Biomedicine & Pharmacotherapy 107 (2018) 359–367

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

MiR-148b-3p inhibits renal carcinoma cell growth and pro-angiogenic phenotype of endothelial cell potentially by modulating FGF2

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ARTICLE INFO

Keywords:
MiR-148b-3
Renal carcinoma
Invasion
Tube formation
FGF2

ABSTRACT

MicroRNAs (miRNAs) have been implicated in a large number of biological processes such as tumor angiogenesis. MiR-148b-3p has been identified as a tumor suppressor in multiple cancer types and the function of miR-148b-3p in renal carcinoma remains unidentified. In this study, we found that the expression of miR-148b-3p was decreased in renal carcinoma based on GEO analysis and the gain-of-function experiments revealed that miR-148b-3p promoted renal carcinoma cell apoptosis and suppressed cell proliferation, migration *in vitro* and tumor growth *in vivo*. Functionally, the tube formation, invasion and migration capabilities of human umbilical vein endothelial cells (HUVECs) were suppressed by conditioned media derived from renal carcinoma 786-O cells that were transfected with miR-148b-3p mimics. Meanwhile, these conditioned media inhibited the proliferation and promoted apoptosis of HUVECs. The key angiogenesis inducer hypoxia inducible factor-1α (HIF-1α) and the pro-angiogenic mediators were decreased in 786-O cells that were transfected with miR-148b-3p mimics. Mechanistically, miR-148b-3p could target fibroblast growth factor-2 (FGF2) and further impaired the activation of fibroblast growth factor receptor 2 (FGFR2). Taken together, our findings demonstrate that miR-148b-3p attenuates renal carcinoma cell growth, the invasion and tube formation of endothelial cell potentially *via* regulating FGF2-FGFR2 signaling pathway.

1. Introduction

Kidney cancer is the ninth most frequent type of cancer and accounts for approximately 3% of adult malignancies worldwide, among which, clear cell renal cell carcinoma (ccRCC) accounts for ~70% [1,2]. As the most common form of kidney tumors, ccRCC is a high-risk metastatic cancer and is also insensitive to chemo/radiotherapies and targeted therapeutic options [3,4]. Therefore, it is urgent to identify novel functional genes and the molecular mechanisms that are involved in the initiation and progression of renal carcinoma. Angiogenesis, the process of formation of new blood vessels from pre-existing vessels or endothelial cell progenitors, plays vital roles in both physiological and pathological progression [5–9]. Angiogenesis is indispensable for tumor growth, clinical drug resistance and metastasis and it has been described as one of the hallmarks of cancer [10,11]. Multiple pro-angiogenic factors and inflammatory cytokines, including vascular endothelial growth factor A (VEGF-A), platelet-derived growth factor (PDGF), placental growth factor (PGF), angiopoietin-1 (Ang-1) and interleukin 6 (IL-6), are involved in tumor angiogenesis [12]. It has been demonstrated that platelet-derived growth factor D (PDGF-D) increases tumor growth and aggressiveness by activating Notch homolog 1 (Notch1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in breast and pancreatic cancer [13,14]. In addition, PDGF-BB is frequently over-expressed in various tumor tissues. PDGF-BB has been found to play important roles in the acquisition of the epithelial-mesenchymal transition (EMT) phenotype of cancer cell and tumor angiogenesis [15]. Meanwhile, a large number of transcriptional factors are closely related with tumor angiogenesis. For instance, as a master regulator of the transcriptional response of angiogenesis, hypoxia-inducible factor 1α (HIF-1α) regulates numerous vital angiogenic genes, such as VEGF and PDGF [16–19]. In addition, many signaling pathways participate in tumor angiogenesis. For example, fibroblast growth factor 2/fibroblast growth factor receptor 2 (FGF2/FGFR2) signaling pathway plays a critical role in promoting basement membrane remodeling, tip cell migration and stalk cell proliferation [20]. Targeting angiogenesis has been supposed to be a therapeutic method for quite a few cancers including renal carcinoma [21,22].By binding to complementary binding sites within the 3′-untranslated region (3′-UTR)

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https://doi.org/10.1016/j.biopha.2018.07.054
Received 3 April 2018; Received in revised form 9 July 2018; Accepted 10 July 2018

of target mRNAs, miRNAs inhibit the translation of mRNAs or promote their degradation [23]. Functionally, cancer-associated miRNAs control tumor cell growth, invasion, migration and metastasis [24,25]. Lots of evidences have demonstrated that miRNAs could modulate the development of tumor angiogenesis by targeting different angiogenic factors and signaling pathways. For instance, miR-93 promotes tumor angiogenesis by enhancing endothelial cells proliferation, migration and tube formation [26]. MiR-126 may fine-tune angiogenic responses by targeting multiple signaling pathways [27]. Hence, investigations of molecular mechanisms underlying the miRNAs-regulated tumor angiogenesis are essential for improving the outcomes of patients with cancer.

MiR-148b-3p has been found as a tumor-suppressor in numerous types of cancers, including gastric cancer and breast cancer [28,29]. In glioma, miR-148b-3p inhibits the biological activities of glioma cell *via* targeting HOX Transcript Antisense RNA (HOTAIR) [30]. In addition, miR-148b-3p is proved to be capable of inducing cell apoptosis by triggering of caspase-3 and caspase-9 activation, inducing S phase arrest by synchronizing cyclin D1 and p21, and restraining cell invasion in hepatocellular carcinoma [31]. In the present study, we demonstrated that miR-148b-3p not only suppressed renal carcinoma tumor growth but also attenuated the invasion and tube formation of human umbilical vein endothelial cells (HUVECs) through regulating the FGF2/FGFR2 signaling pathway.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethics Committee (No: 20161009) of Zhangjiagang Hospital of traditional Chinese Medicine, Jiangsu Province, China. Written consent was obtained from all participants who were involved in the study. All procedures involving experimental animals were performed in accordance with protocols that were approved by the Committee for Animal Research of Zhangjiagang Hospital of traditional Chinese Medicine and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985).

2.2. Cell lines

An immortalized proximal tubule epithelial cell line derived from normal kidney, HK-2, HUVECs and renal carcinoma cell lines (786-O, A-498 and OS-RC-2) were obtained from Nanjing Cobioer Biotechnology Co., Ltd (Nanjing, Jiangsu, China).HK-2 cells were cultured in Keratinocyte Serum Free Medium Kit (17005-042, Invitrogen, Carlsbad, CA, USA). 786-O and OS-RC-2 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. HUVECs and A-498 cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained in the humidified atmosphere of 5% CO_2 at37 °C. NSC12 (a FGF2/FGFR2 interaction inhibitor) was purchased from Selleck Chemicals (#S7940, Selleck, Texas, USA)

2.3. Clinical tissues

56 cases of renal carcinoma tissues and 23 cases of normal tissues were obtained from the Zhangjiagang Hospital of Traditional Chinese Medicine who underwent surgical treatment between January 1, 2012 and May 30, 2017. The written informed consents for participations in the study were obtained from all patients. Parts of tissue samples were immediately snap-frozen in liquid nitrogen, and parts were fixed in formalin for histological examination.

2.4. Cell transfection

After cultivating in six-well plates at 2×10^5 per well, the cells were subsequently transfected with concentration of 100 nM or 50 nM of miR-185 mimics, or negative control miRNA (miR-NC) (Invitrogen, Carlsbad, CA, USA) for 24 h using Lipofectamine 2000 (#11668019, Invitrogen) according to the instruction manual. For inhibiting the level of miR-148b-3p, cells were transfected with 100 nM miR-148b-3p inhibitor (miR-148b-3p^{inhi}) using Lipofectamine 2000 (#11668019, Invitrogen). MiR-185 mimics, negative control miRNA (miR-NC) and miR-148b-3p inhibitor (miR-148b-3p^{inhi}) were purchased from GenePharma (Shanghai, China). The expression construct of FGF2 was produced by subcloning PCR-amplified full-length human FGF2 complementary DNA (cDNA) into the pcDNA3.1 vector (pcDNA3.1-FGF2). Cells were transfected with pcDNA3.1-FGF2 (0.1 μg/well for 96 well culture plates and 2 μg/well for 6 well culture plates). In co-transfection, miR-148b-3p mimics transfected cells were transfected with pcDNA3.1-FGF2 or empty vector (used as a negative control) for 24 h. After that, the proliferation, migration and invasion assays of the transfected cells were conducted.

2.5. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay

Cells (2×10^4) were seeded into 96-well plates and were cultured for 24 h, 48 h, 72 h or 96 h, respectively. 10 μl of MTT solution (#G4000, Promega, Madison, WI, USA) was added into 96-well plates. The purple formazan crystal was dissolved in 200 μl dimethylsulfoxide (DMSO) and the optical density (OD) value was measured at 490 nm.

2.6. Colony formation assay

1000 cells/well cells were cultured in 6 well culture plates for 4 weeks [32]. Then, cell colonies were stained with crystal violet and the number of cell colonies was counted.

2.7. Tube formation assay

3×10^4 HUVECs were seeded into 48-well plate that has been precoated with reduced growth factor basement membrane matrix (50 μl/well, #A1413201, Invitrogen). After culturing for 24 h with conditioned media, the total pipe length of the tube-like structure was calculated using image-Pro Plus software. Tracks of HUVECs organised into networks of cellular cords were counted in 5 randomly selected view fields. The tube formation indexes were expressed as tube length (mm)/mm^2 area [33].

2.8. Wound healing assay

Cells were seeded into six-well and were treated with 10 μg/ml Mitomycin C (#BP25312, Fisher Scientific, Hampton, NH, USA) for 2 h to eliminate proliferation. Next, confluent monolayer was scratched using a pipette tip and cells were cultured with conditioned media for 24 h. The area of cell-free scratch was photographed at 0 h and 24 h after scratching. The wound healing effect was determined by measuring the percentage of the remaining cell-free area compared with the area of the initial wound.

2.9. Transwell invasion assay

2×10^3 HUVECs in 100 μl basic media was placed into the upper chamber of an insert (#353097, BD Biosciences, Franklin Lakes, NJ, USA) in a 24-well plate (8 μM pore size), and 500 μl conditioned media derived from 786-O cells was added into the lower chamber. 24 h later, cells on the surface of the membrane were removed using a cotton swap, while invaded cells on the bottom side of membrane were stained