucleotides were examined for apoptosis induction by FCM. As shown in Table 1, pShRNA-met1, pShRNA-met2 and pShRNA-met3 induced significant apoptotic response after transfection, about  $24.85\% \pm 4.26\%$ ,  $14.63\% \pm 3.83\%$ , and  $19.46\% \pm 4.05\%$  for 24 h ( $P < 0.05$ ) and  $29.25\% \pm 4.63\%$ ,  $19.81\% \pm 4.14\%$ , and  $24.34\% \pm 4.21\%$  for 48 h ( $P < 0.01$ ), respectively. However, pShRNA-GFP did not induce any significant apoptotic response until 48 h after transfection ( $P > 0.05$ ).

#### Effects of the c-Met shRNA on U251 cell adhesion

Suppressing c-Met expression had a clear inhibitory effect on the adhesion of transfected U251 cells to the extracellular matrix (ECM) [Matrigel and Fn] and to ECV304. The percentages of adhesion to ECM were as follows: pShRNA-GFP, 38.5% (Fn) and 88.2% (Matrigel); pShRNA-met1, 9.1% (Fn) and 40.1% (Matrigel); pShRNA-met2, 26.6% (Fn) and 65.2% (Matrigel); and pShRNA-met3, 18.2% (Fn) and 51.5% (Matrigel) (Fig. 5a). The tumor cell lines showed different absorbance abilities: pShRNA-GFP,



**Fig. 2** Immunofluorescence staining for pShRNA-GFP (a), pShRNA-met1 (b), pShRNA-met2 (c), pShRNA-met3 (d), and the inhibition rate of pShRNA-met1, pShRNA-met2 and pShRNA-met3 (e)

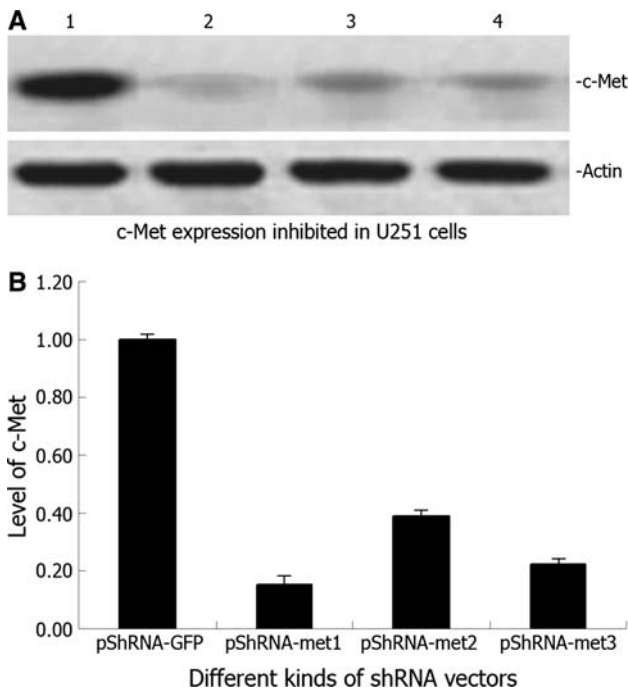
0.596; pShRNA-met1, 0.276; pShRNA-met2, 0.412; and pShRNA-met3, 0.328 (Fig. 5b). Thus, the adhesion of pShRNA-met1 to ECM and to ECV304 cells was significantly suppressed ( $P < 0.001$ ).

#### Effects of c-Met shRNA on U251 cell invasion

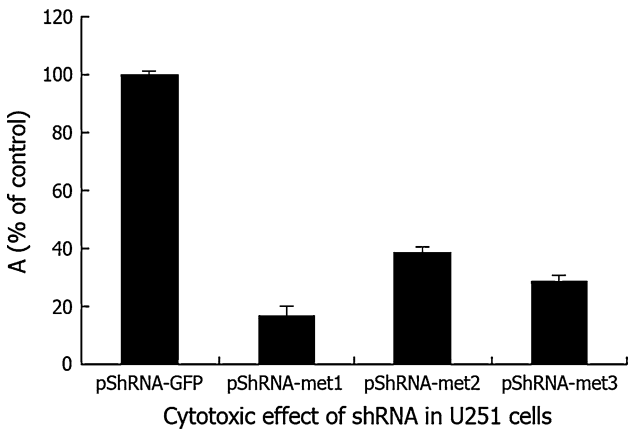
As shown in Fig. 6a, for each 400 $\times$  field under the microscope, the number of migrated pShRNA-met1 ( $245 \pm 10$ ), pShRNA-met2 ( $367 \pm 13$ ) and pShRNA-met3 ( $306 \pm 12$ ) cells was significantly lower than the number of migrated pShRNA-GFP cells ( $442 \pm 15$ ) ( $P < 0.001$ ).

#### Effects of c-Met shRNA on angiogenesis in vitro

As shown in Fig. 6b, in vitro tube formation of ECV304 cells transiently transfected with pShRNA-met1, pShRNA-met2, and pShRNA-met3 was  $34 \pm 3$ ,  $52 \pm 5$ , and  $42 \pm 4$  per 100 $\times$  field, which was significantly lower ( $P < 0.001$ )



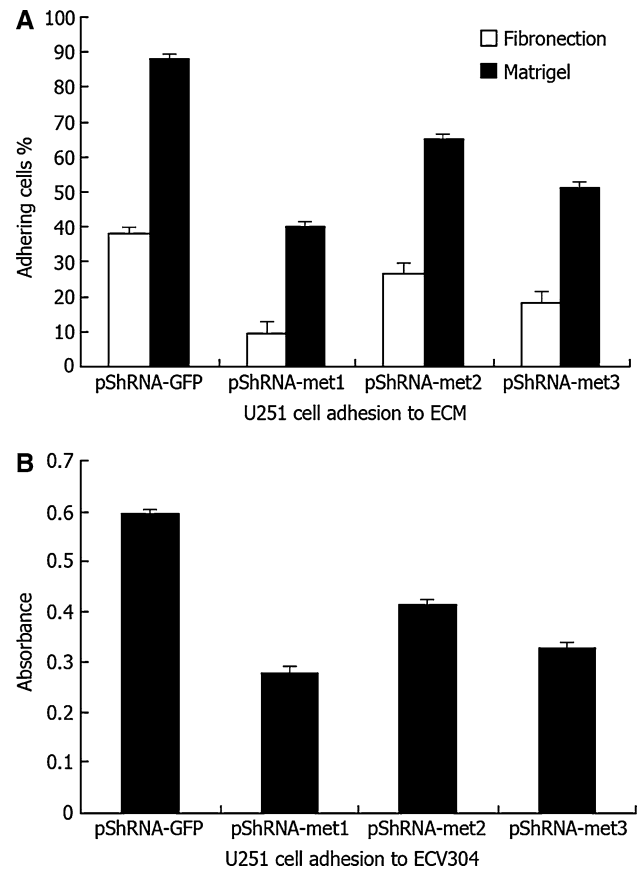
**Fig. 3** Western blotting for c-Met (a) and the inhibition rate of pShRNA-met1, pShRNA-met2 and pShRNA-met3 (b) in U251 cells. Lane 1 pShRNA-GFP; lane 2 pShRNA-met1; lane 3 pShRNA-met2; lane 4 pShRNA-met3



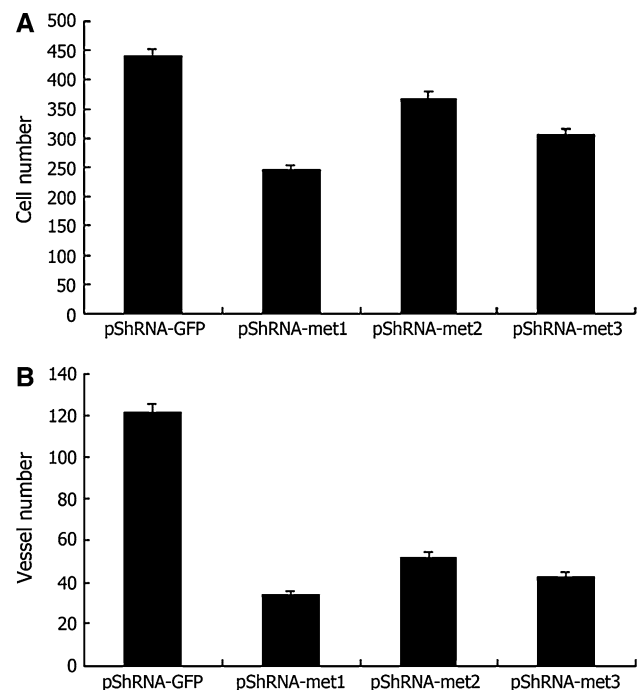
**Fig. 4** Cytotoxic effect of shRNA targeting region of c-Met mRNA. Cells were cultured in plastic 96-well plates and quantitated using MTT assay

**Table 1** Apoptosis rates of each group 24 and 48 h after transfection (n = 6)

Group	24 h (%)	48 h (%)
Untreated	1.96 ± 0.37	3.72 ± 0.21
pShRNA-GFP	1.96 ± 0.40	3.76 ± 0.24
pShRNA-met1	24.85 ± 4.26	29.25 ± 4.63
pShRNA-met2	14.63 ± 3.83	19.81 ± 4.14
pShRNA-met3	19.46 ± 4.05	24.34 ± 4.21



**Fig. 5** Effects of c-Met shRNA on U251 cell adhesion. a U251 cell adhesion to ECM (Fn and Matrigel). b U251 cell adhesion to ECV304



**Fig. 6** Effects of c-Met shRNA on U251 cell invasion and angiogenesis in vitro. a U251 cell invasion. b Angiogenesis in vitro



compared with ECV304 transiently transfected with pShRNA-GFP cells ( $122 \pm 6$ ).

## Discussion

Angiogenesis is a process of generating new capillaries from pre-existing blood vessels, which involves multiple gene products expressed by various cell types. This uncontrolled process of new blood vessel growth from the preexisting circulation network is an important pathogenic cause of tumor growth [22, 23]. Although several proteins such as tumor necrosis factor- $\alpha$ , and fibroblast growth factor 2 (FGF2) have been identified as stimulators of angiogenesis in various settings, HGF is the very important angiogenic growth factor, which is over-expressed in many human cancers. The receptor for HGF is a protein product of a proto-oncogene, c-Met [3], which encodes a transmembrane tyrosine kinase with structural and functional features of a growth factor receptor [4]. Autophosphorylation of this receptor by ligand binding stimulates its intrinsic tyrosine kinase activity with resultant changes in cellular motility, growth, invasion, and angiogenesis. Overexpression of this oncogene was shown in different human solid tumors [5, 6].

RNA interference represents a useful experimental approach for manipulating gene expression, and has shown anticancer efficacy in numerous preclinical studies [24, 25]. In this study, shRNAs targeting c-Met efficiently reduced the transcript levels of c-Met mRNAs, and ultimately resulted in the reduction in c-Met protein levels. Furthermore, this inhibition was shown to be highly selective and sequence-specific. PShRNA-met1 caused a statistically significant reduction of cell viability and inhibited cell growth and caused apoptosis of human glioma cell line U251 significantly. Preclinical study showed that ASODNs against the c-Met had not such good effect [7]. On the other hand, suppressing c-Met expression had a clear inhibitory effect on the adhesion, invasion, and angiogenesis in vitro of glioma U251 cells.

Some researchers found that adenovirus-based U1/ribzyme gene delivery led to a reduction of c-Met mRNA levels by 75% and c-Met protein levels were decreased by 50%, and inhibition of SF/HGF expression by Ad-U1/SF reduced c-Met tyrosine phosphorylation by 45% relative to total c-Met protein for 48 h in U-87 MG cells. And they also found that Ad-U1/Met inhibited cell migration by 56%, and inhibited colony formation in soft agar by 67% in U-87 MG cells [26]. In the present study, we identified that pShRNA-met1 could effectively inhibit cell growth, adhesion, invasion, and angiogenesis of glioma U251 cells in vitro, then we detected apoptosis by FCM when pShRNA-met1 was transfected. It demonstrated that transfecting pShRNA-

met1 was sufficient to trigger apoptosis, indicating that decrease of cell viability was due to apoptosis. It seemed plausible that the effects of pShRNA-met1 varied depending on the expression profile of treated cells. It was also probably due to the complexity of apoptotic pathway in which other antiapoptotic genes might play more important roles in human glioma cell line U251 [7].

In conclusion, the present study demonstrated that vector-mediated RNA interference of c-Met successfully inhibited the expression of c-Met protein and mRNA in human glioma U251 cells in vitro, leading to several antitumor activities such as inhibitory effects on cell proliferation, adhesion, invasion, and angiogenesis of glioma U251 cells. These findings suggest that the RNAi approach can be an effective therapeutic strategy for human glioma. Perhaps, in future work, newer methods of blocking c-Met expression (such as with pShRNA-met1) combined with direct intraparenchymal convection enhanced microinfusion will be able to demonstrate an effect of such targeted gene strategies for gliomas in vivo. Otherwise, it is meaningful to investigate further whether pShRNA-met1 can enhance the sensitivity of resistance to DNA-damaging agents by decreasing apoptosis thresholds [7, 27].

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