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Original Article

Role of microRNA in spinal metastatic cancer angiogenesis

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Abstract: Spine is the third common body site for tumor metastasis via blood-borne transmission. MicroRNA (miR) can regulate tumor progression via affecting hypoxia inducible factor-1α (HIF-1α) and vascular epithelial growth factor (VEGF). This study generated VX2 tumor bearing rabbits, on which tumor cells were isolated and were transfected with miR-18a, in order to analyze the role of miR-18a in spinal metastatic tumor's angiogenesis process. VX2 tumor bearing rabbits were prepared and isolated for tumor cells, which were prepared for single cell suspension. Tumor cells were transfected with miR-18a mimics or inhibitors, and were tested by RT-PCR. MTT assay was used to detect proliferation ability of tumor cells after transfection. Immunohistochemistry (IHC) staining was used to quantify the expression of CD31, Ki67 and micro-vessels in spinal metastatic tumors. The expression of VEGF and HIF-1α were determined by Western blotting. Compared to control group, those cells transfected with miR-18a mimics had elevated miR-18a expression. Those cells had lower proliferation ability starting from 96 hours after transfection ($P<0.05$). The positive rates of CD31, Ki67 and micro-vessel number were 60%, 50% and 62.36 ± 10.22, respectively. All of those parameters were significantly lower than control group ($P<0.05$). Protein levels of HIF-1α and VEGF were decreased in miR-18a transfected tumor cells ($P<0.05$ compared to control cells). MiR-18a over-expression decreased cell proliferation ability, decreased level of CD31, Ki, and micro-vessel regeneration, and inhibited HIF-1α and VEGF expression for blocking angiogenesis.

Keywords: MicroRNA-18a, spinal metastatic tumor, VEGF, HIF-1α

Introduction

Spine is one common site for tumor metastasis, and is the third popular organ for distal metastatic tumors, only next to lung and liver. In all cancer-related death, there are more than 70% patients with spinal metastasis. Such metastatic tumors may cause compression of the spinal cord in 5%~14% patients, and with vessel-dependency [1, 2]. In clinics, such spinal metastasis is frequently discovered at the terminal stage. Currently few studies have been performed regarding its pathogenesis. In general, no major breakthrough has been obtained in treating spinal metastatic tumors, causing relatively shorter survival period and unfavorable prognosis of patients [3]. As the basis for tumor pathogenesis, angiogenesis is crucial for growth, proliferation and metastasis of tumors by providing oxygen and nutrients [4]. Vascular endothelial growth factor (VEGF) and its receptor play various roles in modulating angiogenesis, by enhancing permeability of micro-vessels and facilitating proliferation and migration and endothelial cells after specific binding, in addition to the close relationship with anorexic micro-environment [5]. As one endogenous non-coding single stranded small RNA molecule with conserved sequence, microRNA (miR) modulates gene regulation, with its functions drawing increasing attentions [6]. Previous study has revealed the participation of miR in affecting tumor angiogenesisi by modulating VEGF, hypoxia inducible factor-1 (HIF-1) and other factors [7]. This study generated VX2 tumor-bearing spinal metastatic tumor rabbit model, from which tumor cells were isolated and were transfected with miR-18a. After examining miR-18a expression by RT-PCR, MTT was used to detect proliferative activity of transfected cells, while CD31, Ki67 and micro-vessel density were checked by immunohistochemistry (IHC). The expression of VEGF and HIF-1α were further examined by Western blotting, in

order to illustrate the role of miR-18a in angiogenesis of spinal metastatic tumor and related mechanisms.

Materials and methods

Animals and reagents

A total of 10 VX2 tumor bearing rabbits (5 males and 5 females, body weight between 500 g and 700 g) were provided by Lanji Biotech (China). Matrigel was produced by Becton Dickinson (UK). MiR-18a mimics and inhibitor were synthesized by GenePharma (US). Lipofectamine TM2000 and Trizol reagents were products for Invitrogen (US). Goat anti-rabbit HIF-1α polyclonal antibody, goat anti-rabbit VEGF monoclonal antibody, alkaline phosphatase (AP) conjugated goat anti-rabbit and goat anti-mouse secondary antibody, and goat anti-rabbit β-actin were purchased from Santa Cruz (US).

Rabbits were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Luoyang Orthopedic Hospital of Henan Province.

Tumor cell suspension

Solid lesion at the leg was removed from VX2 tumor bearing rabbits were removed after sterilization. Tumor adjacent tissues were removed to expose tumor tissue, which was rinsed in PBS and incubated in Hanks solution. Tissues were cut into small pieces and were mixed with DMEM culture medium. In a digestion for 3 hours at 37°C water-bath, cell mixture was filtered, centrifuged at 2,500 g for 10min, and was rinsed in DMEM containing 10% fetal bovine serum (FBS). Cells were adjusted to 1×10^8 per mL for further use.

Cell transfection

Cell suspensions were cultured in a humidified chamber with 5% CO_2 at 37°C, using DMEM medium containing 10% FBS, 100 u/mL penicillin and 100 u/mL streptomycin. Tumor cells were then seeded into culture plate until reaching 60% confluence. Lipofectamine 2000 was used to transfect miR-18a mimics (10 nmol/L) or miR-18a inhibitor (20 nmol/L) to tumor cells. 4~6 hours after transfection, the medium was changed. Cells were observed after 48 hours to confirm 100% transfection efficiency.

Tumor model

Experimental animals were randomly divided into experimental and control group. MiR-18a transfected tumor cells (2×10^6 cells per animal, 10^6 cells per 0.1 mL) or un-transfected tumor cells were subcutaneously injected. Animals were anesthetized by 3% pentobarbital sodium (1 mg/kg) via intravenous injection, and were fixed in supine position. A middle incision was made using L3 spine as the center to expose abdominal aorta and superior vein, the former of which was blocked at L3 artery posterior entry. Tumor cell suspension was slowly infused into the blood vessel. After suturing, penicillin (400,000 U) was applied daily for 3 days. The respiration, incision, urination and muscle tension were continuously monitored until complete paralysis for forelimbs. Animals were sacrificed and extracted for spinal vertebrates. The size, morphology, color and texture of tumors were observed, followed by HE staining.

RT-PCR

48 hours after transfecting miR-18a mimics or inhibitor, total RNA was extracted by TRIzol reagent following manual instruction. Total RNA concentration and purity were determined by D260 nm/280 nm under UV spectrometer. 200 ng total RNA were used as the template to synthesize cDNA based on polyA tail. Using cDNA as the template, PCR amplification was performed using specific primers (miR-18a, 5'-UAAGG UGCAU CUAGU GCAGA UAG-3'; U6, 5'-GTGCT CGCTT CGGCA GCACA TATAC TAAAA TGTGC TCGCT TCGGC AGCAC ATATA CTAAA ATTGG AACGA TACAG AGAAG ATTAG CATGG CCCCT GCGCA AGGAT GACAC GCAAA TTCGT GAAGC GTTCC ATATT TT-3'). PCR conditions were: 95°C denature for 30 sec, followed by 95°C for 5 sec and 60°C for 30 sec, repeating for 40 cycles.

MTT assay

All cells after transfection at log-phase were seeded into culture plate at 8×10^4 cells per well, and were kept in a humidified chamber with 5% CO_2 at 37°C. Cell viability was detected at 24 hour, 48 hour, 72 hour and 96 hour after inoculation. 20 μL MTT solutions (5 mg/mL)

Table 1. MiR-18a expression in tumor cells after transfection

Group	Mimics NC	MiR-18a mimics	Inhibitor NC	MiR-18a inhibitor
MiR-18a	0.002 ± 0.01	3.51 ± 1.45*	4.32 ± 0.86	1.07 ± 0.11#

Note: *, P<0.05 compared to mimics NC control group; #, P<0.05 compared to inhibitor NC control group.

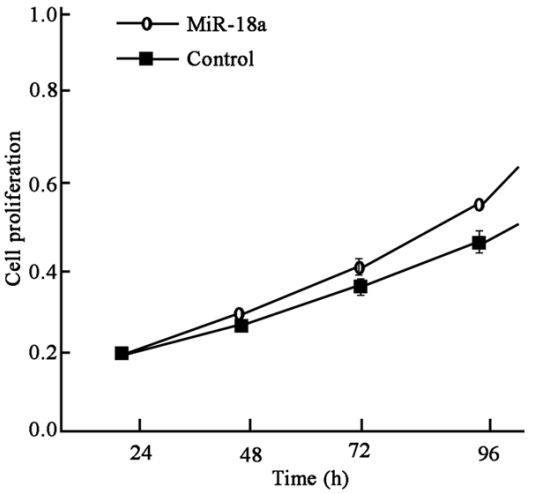

Figure 1. Cell proliferation activity of cancer cells transfected with miR-18a.

were added into each well, and were incubated for 4 hours. 0.15 mL DMSO was then applied for 10-min vortex until complete resolving of crystals. Absorbance value (A) was measured at 570 nm of each well. Using A value as the vertical axis, time-dependent growth curve of cells was plotted.

IHC staining

Tumor samples were fixed in formalin and dehydrated for making paraffin blocs, which were sectioned into tissues slices. Slides were dewaxed and rehydrated. Antigen retrieval was performed using 0.01% citric acid buffer under heating. Hydrogen peroxide was used for quenching hydrogen peroxidase activity. Normal goat serum (NGS) was used to block tissues. Primary antibody was applied for 1-hour incubation, followed by secondary antibody with horseradish peroxidase (HRP) labeled secondary antibody for 10-min incubation. Streptomycin-hydrogen peroxidase was then added for 10-min incubation. DAB substrate was used to develop the signal. After quenching, hematoxylin was used to counter-stain slices, which were then dehydrated and mounted with coverslips. Five fields were randomly selected from each slide and were observed. Both positive control and negative (PBS) control were performed in parallel.

Positive signal for CD31 was identified as brown or brown-yellow granules in cytoplasm or membrane. Negative (-): staining cells ≤10%; Weak positive (+): staining cells between 11% and 25%; Positive (++): staining cells between 26% and 50%; Strong positive (+++), staining cells >50%.

Positive signals for Ki67 were identified as brown or brown-yellow granules in nucleus. Negative (-): No staining or <1% positive nucleus; Weak positive (+): nuclear staining between 1% and 10% and/or weak cytoplasmic staining; Positive (++): staining nucleus between 11% and 50% and/or significant cytoplasmic staining; Strong positive (+++), staining nucleus ≥50% and/or strong cytoplasmic staining.

Weidner method was used to count micro-vessel density. Under low magnification, the density zone of vascular endothelial cells with CD31 labels was observed. Five hotspots were selected to calculate micro-vessel number under high magnification filed. The mean vascular density (MVD) was calculated.

Western blotting

Tumor samples were homogenized and added with lysis buffer. Proteins were separated under 8% SDS-PAGE, and were transferred to NC membrane. After blocking 1 hour, primary antibody (1:200 for VEGF and HIF-1α, and 1:500 for β-actin) was added for overnight incubation at 4°C. TBST was used to rinse the membrane, which was incubated in secondary antibody (1:2,000) for 1 hour. After TBST rinsing, chromogenic substrate A and B were added to develop the membrane. Quantity One software was used to analyze the optical density of protein bands in triplicates.

Statistical analysis

SPSS17.0 software was used to process all collected data, of which enumeration data were compared by chi-square test, while measurement data were compare by analysis of vari-

Table 2. CD31, Ki67 expression and MVD in spinal metastatic tumors

Group	N	CD31				Ki67				MVD
		-	+-++	+++	Positive rate (%)	-	+-++	+++	Positive rate (%)	
miR-18a	10	4*	4*	2*	60*	5*	3*	2*	50*	62.36 ± 10.22*
Control	10	1	3	6	90	2	4	4	80	98.73 ± 15.67

Note: *, P<0.05 compared to control group.

ance (ANOVA) and were presented as mean ± standard deviation (SD). A statistical significance was defined when P<0.05.

Results

MiR-18a expression in tumor cells

RT-PCR was used to detect the expression of miR-18a in tumor cells after transfection. Results showed that, compared to control group, those tumor cells transfected with miR-18a mimics had elevated expression, while those transfected with miR-18a inhibitor had lowered miR-18a (P<0.05, **Table 1**).

Proliferation activity of tumor cells

All tumor cells after transfected with miR-18a were continuously cultured and detected for viability using MTT assay. Results showed gradually depressed growth curve of miR-18a-transfected cells from 24 hour till 96 hour (P<0.05, **Figure 1**).

CD31, Ki67 expression and MVD

IHC staining was employed to detect the expression of CD31, Ki67 and MVD. Results showed 6 cases of CD31-positive tumors (60%), 5 cases of Ki67-positive (50%) in miR-18a transfected cells. Both of these two parameters were significantly lower than control group (P<0.05). MVD was 62.36 ± 10.22 in transfected group, which was significantly decreased compared to control group (P<0.05, **Table 2**; **Figure 2**).

VEGF and HIF-1α expression

We further employed Western blotting to show the expression of VEGF and HIF-1α in tumor cells. Results showed significantly depressed VEGF and HIF-1α protein levels in miR-18a transfected cells compared to control group (P<0.05, **Table 3**; **Figure 3**).

Discussion

The metastasis of malignant tumor includes both lymph node and blood borne transmission. Blood-borne metastasis consists of a series of process including detachment of tumor cells from primary lesion, invasion of adjacent vessels and lymph nodes, entry into circulation, seeding at distal sites, penetrating endothelial wall of micro-vessels, local growth, proliferation and metastatic lesion formation [8]. Study has indicated the formation of spinal metastatic tumor by the seeding of detached tumor cells and infiltration/growth after pulmonary circulation [9]. Previous study on mouse models for bone metastasis of breast cancer had found more than 70% incidence of bone metastatic lesion after injection of breast carcinoma cells via pulmonary aorta. However, most animals died within short time due to multiple metastasis [10]. In this study, as the entry of renal artery of rabbit locates at L2 spine level, we blocked the abdominal aorta at posterior site of L3 segment, and injected tumor cell suspension. Such method of generating VX2 spinal metastatic tumor model was successful.

During the process of tumor angiogenesis, the endothelial cells need to be activated for proliferation, migration and scaffolding roles along with peripheral cells, vascular smooth muscle cells and endothelial cells within a complicated blood vessel network which can be regulated by multiple factors [11, 12]. MicroRNA is one kind of single stranded RNA consisting of 20~22 nucleotides, and plays an important role in gene regulation. Single miRNA may modulate hundreds of mRNA transcription via translation inhibition, mRNA degradation and induction of mRNA instability [13]. MiR-17-92 gene family has potent angiogenesis function, including miR-17, miR-18a, miR-20a, miR-19b and miR-92a [14]. MiR mainly exerts its angiogenesis functions via targeting effector proteins. Among all miRs, miR-18 plays an important role in inhibiting the connection of tissue growth factors [15]. This study firstly prepared VX2 rabbit model from which tumor cell suspension was prepared and was transfected with miR-18a. RT-PCR study revealed the expression of

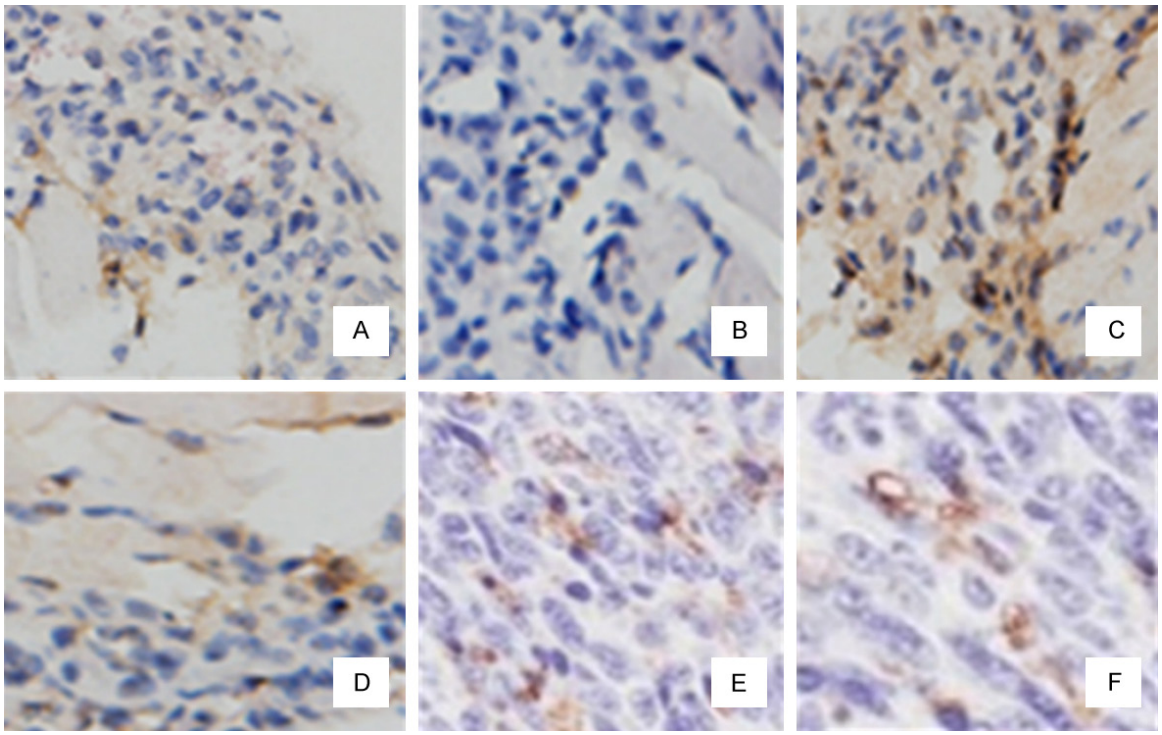

Figure 2. IHC staining of spinal metastatic tumors. A, C and E. Control group; B, D and F. MiR-18a transfected group. A and B. CD31 IHC; C and D. Ki67 IHC; E and F. Micro-vessel staining.

Table 3. VEGF and HIF-1α protein level

Group	N	VEGF	HIF-1α
MiR-18a	10	13.71 ± 4.28*	12.82 ± 4.12*
Control	10	19.53 ± 6.43	19.24 ± 5.98

Note: *, P<0.05 compared to control group.

miR-18a in all transfected cells. Results showed higher miR-18a expression in those cells transfected with miR-18a mimics while miR-18a expression level was decreased in cells transfected with miR-18a inhibitor. When cultivating those tumor cells with miR-18a transfection. At 24 hour, we found slightly retard of those tumor cells compared to control group. Until 96 hour, the activity of transfected cells was significantly depressed. These data collectively suggested the participation of miR-18a in the pathogenesis/progression of malignant tumors. The over-expression of miR-18a may inhibit proliferation of tumor cells. Previous study has found the relationship between MiR-18a overexpression and lowered cell cycle of SW620 cells. In a rectal carcinoma model, we found the tumor inhibition rate as high as 71.8% [16], a result consistent with our results.

CD31, also named as platelet endothelial cell adhesion molecule-1, plays a critical role in inducing adhesion between endothelial cells, especially in the angiogenesis by endothelial tissues [17]. MVD is one golden standard for reflecting tumor angiogenesis. It has been reported to have close relationship between MVD in gastrointestinal mesenchymal tissues and tumor recurrence and metastasis, in addition to prognosis [18]. In this study we identified CD31-positive rate in miR-18a transfection group at 60% and Ki67 positive rate at 50%, with MVD at 62.36 ± 20.33. All of these parameters suggested the down-regulation of CD31, Ki67 and *de novo* angiogenesis for effectively inhibiting spinal metastatic tumors growth.

As the receptor of VEGF, VEFGR can form dimer form for auto-phosphorylation involving multiple cascades including membrane-cytoplasm kinase reaction, transduction of signals, *de novo* angiogenesis, and inhibition of apoptosis and integrity of new vessels. In addition, VEGF can stimulate DNA synthesis of endothelial cells, whose proliferation were observed, and change permeability of blood vessels [19]. The response and relevant modulation of body tis-

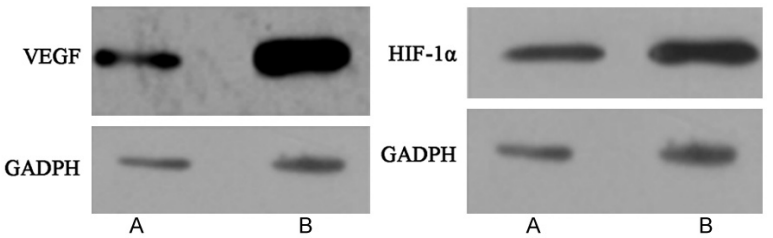

Figure 3. VEGF and HIF-1α expression. A. MiR-18a transfected tumor cells; B. Control cells.

sues and cells under anorexic condition was mainly accomplished by altering HIF-1 factor. The expression of HIF-1α is closely correlated with oxygen status of cells, as depressed expression existed when sufficient oxygen was supplied. When cells are undergone severe hypoxic status, HIF-1α was up-regulated. Such activated HIF-1α will then bind with HIF-1β for translocation into cell nucleus, where the response of tumor cells in hypoxic status was achieved by regulating target gene expression [20]. In this study, Western blotting revealed significantly lower levels of VEGF and HIF-1α in miR-18a transfected tumors. These results indicated the inhibition of VEGF expression by miR-18a, whose over-expression may also inhibit HIF-1α expression. This might be one novel signal regulatory pathway for miR-18a in inhibiting angiogenesis of spinal metastatic vessels.

In summary, miR-18a participates in all process of malignant tumors. The over-expression of miR-18a may decrease the proliferation activity of tumor cells; decreased CD31 and Ki67 levels lowered the number of novel microvessels. It can also inhibit VEGF and HIF-1α expression for impeding the angiogenesis of spinal metastatic tumors. The elevation of miR-18a thus may provide new insights for treating spinal metastatic tumors.

Disclosure of conflict of interest

None.

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