Biomedicine & Pharmacotherapy 97 (2018) 616–623

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Original article

MicroRNA-543 promotes the proliferation and invasion of clear cell renal cell carcinoma cells by targeting Krüppel-like factor 6

Fan Yang[a], Jianjun Ma[a], Qisheng Tang[a], Wei Zhang[a], Qiang Fu[a], Jinbo Sun[b], He Wang[a,*], Bin Song[a,*]

[a] Department of Urology, Tangdu Hospital, The Fourth Military Medical University, Xi'an, Shaanxi 710038, China
[b] Department of Urology, The Fourth Military Medical University, Xi'an, Shaanxi 710032, China

ARTICLE INFO

Keywords:
Clear cell renal cell carcinoma
KLF6
miR-543
p21

ABSTRACT

MicroRNA-543 (miR-543) has been suggested as an important regulator of the development and progression of various cancer types. However, the role and biological function of miR-543 in clear cell renal cell carcinoma (ccRCC) remains unclear. Here, we found that miR-543 expression was significantly increased in tumor tissues from ccRCC patients and ccRCC cell lines. We found that overexpression of miR-543 markedly promoted the proliferation and invasion of ccRCC cells, whereas suppression of miR-543 had the opposite effects. Krüppel-like factor 6 (KLF6) was identified as a target gene of miR-543. Furthermore, we found that miR-543 negatively regulates the expression of KLF6 and p21 in ccRCC cells. Overexpression of KLF6 markedly attenuated the oncogenic effect of miR-543 overexpression. Moreover, knockdown of KLF6 significantly reversed the antitumor effect of miR-543 inhibition. Overall, our results demonstrate that miR-543 promotes the proliferation and invasion of ccRCC cells by targeting KLF6 and suggest that miR-543 may serve as a potential therapeutic target for treatment of ccRCC.

1. Introduction

Renal cell carcinoma is one of the most prevalent cancers of the urological system and is the second leading cause of cancer-related death in patients with urological malignancies, with increasing incidence in recent years [1,2]. Clear cell renal cell carcinoma (ccRCC) is the most common histological type of renal cell carcinoma, accounting for ~75% of renal cell carcinoma cases [3]. Despite the advance in cancer treatments, the 5 year survival rate for renal cell carcinoma remains low [4,5]. Therefore, it is important to investigate the underlying mechanism of renal cell carcinoma and develop novel therapeutic approaches for renal cell carcinoma.

MicroRNAs (miRNAs) are endogenous small, non-coding RNAs and are emerging as novel tools for control gene expression [6]. miRNAs negatively regulate gene expression by the binding of a seed sequence to the 3′-untranslated region (3′-UTR) of target mRNA, which leads to the degradation of mRNA and inhibition of translation [6]. miRNAs are involved in the regulation of various biological events, including proliferation, differentiation, and apoptosis. Therefore, miRNAs are involved in the pathogenesis of numerous human diseases, including tumorigenesis [7]. Studies have shown that miRNAs are involved in the development and progression of ccRCC [8]. Dysregulation of miRNAs is associated with the biological characteristics of ccRCC and may serve as a novel and potential biomarkers for prognosis and treatment [9]. Therefore, miRNA-targeted therapy may have potential application for treatment of ccRCC.

Krüppel-like factor (KLF) family consists of zinc finger transcription factors that regulate various biological processes, including proliferation, invasion, and differentiation [10]. KLF6 is a member of the KLF family located on chromosome 10p15, which regulates the expression of pregnancy-specific glycoprotein genes [11]. KLF6 is highly expressed during placental development and is essential for fetus development [12]. The inactivation of KLF6 has been implicated in various cancers, including colorectal cancer [13], gastric cancer [14], hepatocellular carcinoma [15], and prostate cancer [16]. KLF6 is frequently downregulated in tumor tissues, and overexpression of KLF6 promotes apoptosis and inhibits the proliferation of tumor cells [14,17]. Studies also have shown that KLF6 expression is significantly decreased in ccRCC tissues and cell lines and that KLF6 is associated with the regulation of the metastasis and proliferation of ccRCC cells [18,19].

Abbreviations: ccRC, Cclear cell renal cell carcinoma; CCK-8, cell counting kit-8; KLF6, Krüppel-like factor 6; miRNAs, microRNAs; miR-543, microRNA-543; 3′-UTR, untranslated region; FBS, fetal bovine serum; RT-qPC, Rreal-time quantitative polymerase chain reaction

* Corresponding authors.
E-mail addresses: wang_hewh@163.com (H. Wang), binsong_bs@163.com (B. Song).

http://dx.doi.org/10.1016/j.biopha.2017.10.136
Received 11 August 2017; Received in revised form 17 October 2017; Accepted 24 October 2017

Therefore, KLF6 is a candidate tumor suppressor gene that may serve as a potential target for treatment of cancers.

An increasing number of studies have shown that miR-543 is a tumor-associated miRNA involved in many types of cancers, including colorectal cancer [20], gastric cancer [21], osteosarcoma [22], and prostate cancer [23]. However, little is known about the role of miR-543 in ccRCC. In this study, we aimed to investigate the potential role and underlying mechanism of miR-543 in the progression of ccRCC. Here, we found that miR-543 expression was significantly increased in tumor tissues from ccRCC patients and ccRCC cell lines. Overexpression of miR-543 markedly promoted the proliferation and invasion of ccRCC cells, whereas suppression of miR-543 showed the opposite effects. KLF6 was identified as a target gene of miR-543. KLF6 expression was inversely correlated with miR-543 expression in ccRCC specimens. Furthermore, we found that miR-543 negatively regulates the expression of KLF6 and p21 in ccRCC cells. Overexpression of KLF6 markedly attenuated the oncogenic effect of miR-543 overexpression. Moreover, knockdown of KLF6 significantly reversed the antitumor effect of miR-543 inhibition. Taken together, our study suggests that miR-543 promotes the proliferation and invasion of ccRCC cells by inhibiting KLF6, thus providing a potential therapeutic target for treatment of ccRCC.

2. Materials and methods

2.1. Tissue specimens

Twenty paired tissue specimens of ccRCC tissues and adjacent noncancerous tissues were obtained from twenty ccRCC patients undergoing surgery at Tangdu Hospital. The fresh tissues was frozen in liquid nitrogen immediately after dissection during the surgery. All tissue samples were clinically and pathologically confirmed to be of the clear cell type and classified according to the 2011 Union for International Cancer Control TNM classification. Adjacent tissues (located > 3 cm away from the tumor) were used as normal tissues for control. All tissue samples were stored at −80 °C until RNA extraction. The written informed consents regarding tissue specimens for research purposes were obtained from all the participants. This study was approved by the Institutional Human Experiment and Ethic Committee of Tangdu Hospital and was conducted in accordance with Helsinki Declaration. The characteristics of ccRCC clinical specimens were as follows: Sex (male, n = 13 and female, n = 7); age (> 55, n = 15 and ≤55, n = 5); tumor size (> 5 cm, n = 14 and ≤5 cm, n = 6); TNM stage (T1a, n = 4; T1b, n = 7; T2a, n = 3; T2b, n = 1; T3, n = 3; T4, n = 2); distant metastasis (yes, n = 8 and no, n = 12).

2.2. Cell cultures

Human normal kidney tubule epithelial cell line HK-2 and ccRCC cell lines including 786-O, A-498, Caki-1, and Caki-2 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HK-2 cells were grown in Keratinocyte Serum Free Medium supplemented with bovine pituitary extract and human recombinant epidermal growth factor. 786-O cells were grown in RPMI-1640 medium (Gibco, Rockville, MD, USA); A-498 cells were grown in Eagle's Minimum Essential Medium (ATCC); Caki-1 and Caki-2 cells were cultured in McCoy's 5a Medium Modified medium (ATCC). In addition, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin mix (Sigma-Aldrich, St. Louis, MO, USA) were supplemented in the medium. Cells were maintained at 37 °C in an atmosphere of 95% air and 5% carbon dioxide (CO_2).

2.3. Real-time quantitative PCR (RT-qPCR)

The total RNAs from freshly frozen tissues or cultured cells were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The reverse transcription of miRNA and mRNA was conducted using TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and M-MLV Reverse Transcriptase (Thermo Fisher Scientific, Inc., Waltham, MA, USA), respectively. PCR amplification was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) on an MX3005P QPCR system (Stratagene, La Jolla, CA, USA) following PCR amplification procedure: denaturing (95° C, 10 s), annealing (55° C, 15 s), and extension (72° C, 30 s) for 45 cycles. PCR amplification was performed using the following primers: miR-543 forward: 5′-CCAGCTACACTGGGCAGCAGCAATTCATGTTT-3′ and reverse: 5′-CTCAACTGGTGTCGTGGA-3′; U6 snRNA forward: 5′-GCGCGTCGTGAAGCGTTC-3′ and reverse: 5′-GTGCAGGGTCCGAGGT-3′; KLF6 forward: 5′-CTCTCAGCCTGGAAGCTTTTAGCCTAC-3′ and reverse: 5′-ACAGCTCCGAGGAACTTTCTCCCA-3′; p21 forward: 5′-TTAGCAGCGGAACAAGGAGT-3′ and 5′-AGCCGAGAGAAAACAGTCCA-3′; GAPDH forward: 5′-AATGGGCAGCCGTTAGGAAA-3′ and reverse: 5′-TGAAGGGGTCATTGATGGCA-3′. U6 snRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal controls. Relative gene expression analysis was performed using the comparative $2^{-\Delta\Delta Ct}$ method.

2.4. Cell transfection

The miR-543 mimics (5′-AAACAUUCGCGGUGCACUUCUU-3′), miR-543 inhibitor (5′-AAGAAGUGCACCGCGAAUGUUU-3′) and negative controls (NC; 5′-UCACAACCUCCUAGAAAGAGUAGA-3′) were purchased from Genepharma (Shanghai, China) and transfected into cells for a final concentration of 20 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. Specific siRNAs targeting KLF6 and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and transfected into cells according to the manufacturer's instructions. The open reading frame of KLF6 was inserted into the pcDNA3.1 vector to generate the KLF6 expression vector. KLF6 vectors (200 ng) were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. After 48 h of transfection, the transfection efficacy was confirmed by RT-qPCR or western blot analysis and cells were collected for the following experiments.

2.5. Cell counting kit-8 (CCK-8) assay

Cell proliferation was determined using the CCK-8 assay. Briefly, cells were seeded into 96-well plates at a density of 1×10^4 cells/well and grown until 80% confluence. After transfection of miRNAs or vectors for 48 h, 10 μl CCK-8 solution (Sigma-Aldrich) was added to the cells and cultured for 2 h at 37 °C. The optical density value was then measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

2.6. Cell cycle assay

Cell cycle distribution was measured by flow cytometer. Cells were serum-free starved for 24 h to synchronized cell cycle. After transfection of miR-543 mimics or inhibitor for 48 h, a total of 1×10^6 cells were harvested by trypsinization (0.25% trypsin) and fixed with 70% ethanol at 4 °C for 1 h. Then, cells were washed with ice-cold phosphate buffer solution (PBS) and centrifuged at 1,000 *g* for 5 min. The palleted cells were re-suspended in 500 μl binding buffer containing 50 μg/ml PI and 50 μg/ml RNase and incubated for 15 min in the dark at room temperature. Then, cells were detected by flow cytometry (BD Biosciences, San Jose, CA, USA). The data were analyzed by BD CellFIT software.

2.7. Transwell invasion assay

Cell invasive ability was evaluated by the Transwell invasion assay.