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Original Article

Inhibitory effect of midkine-binding peptide on tumor proliferation and migration

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Abstract: Background: To investigate the inhibitory effect of midkine-binding peptides on human umbilical vein endothelial cells (HUVECs) proliferation and angiogenesis of xenograft tumor. Methods: The midkine-binding peptides were panned by Ph.D.-7™ Phage Display Peptide Library Kit, and the specific binding activities of positive clones to target protein were examined by phage ELISA. The effect of midkine-binding peptides on proliferation of HUVECs was confirmed by MTT test. The xenograft tumor model was formed in BALB/c mice with the murine hepatocarcinoma cells H22 (H22). Microvessel density (MVD) was analyzed by immunohistochemistry of factor VIII staining. Results: Midkine-binding peptides have the inhibitory effects on tumor angiogenesis, a proliferation assay using human umbilical vein endothelial cells (HUVECs) indicated that particular midkine-binding peptides significantly inhibited the proliferation of the HUVECs. Midkine-binding peptides were also observed to efficiently suppress angiogenesis induced by murine hepatocarcinoma H22 cells in BALB/c nude mice. Conclusion: The midkine-binding peptides can inhibit solid tumor growth by retarding the formation of new blood vessels. The results indicate that midkine-binding peptides may represent potent anti-angiogenesis agents in vivo.

Keywords: Midkine, angiogenesis, peptide

Introduction

The realization that angiogenesis is an essential step for tumor growth and initiation of metastasis [1] has led to significant interest in the discovery of angiogenic factors [2, 3] and the development of drugs that target these molecules. Several inhibitors of angiogenesis have been identified, including angiostatin [4] and endostatin [5]. These agents are able to inhibit tumor growth by suppressing angiogenesis in xenograft models.

Midkine (MK) is a secreted heparin-binding growth factor identified as a product of a retinoic acid response gene [6, 7]. The pathophysiological effects of midkine include enhanced plasminogen activator activity [8], oncogenic transformation of fibroblasts [9], antiapoptotic activity [10] and angiogenic activity [11]. MK is overexpressed in a variety of cancers such as esophageal [12], gastric [13], colon [14], pancreatic [15], lung [16] and breast cancers [17], whereas its expression is usually low or undetectable in normal adult tissues. Additionally, MK is expressed in bladder cancer and overexpression correlates with a poor outcome in patients with invasive cancers [18].

There is evidence that MK plays an important role in angiogenesis, which is a crucial event for tumor development and progression. It has been reported that enhanced tumor growth in MCF-7 breast carcinoma cells due to high levels of MK expression correlate with an increase in vascular density and endothelial proliferation. Moreover, midkine was found to induce a strong angiogenic response in a rabbit corneal assay [19]. An anti-midkine antibody was shown to inhibit the growth of Wilm's tumor cells in vitro [20], and antisense oligo DNA to MK inhibits the growth of colon carcinoma cells in vivo [21]. Consequently, MK represents a suitable molecular target of tumor therapy.

We have confirmed that antisense oligo DNA to MK can significantly inhibit tumor growth in a human hepatocellular carcinoma (HCC) model

Table 1. Enrichment of phages for each round of selection from phage library

rounds	Ph.D.-C7C™ phage display library			Ph.D.-7™ phage display library		
	Selected phages (pfu)[a] (input)	Eluted phages (pfu) (output)	Ratio (%)	Selected phages (pfu) (input)	Eluted phages (pfu) (output)	Ratio (%)
I	1×10^{11}	4×10^{5}	4×10^{-4}	1×10^{11}	2×10^{5}	2×10^{-4}
II	3×10^{10}	1×10^{6}	3.3×10^{-3}	3×10^{10}	6×10^{6}	2×10^{-3}
III	2×10^{11}	1×10^{8}	5×10^{-2}	2×10^{11}	1×10^{8}	5×10^{-2}

[a]pfu: Plaque forming units.

[22] and suppress the angiogenesis both in HepG2-induced CAM and in situ human HCC tissues [23]. In contrast to large molecules such as antibodies and antisense oligo DNA, peptides are known to exhibit less toxicity and posses better pharmacokinetic properties [24]. In this study, inhibition of tumor growth and suppression of angiogenesis in a murine hepatocellular carcinoma model using peptides that bound to MK was examined.

Materials and methods

Cell lines, animals, antibodies and other reagents

Human umbilical vein endothelial cells (HUVECs) and murine hepatocarcinoma cells H22 (H22) were purchased from American Type Culture Collector (ATCC). All cells were cultured in DMEM medium (Gibco) supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum (Gibco). Six-week-old female BALB/c nude mice were purchased from Shanghai Bikai Experimental Animal Center and kept in the local central animal facility. The mice were housed under standard conditions and had free access to water and food. Animal procedures were performed in accordance to approved protocols and followed recommendations for proper care and use of laboratory animals. Recombinant human midkine (rh-midkine) was purchased from BioVendor Laboratory Medicine Inc. Anti-M13 phage antibody was purchased from New England Biolabs Inc. (Beverly, MA, USA). Anti-factor VIII antibody was purchased from DAKO (Denmark). All of the other reagents used were analytical grade and commercially available.

Phage peptide library and bacterial strain

The Ph.D.-7™ Phage Display Peptide Library Kit (2×10^{13} pfu/ml), Ph.D.-C7C™ Phage Display Peptide Library Kit (2×10^{13} pfu/ml) and the host bacterial strain *E. coli* ER2738 were purchased from New England Biolabs Inc.

Phage selection with rh-midkine and the amplification of the peptide library

The biopanning and amplification procedures were essentially performed according to procedures provided by the manufacturer. Three rounds of biopanning were performed. Briefly, 100 μl of rh-midkine (100 μg/ml in 0.1 mol/L $NaHCO_3$, pH 8.6) was coated onto 96-well microtiter plates and incubated overnight at 4°C with gentle agitation in a humidified container. Following blocking with an appropriate buffer (TBS, pH 7.2, containing 5% BSA), each well was filled with the phage library (100 μl, approximately 2.0×10^{10} pfu), which was 10-fold diluted with TBST (TBS, 0.05% Tween-20) and incubated at room temperature for 1 h. To remove non-specific phage binding, the wells were washed 10 times with TBST (TBS, 0.1% Tween-20) during the first round of biopanning and the concentration of Tween-20 was raised to 0.5% in the second and third rounds of biopanning. The bound phage were eluted with 100 μl of 0.1 mol/L glycine (pH 2.2) at room temperature for 15 min and neutralized with 15 μl of 1 mol/L Tris-HCl (pH 9.1). The titer of the phage-solution was determined by infecting *E. coli* ER2738, followed by counting surviving colonies on LB/IPTG/X-gal agar plates after incubation at 37°C overnight. The rest of the solution was used for amplification. The amplified phage particles were purified using PEG/NaCl.

Peptide-protein binding assays

The binding activity of monoclonal phages for rh-midkine was detected by ELISA, which was essentially performed according to the manufacturer's instructions. Briefly, 100 μl of rh-midkine (100 μg/ml in 0.1 mol/L $NaHCO_3$, pH 8.6) was coated onto 96-well microtiter plates and incubated overnight at 4°C with gentle agitation in a humidified container. Following blocking (TBS, pH 7.2, containing 5% BSA), approximately 2×10^{10} pfu amplified monoclonal phages were added to each well and then incubated with rh-midkine for two hours at room temperature. The wells were washed with TBST

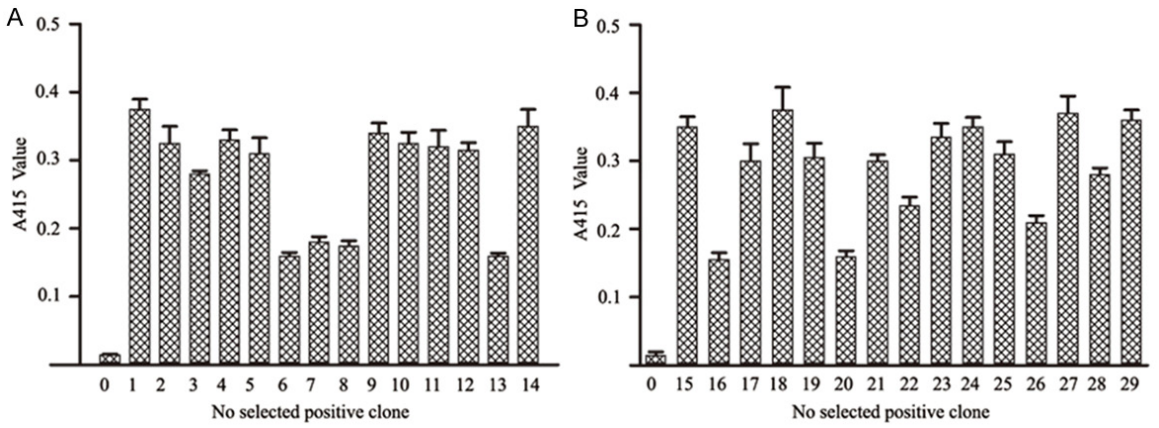

Figure 1. Specific binding of selected phage clones to the anti-M13 antibody. 29 clones bound to the target protein; there were 14 cyclic peptides (No. 1-14) (A) and 15 linear peptides (No. 15-29) (B). The original phage peptide library without selection, represented as No. 0, the original library without selection was used as negative control. All of the treatments were performed in triplicate.

Table 2. Amino acid sequences deduced from the DNA sequences

Groups	Clones	Sequences	Frequency
Cyclic peptides	Phage 1, phage 2, phage 3, phage 4, phage 10, phage 11	ACVIHWDFIC	6
	Phage 5, phage 12	ACTWLHWWAC	2
	Phage 9, phage 14	ACTSTAMDNC	2
	Phage 8	ACNSHTDTNC	1
	Phage 13	ACMPKVGLNC	1
	Phage 6	ACKNTFANVC	1
	Phage 7	ACTIPRMPYC	1
Linear peptides	Phage 17, phage 19, phage 21, phage 23, phage 24	LLWYDEI	5
	Phage 15, phage 18, phage 27, phage 29	HAIYPRH	4
	Phage 25, phage 28	PVPRSRP	2
	Phage 16	PISSYQR	1
	Phage 20	RPPGYIP	1
	Phage 22	VPFYSHS	1
	Phage 26	RPPGYI P	1

Table 3. Name and sequence of the midkine-binding peptides

Name	Amino acid sequence
MK-P1	CVIHWDFIC
MK-P2	CTWLHWWAC
MK-P3	CTSTAMDNC
MK-P4	LLWYDEI
MK-P5	HAIYPRH
MK-P6	PVPRSRP

(TBS, 0.1% Tween-20) five times and the amount of bound phages was detected with horseradish peroxidase (HRP)-conjugated anti-M13 phage antibody (1:5000, Pharmacia #27-9411-01). After the addition of the substrate, the antibody reaction was analyzed in a microtiter plate reader at 415 nm. The original phage library without selection was used as the negative control.

Peptides sequencing

Single-stranded DNA (ssDNA) was prepared from the identified phage clones as described in the kit guidelines and sequencing was carried out by Invitrogen. The primer used for sequencing was: 5'-CCCTCATGTTAGCGTAACG-3'. The corresponding amino acid sequences were deduced from the DNA sequences and multiple sequence alignments were performed using the BLAST software package to determine which peptides were related by sequence.

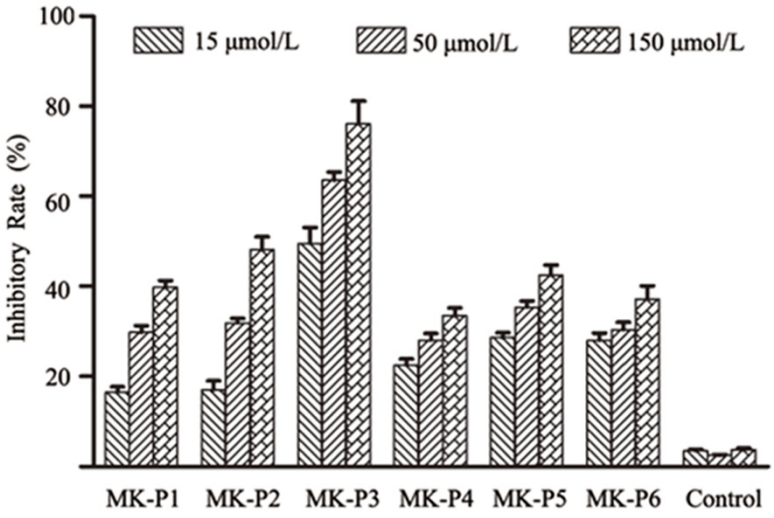

Figure 2. Effects of midkine-binding peptides on the proliferation of HUVECs. Midkine-binding peptides significantly inhibited the proliferation of HUVECs. Among the six peptides examined, MK-P3 showed the highest inhibitory activity, the non-specific peptide was examined as the negative control. All of the treatments were repeated six times.

Table 4. Inhibitory effects of midkine-binding peptides on xenograft tumors

Groups	n	Tumor weight (g)	Inhibitory rate (%)
PBS	12	3.099 ± 0.452[a]	-
Control	12	2.897 ± 0.316	6.5
5-FU 10 mg kg^{-1}	12	1.692 ± 0.563*	45.4
MK-P1 0.5 mg kg^{-1}	12	2.42 ± 0.56*	22.0
MK-P2 0.5 mg kg^{-1}	12	2.31 ± 0.645*	25.4
MK-P3 0.5 mg kg^{-1}	12	1.35 ± 0.88**	56.4
MK-P4 0.5 mg kg^{-1}	12	2.31 ± 0.49*	25.5
MK-P5 0.5 mg kg^{-1}	12	2.26 ± 0.34**	27.0
MK-P6 0.5 mg kg^{-1}	12	2.50 ± 0.16*	19.3

[a]Data are presented as means (± SD), n = 12, *P < 0.05, **P < 0.01, compared with PBS control, the negative control group was treated with non-specific peptide.

Peptide synthesis

Peptides were synthesized chemically by the standard N-(9-fluorenyl-methoxycarbonyl) (Fmoc) protocol and purified by reversed-phase high-performance liquid chromatography (RH-HPLC) (Zhongtai, Hangzhou, China). The purity of all products was > 95%.

Cell proliferation assay

HUVEC cells were seeded into 96-well plates (3000 cells/well) in 100 μl DMEM medium. The cells were treated with or without the peptides at concentrations of 15, 50 and 150 μg/ml for 72 h, 10 μl of MTT stock solution (5 mg/ml) was added into each well and incubated for another 4 h at 37°C. The solution was removed and 150 μl DMSO was added per well. After 10 min incubation at 37°C, the optical density at 570 nm was measured using an enzyme-linked immunosorbent assay reader (Bio-Rad Model 550).

Xenografts in BALB/c mice and treatment

H22 cells were suspended in DMEM at a concentration of 5×10^6 cells/ml. 100 μl of the suspension was injected into the subcutaneous on the right side of the back of each mouse. The mice were randomly divided into eight groups (12 mice per group). The peptides and 5-FU were injected daily into the peritoneal cavum for the seven treated groups following inoculation. The same volume of a PBS solution was injected into the mice of the negative control group. After twenty-one days of treatment, the mice were sacrificed and tumors were removed and weighted. The percentage of tumor growth inhibition was calculated as: Inhibitory rate (%) = $(W_{control}-W_{treat})/W_{control}\times100$.

Immunohistochemistry analysis for microvessel formation

Tumors were fixed and frozen in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC). 5-μm-thick sections were cut and stained with hematoxylin and eosin for histopathological analysis. To analyze the microvessel formation in tumors, sections were stained with anti-factor VIII monoclonal antibody (DAKO Corp., Carpinteria, CA) and subsequently with the avidin-biotin-peroxidase (ABC) method. Positively stained vascular endothelial cells (brown) were visualized and imaged using a digital camera attached to an Olympus microscope. Microvessel density (MVD) was determined according to methods described previ-

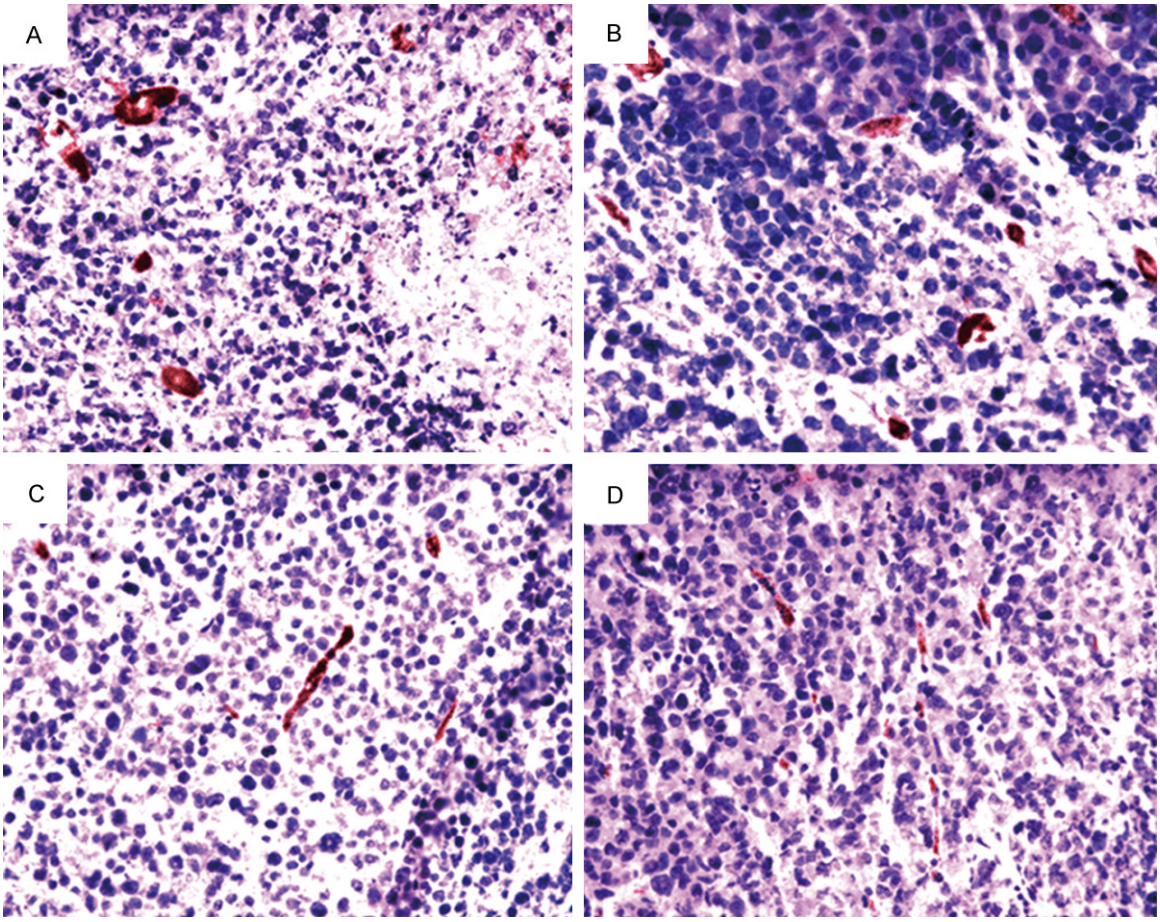

Figure 3. Effects of midkine-binding peptides treatment on tumor angiogenesis. The representative images of immunohistochemistry stained for microvessels (factor VIII) in a xenograft tumor (×200). A. Sections of tumors from PBS treatment. B. Sections of tumors from non-specific peptide treatment as negative control. C. Sections of tumors following treatment with MK-P3. D. Sections of tumors from treatment with MK-P.

ously [25]. Briefly, regions of the highest vessel density ("hot spot" regions) were scanned at low magnification (×40-100) and counted at higher magnification (×200). Three such "hot spot" fields were counted in each tumor section and the mean microvessel density value was recorded. Any endothelial cell or endothelial cell clusters that was clearly separated from adjacent microvessels were considered a single, countable microvessel. Positively stained vascular endothelial cells were visualized and imaged using a Magnifire camera (Olympus, Melville, NY) attached to an Olympus Provis microscope.

Statistical analysis

Data were described as the mean ± SD for general characteristics of the subjects. The SPSS13.0 software was used for all statistical analyses. Two-tailed *P* values with composite results $P < 0.05$ were considered statistically significant.

Results

Specific enrichment of positive phages

To enrich rh-midkine binding phages from the Ph.D.-C7C and Ph.D.-7 phage display libraries, three rounds of selection with the rh-midkine protein were performed. The enrichment was determined by the use of the output/input ratio of phages after each round of selection. The ratio increased approximately 10-fold after the second round of selection. After the third round of selection of the Ph.D.-C7C and Ph.D.-7 phage display libraries, the output/input ratio of phages increased 125-fold and 250-fold respectively, which indicated that both linear and cyclic peptide libraries had an obvious enrichment for the specific binding of phages to rh-midkine (**Table 1**).

Table 5. Effects of midkine-binding peptides on microvessel density in tumor xenografts

Groups	MVD
MK-P1 0.5 mg kg^{-1}	18.8 ± 2.9*
MK-P2 0.5 mg kg^{-1}	19.2 ± 2.2*
MK-P3 0.5 mg kg^{-1}	15.3 ± 1.2**
MK-P4 0.5 mg kg^{-1}	17.6 ± 3.0*
MK-P5 0.5 mg kg^{-1}	15.7 ± 3.2**
MK-P6 0.5 mg kg^{-1}	16.1 ± 2.4**
PBS	25.1 ± 0.7
Control	22.7 ± 1.6

†Data are presented as means ± SD, n=12; *P < 0.05, **P < 0.01, compared with PBS control, the negative control group was treated with non-specific peptide.

Estimation of binding specificities of selected phage clones

The positive clones were chosen from the samples after the third round of selection with the rh-midkine protein and the specificity was examined by phage ELISA. 29 clones bound to the target protein and were chosen for further sequencing (**Figure 1**).

Analyses of sequences of positive phage clones

The ssDNA was prepared for positive phage clones and sequenced and the amino acid sequences of the midkine-binding peptides were deduced from the DNA sequences. Analysis of the 29 clones from the affinity-selected phage population revealed that the majority of clones fell into six groups with different sequences (**Table 2**). The six peptides were designated and synthesized (**Table 3**).

Effects of midkine-binding peptides treatment on HUVEC growth

To investigate the activity of midkine-binding peptides on the vascular endothelial cell proliferation, we analyzed the growth of HUVECs treated with midkine-binding peptides. From the results presented in **Figure 2**, the midkine-binding peptides were observed to significantly inhibit the proliferation of HUVECs. The inhibition level ranged from 15.6 to 76.6%. Among the six peptides, MK-P3 showed the highest inhibitory activity. The percentage of the inhibition was calculated as:

Inhibitory rate (%) = $(OD570_{control}-OD570_{treat})/OD570_{control}\times100$

Effects of midkine-binding peptides treatment on xenograft tumor growth

Treatment with the midkine-binding peptides resulted in a significant reduction in the weight of tumors when compared to PBS-treated mice (**Table 4**). The inhibitory efficacy of MK-P3 was 56.4%, which was the highest. Moreover, the 5-FU (5-Fluorouracil) group (positive control) had an inhibitory efficacy of 45.4% when compared to the PBS treated group.

Effects of midkine-binding peptides treatment on tumor angiogenesis

Sections of xenograft tumors from the mice in all eight groups were stained for endothelial cell specific markers to detect the number of endothelial cells as a measure of tumor angiogenesis. A single microvessel was defined as any immunohistochemistry stained endothelial cell distinguished from adjacent tumor cells and other connective tissue elements. A representative result of immunostaining is shown in **Figure 3**. A high density of microvessels was observed in the untreated tumor sample, whereas treatment with the midkine-binding peptides resulted in a significant reduction in vascularization (**Table 5**; **Figure 3**).

Discussion

Angiogenesis is a process of remodeling of a primitive vascular network into mature vasculature through sprouting, branching and differential growth of blood vessels. This complex process involves endothelial cell proliferation, chemotactic migration and functional maturation, as well as differential recruitment of supporting cells. Normal or physiological angiogenesis occurs during embryonic development, wound healing, menstruation and pregnancy. Angiogenesis is controlled and regulated by many molecules including stimulators and inhibitors. An appropriate balance between stimulators and inhibitors is pivotal for the maintenance and regulation of angiogenesis. Tumor-associated angiogenesis is critical for tumor growth and is controlled by a balance between pro- and anti-angiogenic factors. Growing evidence demonstrates the heterogeneity of tumor angiogenesis, probably arising from the vastly different microenvironments of individual tumors [26-30]. Due to the heterogeneous nature of tumor angiogenesis, effective anti-angiogenic therapies should be optimized and may require interference with multiple

angiogenic pathways. A large number of natural proteins are angiogenic inhibitors and various inhibitors of angiogenesis exhibit antitumor effects [31, 32]. Selection of target proteins is very important in designing and screening anti-angiogenic peptides. Novel targets could provide unique characteristics and new desirable features for further development into peptide drugs.

In this paper, we initially analyzed the effects of midkine-binding peptides on endothelial cell proliferation. The results indicated that midkine-binding peptides could retard HUVEC growth, suggesting that these peptides may also inhibit angiogenesis in vivo. Furthermore, we developed an *in situ* mouse hepatocellular carcinoma model to determine the influence of the midkine-binding peptides on tumor growth and angiogenesis in vivo. The results indicated that midkine-binding peptides significantly inhibited the mouse hepatocellular carcinoma model. Furthermore, midkine-binding peptides also suppressed tumor angiogenesis.

It is well established that tumor growth and metastasis depend on the induction of a new blood supply [33, 34]. Angiogenic activity, as determined by MVD, has been shown to correlate with an unhealthy prognosis in a number of solid tumors [35-37]. Therefore, anti-angiogenic agents targeting either of these factors or vascular endothelial cells are promising therapeutic modalities as tumor dormancy therapies [38]. In the present study, we have addressed the potential therapeutic role of midkine-binding peptides on angiogenesis. Significant inhibition of angiogenesis was achieved by the midkine-binding peptide MK-P3, indicating that MK-P3 represents a possible effective anti-angiogenesis agent.

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Disclosure of conflict of interest

None.

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