

## Original Article

# MicroRNA-125a down-regulates the expression of smoothened protein and the activity of tumor cells in hepatocellular carcinoma

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**Abstract:** Aims: The present study was to investigate the expression of microRNA (miRNA or miR)-125a in hepatocellular carcinoma tissues, as well as its mechanism of action in hepatocellular carcinoma. Methods: A total of 20 patients with hepatic cancer who received surgical resection between August 2015 and December 2015 in our hospital were enrolled in the present study. The expression of miR-125a and smoothened (SMO) protein mRNA was measured by quantitative real-time polymerase chain reaction. The expression of SMO protein was determined using Western blotting. Hepatoma G2 (HepG2) cells were transfected with miR-125a mimics or inhibitor. The expression of miR-125a and SMO mRNA and protein was investigated. The invasion and migration of HepG2 cells were studied using transwell assay, and the proliferation of the cells was examined using MTT assay. Results: The expression of miR-125a in hepatocellular carcinoma tissues was decreased, while the expression of SMO mRNA and protein was enhanced. Overexpression of miR-125a inhibited the transcription and translation of SMO gene in HepG2 cells. In addition, expression of miR-125a decreased the invasion, migration and proliferation of HepG2 cells. Conclusions: The present study demonstrates that miR-125a exerts its anti-tumor effect by down-regulating SMO expression and the invasion, migration and proliferation of hepatocellular carcinoma cells. In addition, miR-125a can be a potential target in the molecular treatment and prognosis of hepatocellular carcinoma.

**Keywords:** Hepatocellular carcinoma, miR-125a, SMO

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in human [1]. HCC has a high mortality rate because of lack of effective early diagnosis, and metastasis and recurrence [2, 3]. Hedgehog (Hh) signaling pathway is a highly conserved signaling pathway that plays important roles in human embryonic development [4], organogenesis [5], and tissue repair [6]. Smoothened (SMO) protein is a membrane protein in Hh signaling pathway. It acts as a converter that transforms extracellular Hh signals into intracellular glioma-associated oncogene homolog (Gli) signals, finally leading to the activation of transcription factor Gli1 [7]. Studies show that human SMO-mediated abnormal activation of Hh signaling pathway is closely associated with the occurrence and development of various malignant tumors [8, 9].

MicroRNA (miRNA or miR) is a kind of non-coding single-strand RNA with 20-22 nucleotides. It widely exists in eukaryotic cells, and affects cell physiological functions such as proliferation, differentiation, and metabolism by regulating the expression of specific genes [10]. Studies show that abnormal regulation of miRNA is an important reason that causes the occurrence and metastasis of tumors [11, 12]. miR-125a is located at human chromosome 19q13 [13], and participates in the occurrence and development of breast cancer [14], ovarian cancer [15], lung cancer [16], and gastric cancer [17] by regulating the expression of multiple target genes. It is also reported that miR-125a inhibits the secretion of surface antigen by hepatitis B virus [18]. However, the expression of miR-125a and its mechanism of action in lung cancer are rarely studied. In the present study, we measure the expression of miR-125a in lung

cancer tissues and try to elucidate the relationship between miR-125a and the occurrence of lung cancer. In addition, the molecular mechanism of action of miR-125a in cancer cell proliferation and metastasis is also investigated.

### Materials and methods

#### Patients

A total of 20 patients with hepatic cancer who received surgical resection between August 2015 and December 2015 in our hospital were enrolled in the present study, including 13 males and 7 females with an average age of 55.3 years. Before surgery, none of the patients received antitumor treatments such as radiotherapy and chemotherapy. Tumor and its adjacent tumor-free tissues were resected and frozen in liquid nitrogen within 10 min, and stored at -80°C until use. All procedures were approved by the Ethics Committee of The Second Military Medical University, China. Written informed consents were obtained from all patients or their families.

#### Bioinformatics

Bioinformatics prediction is the basis for functional studies of miRNAs. Using TargetScan ([www.targetscan.org](http://www.targetscan.org)), miRNAs that could potentially regulate SMO by targeting the 3'-untranslated (UTR) of its mRNA were predicted.

#### Cells

Human hepatoma G2 (HepG2) cells (Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM medium supplemented with 10% fetal bovine serum. One day before transfection, cells in log-phase growth were seeded into 24 well plates ( $2 \times 10^5$ ) and divided into has-miR-125a mimics group, has-miR-125a inhibitor group, negative control (NC) group and untransfected (blank) group. When reaching 70% confluency, 7.5  $\mu$ L small RNA fragments and 7.5  $\mu$ L Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) were added into two individual vials containing 125  $\mu$ L serum-free DMEM medium, respectively. Five minutes later, the liquids in the two vials were mixed before standing still for another 5 min. Then, the mixture was added onto the cells for an incubation of 6 h. After changing

fresh medium, the cells were cultured under normal condition for 48 h before use.

#### Quantitative real-time polymerase chain reaction (qRT-PCR)

Tissues (100 mg) were ground into powder using liquid nitrogen before addition of 1 ml Trizol (Thermo Fisher Scientific, Waltham, MA, USA) for lysis. Similarly, cells ( $2 \times 10^5$ ) were collected and added into 1 ml Trizol (Thermo Fisher Scientific, Waltham, MA, USA) for lysis. After lysis, total RNA was extracted using phenol chloroform method. The purity of RNA was determined by A260/A280 using ultraviolet spectrophotometry (Nanodrop ND1000, Thermo Scientific, Waltham, MA, USA). Then, cDNA was obtained by reverse transcription using Reverse Transcription System (Takara, Dalian, China) from 1  $\mu$ g RNA and stored at -20°C. Reverse transcription of miRNA was performed by adding polyA tail.

To test the expression of miR-125a in tissues and cells, SYBR® PrimeScript™ miRNA RT-PCR Kit (Takara, Dalian, China) was chosen, using U6 as internal reference. The qRT-PCR reaction system (25  $\mu$ L) contained 12.5  $\mu$ L SYBR Premix Ex Taq, 1  $\mu$ L forward primer (miR-125a: 5'-GTCCTCACAACGATTCCACAAG-3'), 1  $\mu$ L Uni-miR qPCR Primer, 2  $\mu$ L template and 8.5  $\mu$ L ddH<sub>2</sub>O. Each sample was tested in triplicate. The PCR protocol was: initial denaturation at 95°C for 30 s; 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 20 s (iQ5; Bio-Rad, Hercules, CA, USA). The  $2^{-\Delta\Delta Ct}$  method was used to calculate the relative expression of miR-125a against U6.

To measure the expression of SMO mRNA in tissues and cells, mRNA SYBR Green (KAPA BIOSYSTEMS, Boston, MA, USA) qRT-PCR was performed, using GAPDH as internal reference. The qRT-PCR reaction system (20  $\mu$ L) contained 10  $\mu$ L SYBR EX Taq-Mix, 0.5  $\mu$ L upstream primer (5'-GAGTCGTGCATCCCGTTCC-3'), 0.5  $\mu$ L downstream primer (5'-GCCAACTCAGCAAAA-GCCCC-3'), 1  $\mu$ L cDNA and 8  $\mu$ L ddH<sub>2</sub>O. Each sample was tested in triplicate. The PCR protocol was: initial denaturation at 95°C for 10 min; 40 cycles of 95°C for 1 min, 60°C for 40 s, 72°C for 30 s, and 72°C for 1 min (iQ5; Bio-Rad, Hercules, CA, USA). The  $2^{-\Delta\Delta Ct}$  method was used to calculate the relative expression of mRNA against GAPDH.

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### Western blotting

Tissues (50 mg) were ground using liquid nitrogen, and cells were trypsinized and collected. Then, precooled Radio-Immunoprecipitation Assay (RIPA) lysis buffer (600  $\mu$ l; 50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% TritonX-100, and 1% sodium deoxycholate; Beyotime Institute of Biotechnology, Shanghai, China) was added to the samples. After lysis for 50 min on ice, the mixture was centrifuged at 12,000 g/min and 4°C for 5 min. The supernatant was used to determine protein concentration by bicinchoninic acid (BCA) protein concentration determination kit (RTP7102, Real-Times Biotechnology Co., Ltd., Beijing, China). Protein samples (50  $\mu$ g) were then mixed with equal volume of 2 $\times$  sodium dodecyl sulfate loading buffer before denaturation in boiling water bath for 5 min. Afterwards, the samples (10  $\mu$ l) were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (300 mA, 1.5 h) and blocked with 50 g/L skimmed milk at room temperature for 1 h. Then, the membranes were incubated with rabbit anti-human SMO polyclonal primary antibody (1:2,000; Abcam, Cambridge, UK) and rabbit anti-human GAPDH primary antibody (1:2,000; Abcam, Cambridge, UK) at 4°C overnight. After extensive washing with phosphate-buffered saline with Tween 20 for 3 times of 15 min, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:1,000; Abcam, Cambridge, UK) for 1 h at room temperature before washing with phosphate-buffered saline with Tween 20 for 3 times of 15 min. Then, the membrane was developed with enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA) for imaging. Image lab v3.0 software (Bio-Rad, Hercules, CA, USA) was used to acquire and analyze imaging signals. The relative content of SMO protein was expressed as SMO/GAPDH ratio.

### Transwell assay

Transwell chambers (Corning Inc., Corning, NY, USA) were used to evaluate the migration ability of HepG2 cells. Transfected cells were collected by trypsin digestion, and resuspended to a density of  $5 \times 10^5$  cells/mL using DMEM con-

taining 0.1% bovine serum albumin. The cell suspension (200  $\mu$ L) was added into the migration chamber. In the lower chamber, 750  $\mu$ L DMEM medium supplemented with 20% serum was added. After 4 h, the cells in migration chamber were wiped by cotton swab. Cells that moved to the other side of the chamber were fixed with 100% methanol for 10 min. After being stained with 0.1% crystal violet, the number of cells was counted under a microscope.

Growth-factor depleted Matrigel invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA) were used to determine the invasion ability of cells. In Matrigel chambers, 500  $\mu$ L serum-free DMEM medium was added and kept for 1 h at room temperature. In the lower chamber, 750  $\mu$ L DMEM medium supplemented with 20% serum was added. After trypsinization, the transfected cells were resuspended to a density of  $4 \times 10^5$  cells/ml using DMEM containing 0.1% bovine serum albumin. After adding 500  $\mu$ L cell suspension into the invasion chamber, the cells were incubated at 37°C and 5% CO<sub>2</sub> for 18 h. Then, the cells in invasion chamber were wiped by cotton swab. Cells that moved to the other side of the chamber were fixed with 100% methanol for 10 min. After being stained with 0.1% crystal violet, the number of cells was counted under a microscope.

### MTT assay

At 24, 48, and 72 h after transfection, 20  $\mu$ L MTT was added into 96 plate containing cells of has-miR-125a mimics, has-miR-125a inhibitor, NC and blank groups. After incubation at 37°C for 4 h, the supernatant was discarded. Then, 150  $\mu$ L MTT was added and absorbance of each well was measured at 492 nm.

### Statistical analysis

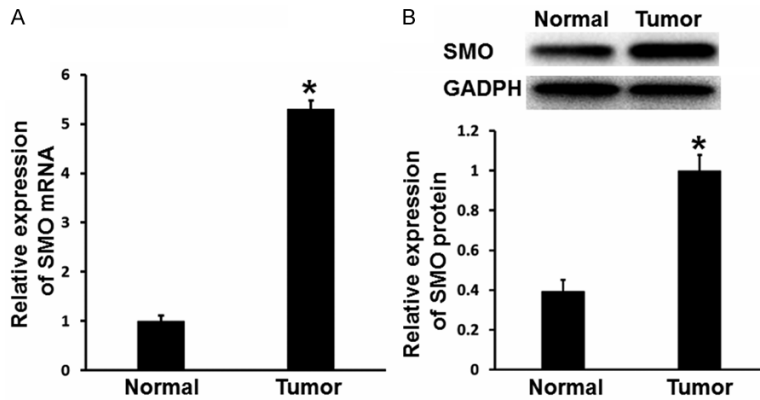
The results were analyzed using SPSS16.0 software (IBM, Armonk, NY, USA). All data were expressed as means  $\pm$  standard deviations. Differences between groups were compared using t-test.  $P < 0.05$  was considered statistically significant.

## Results

### Expression of SMO is elevated in tumor tissues

To measure the expression of SMO mRNA and protein, qRT-PCR and Western blotting were

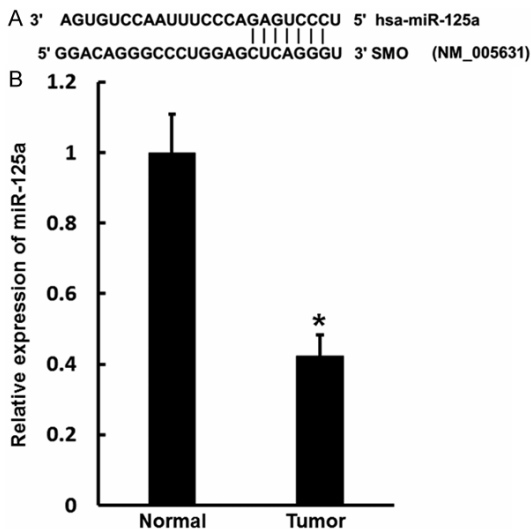
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**Figure 1.** Expression of SMO in tumor and its adjacent normal tissues. A. Relative expression of SMO mRNA determined using qRT-PCR. B. Relative expression of SMO protein determined using Western blotting. \*P < 0.05 compared with normal tissues.

mRNA (**Figure 2A**). To determine the expression of miR-125a, qRT-PCR was employed. The data showed that miR-125a expression in tumor tissues was significantly lower than that in tumor-adjacent normal tissues (P < 0.05) (**Figure 2B**). The result indicates that the decrease of miR-125a expression in tumor tissues is associated with SMO expression.

### *Overexpression of miR-125a inhibits the transcription and translation of SMO gene*



**Figure 2.** Relationship between miR-125a and SMO mRNA in tumor and its adjacent normal tissues. A. Interaction between miR-125a and 3'-UTR of SMO mRNA predicted by bioinformatics. B. Relative expression of miR-125a in tumor and its adjacent normal tissues determined using qRT-PCR. \*P < 0.05 compared with normal tissues.

To identify the regulation of SMO expression by miR-125a, HepG2 cells were transfected with miR-125a mimics and miR-125a inhibitor, and the levels of miR-125a and SMO were measured. The data showed that the level of miR-125a in cells transfected with miR-125a mimics was significantly higher than that in NC group or blank group (P < 0.05), while that in cells transfected with miR-125a inhibitor was significantly lower than that in NC group or blank group (P < 0.05) (**Figure 3A**). Furthermore, the expression of SMO mRNA and protein in cells transfected with miR-125a mimics was significantly lower than that in NC group or blank group (P < 0.05). By contrast, the expression of SMO mRNA and protein in cells transfected with miR-125a inhibitor was significantly higher than that in NC group or blank group (P < 0.05) (**Figure 3B** and **3C**). These results suggest that overexpression of miR-125a inhibits the transcription and translation of SMO gene.

### *Overexpression of miR-125a inhibits the invasion and migration of HepG2 cells*

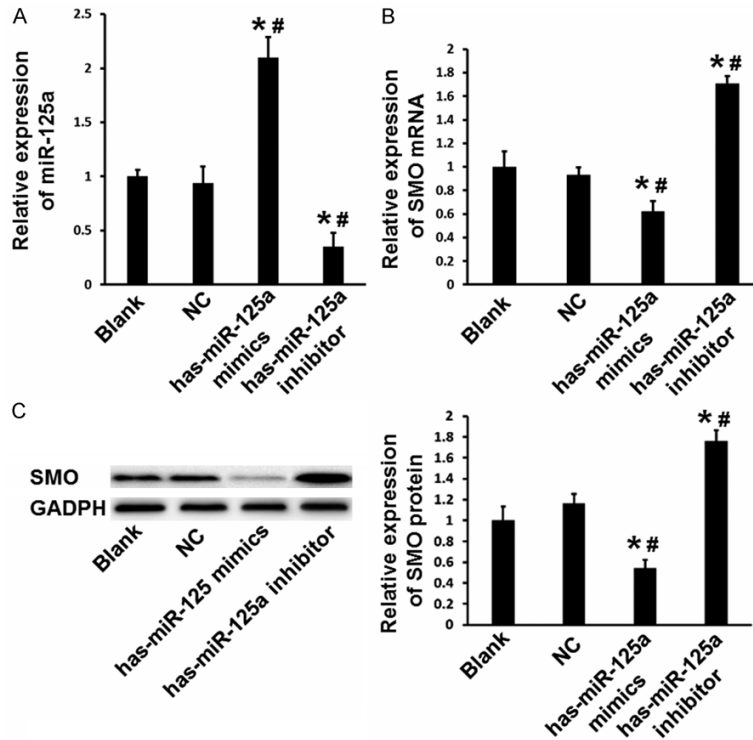
performed. The data showed that SMO mRNA and protein expression in tumor tissues was significantly higher than that in tumor-adjacent normal tissues (P < 0.05) (**Figure 1**). The result suggests that the expression of SMO is elevated in tumor tissues.

### *Decrease of miR-125a expression in tumor tissues is associated with SMO expression*

Using TargetScan, miR-125a was predicted to regulate SMO by targeting the 3'-UTR of its

To test the effect of miR-125a on the invasion ability and migration ability of HepG2 cells, transwell assay was carried out. The data showed that the invasion ability and migration ability of HepG2 cells with overexpression of miR-125a were significantly lower than those of cells in NC group or blank group (P < 0.05) (**Figure 4A** and **4B**). By contrast, invasion and migration of HepG2 cells with inhibited expression of miR-125a were significantly higher than those in NC group or blank group (P < 0.05)

## Effect of miR-125a on SMO and liver cancer



**Figure 3.** Effect of miR-125a expression on SMO. A. Relative expression of miR-125a in HepG2 cells of blank, NC, miR-125a mimic, and miR-125a inhibitor groups. B. Relative expression of SMO mRNA in HepG2 cells of blank, NC, miR-125a mimic, and miR-125a inhibitor groups. Expression of miR-125a and SMO mRNA was measured using qRT-PCR. C. Relative expression of SMO protein in HepG2 cells of blank, NC, miR-125a mimic, and miR-125a inhibitor groups. SMO protein expression was determined using Western blotting. \*P < 0.05 compared with blank group; #P < 0.05 compared with NC group.

(Figure 4A and 4B). The results indicate that overexpression of miR-125a inhibits the invasion and migration of HepG2 cells.

*Overexpression of miR-125a decreases the proliferation of HepG2 cells*

To examine how miR-125a affects HepG2 cell proliferation, we used MTT assay. The data showed that the proliferation of cells with overexpression of miR-125a was decreased compared with that of cells in NC group or blank group, while the proliferation of cells with inhibited expression of miR-125a was enhanced (P < 0.05) (Figure 5). The result suggests that overexpression of miR-125a decreases the proliferation of HepG2 cells.

### Discussion

It has been reported that miRNA can bind with the 3'-UTR of mRNA, and has oncogene or

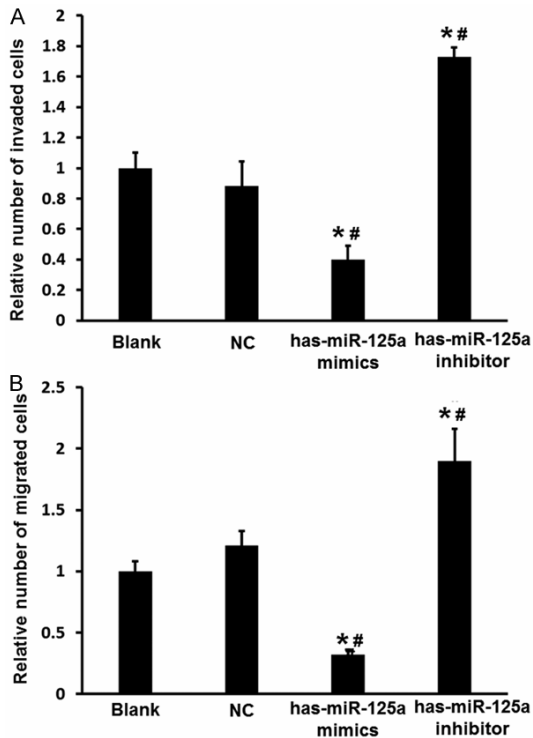
tumor-suppressor functions [10, 19]. A study shows that miR-125a expression in hepatocellular carcinoma tissues is different from that in tumor-adjacent tissues [20]. Another study has discovered that the expression of miR-125a in liver cancer tissues is reduced compared with that in tumor-adjacent tissues [21]. However, there has been no report on the mechanism of action of miR-125a in the occurrence and development of liver cancer.

The present study has demonstrated that the expression of miR-125a in liver cancer tissues is significantly lower than that in tumor-adjacent tissues. Bioinformatics prediction shows that SMO gene is a potential target of miR-125a. SMO protein is a transmembrane protein in Hh signaling pathway, and switches the signaling pathway on and off [7]. It has been widely demonstrated that abnormal activation of Hh signaling pathway is associated with

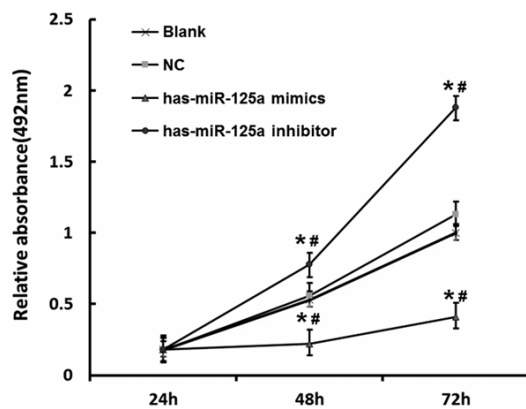
the occurrence and development of various malignant tumors [22], including medulloblastoma [23], lung cancer [24], pancreatic cancer [25], prostatic cancer [26], and breast cancer [27]. In addition, the present study has discovered that SMO mRNA and protein expression in liver cancer tissues is elevated, suggesting that miR-125a is associated SMO gene.

To understand the mechanism by which miR-125a regulates liver cancer, we have transfected HepG2 cells with miR-125a mimics or miR-125a inhibitor to overexpress or inhibit miR-125a. Our results show that overexpression of miR-125a in HepG2 decreases the expression of SMO and vice versa. In addition, overexpression of miR-125a inhibits the invasion, migration and proliferation of HepG2 cells. In recent years, more and more target genes that are regulated by miR-125a have been reported. For example, miR-125a inhibits breast cancer cell

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**Figure 4.** Effect of miR-125a expression on (A) invasion ability and (B) migration ability of HepG2 cells. Relative number of invaded and migrated cells of blank, NC, miR-125a mimic, and miR-125a inhibitor groups was determined using transwell assay. \*P < 0.05 compared with blank group; #P < 0.05 compared with NC group.



**Figure 5.** Effect of miR-125a expression on the proliferation of HepG2 cells. Relative absorbance of wells containing cells in blank, NC, miR-125a mimic, and miR-125a inhibitor groups was determined using MTT assay. \*P < 0.05 compared with blank group; #P < 0.05 compared with NC group.

growth by targeting Hu antigen R (HuR) [14], and suppresses liver cancer cell proliferation

and migration by targeting MMP1 and VEGF [28]. These studies demonstrate that miR-125a plays important roles in the progression of multiple tumors. The present study finds that miR-125a expression is negatively correlated with the invasion ability of liver cancer cells. In conclusion, miR-125a plays important roles in the invasion and metastasis of hepatocellular carcinoma possibly by targeting and regulating SMO gene. The study provides a new clinical marker for the prognosis and treatment of hepatocellular carcinoma, and an experimental basis for its molecular targeted therapy.

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### Disclosure of conflict of interest

None.

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