FEBS Letters 589 (2015) 3148–3153

journal homepage: www.FEBSLetters.org

MicroRNA-490-3p regulates cell proliferation and apoptosis by targeting HMGA2 in osteosarcoma

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ARTICLE INFO

Article history:
Received 24 July 2015
Revised 20 August 2015
Accepted 20 August 2015
Available online 1 September 2015

Edited by Tamas Dalmay

Keywords:
MicroRNA-490-3p
High mobility group a isoform 2
Osteosarcoma
Proliferation

ABSTRACT

MicroRNA-490-3p (miR-490-3p) has been implicated in several human malignancies; however, its potential functions and the underlying molecular mechanisms in osteosarcoma progression remain largely unclear. Here, we showed that miR-490-3p was down-regulated in osteosarcoma cell lines. Ectopic expression of miR-490-3p decreased cell proliferation, induced G1 arrest and apoptosis in vitro and inhibited tumorigenicity in a mouse xenograft model. Furthermore, miR-490-3p bound directly to HMGA2 mRNA 3′UTR and mediated a decrease in HMGA2 mRNA and protein expression. Re-expression of HMGA2 reversed the inhibitory effects of miR-490-3p. Further investigations showed an inverse correlation between low miR-490-3p and high HMGA2 expression in osteosarcoma tissues. Taken together, our results suggest that miR-490-3p functions as a potential tumor suppressor by down-regulating HMGA2 expression directly, and it may represent a potential therapeutic target for patients with osteosarcoma.

1. Introduction

Osteosarcoma is the most common primary bone malignancy and is derived from primitive bone-forming mesenchymal cells; it occurs most frequently in adolescents and young adults [1]. Although the development of adjuvant and neo-adjuvant chemotherapy has improved the survival rate of patients with osteosarcoma from 15% to 60–70% [2,3], overall clinical outcomes remain unsatisfactory, especially for patients with metastasis or recurrent osteosarcoma. In recent years, evidence has accumulated of the molecular alterations involved in osteosarcoma progression. However, the highly complex molecular mechanism of osteosarcoma has not been elucidated fully. Furthermore, it is important to search for novel markers for osteosarcoma to optimize therapeutic strategies and predict clinical outcomes.

Over the past decade, a class of endogenous non-coding small RNAs of ~22 nucleotides, known as microRNAs (miRNAs), have emerged as potential regulators of various critical biological processes, such as the proliferation, differentiation, progression and apoptosis of tumor cells [4,5]. miRNAs function as post-transcriptional regulators, pairing with the 3′ untranslated region (3′UTR) of target mRNAs either by cleaving mRNA molecules or by inhibiting translation [6,7]. Therefore, dysregulation of oncogenic miRNAs (targeting tumor suppressor genes) or tumor-suppressive miRNAs (targeting oncogenes) may lead to dysfunction of cancer cells, inducing proliferation and apoptosis [8].

Alteration in miR-490-3p has been reported in several types of cancer. Reports have shown that miR-490-3p functions as a tumor suppressor in A549 lung cancer cells, gastric cancer and ovarian carcinoma by targeting CCND1, SMARCD1 and CDK1 [9–11]. miR-490-3p plays an oncogenic role in hepatocellular carcinoma cells by targeting endoplasmic reticulum–Golgi intermediate compartment protein 3 (ERGIC3) [12]. Thus, whether miR-490-3p acts as a tumor suppressor or an oncogene may depend on the cellular context. Previously, Nakatani et al. revealed that measurement of miR-490-3p achieved sufficient statistical power to predict prognosis in Ewing's sarcoma [13]. However, the function of miR-490-3p in osteosarcoma pathogenesis, as well as the molecular mechanisms by which miR-490-3p exerts its functions and modulates the malignant phenotypes of osteosarcoma cells, has not been fully understood.

In this study, we tested the hypothesis that miR-490-3p may also function as a tumor suppressor in osteosarcoma. As expected, miR-490-3p was significantly down-regulated in osteosarcoma

Author contributions: Wei Liu and Guoxing Xu contributed to the conception of the study, performed the experiments and edited the manuscript; Wei Liu, Huaqiang Liu, Tingting Li contributed to data analyses and manuscript preparation; Wei Liu and Guoxing Xu contributed to statistical analysis; all authors read and approved the manuscript.

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http://dx.doi.org/10.1016/j.febslet.2015.08.034
0014-5793/

cells, and ectopic expression of miR-490-3p inhibited cell proliferation in vitro and tumorigenicity in vivo. We further demonstrated that miR-490-3p induced G1/S cell cycle arrest and apoptosis in osteosarcoma cells. Interestingly, we screened and identified a novel miR-490-3p target within high mobility group a isoform 2 (HMGA2). Over-expression of HMGA2 may reverse the suppressive function of miR-490-3p in osteosarcoma cells. In addition, a negative correlation between the expression of miR-490-3p and HMGA2 was found in osteosarcoma specimens. Taken together, our results indicate that miR-490-3p, acting as a tumor suppressor and potential therapeutic target against osteosarcoma, may repress tumor proliferation by direct inhibition of HMGA2 expression.

2. Materials and methods

2.1. Cell lines and cell culture

Osteosarcoma cell lines (HOS, Saos2, U2OS, MG63), the immortalized normal osteoblastic cell line hFOB 1.19, and HEK293T were obtained from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Osteosarcoma cell lines were grown in RPMI1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. HEK293T cells were cultured in DMEM with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. All the cell lines were cultured at 37 °C in a humidified incubator with 5% CO_2.

2.2. Osteosarcoma tissues

Osteosarcoma and corresponding adjacent non-tumorous tissues were obtained from 22 Chinese patients who underwent surgery between 2011 and 2013 at Changhai Hospital (Shanghai, China). All surgically resected tissues were immediately preserved in liquid nitrogen. Detailed clinical pathology information for all samples was available. The protocol was approved by the Ethics Committee of the Second Military Medical University, Shanghai, China. Informed consent was obtained from each patient.

2.3. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA, including miRNA, was extracted from tissues or cells using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed to cDNA using a Prime Script RT reagent Kit (Takara, Dalian, China). Real-time PCR was performed with the SYBR green Premix Ex Taq II (Takara, Dalian, China) using an Applied Biosystems Step One Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). β-Actin and U6 were used as the endogenous controls for measurement of mRNA expression level and miRNA expression analysis, respectively. The sequences of the primers for HMGA2 were 5′-CCAACCGGTGAGCCCTCT-3′ (forward) and 5′-TTGAGCTGCTTTAGAGGGAC-3′ (reverse). The sequences of the primers for β-actin were 5′-CTCCATCCTGGCCTC GCTGT-3′ (forward) and 5′-GCTGTCACCTTCACCGTTCC-3′ (reverse).

2.4. Construction of plasmids

The miR-490-3p agomir and negative control were purchased from Ribobio (Guangzhou, China). The 3′UTR segment of HMGA2 was amplified from normal human cDNA and inserted into the psi-CHECK2 vector (Promega, Madison, WI, USA), downstream of the luciferase stop codon. The primers for HMGA2-3′UTR were 5′-AA CTCGAGCGATTTCTACCTCAGCAGCA-3′ (forward) and 5′-ATGCGGC CGCTTTCGCTCCTCCCACCTCAT-3′ (reverse). A mutant form of HMGA2 3′UTR was generated by the Quik Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The HMGA2 fragment was amplified from the *Homo sapiens* full open reading frame cDNA library and cloned into a pcDNA3.1(+) vector to generate pcDNA3.1-HMGA2 recombinant plasmids.

2.5. MTT and colony formation assays

Cells (5×10^3) transfected with miR-490-3p agomir or negative control were seeded into 96-well plates and stained with 0.5 mg/ml sterile MTT dye (Sigma–Aldrich, St. Louis, MO, USA) for 4 h at 37 °C, following which the culture medium was discarded and an extra 150 μl dimethyl sulfoxide (Sigma–Aldrich) added. The absorbance at 490 nm was measured at 24, 48, 72 and 96 h after transfection. The colony formation assay was performed as described previously [14]. Briefly, cells were trypsinized and seeded on six-well plates (400 cells/well). After 2 weeks, the colonies were stained with 1% crystal violet and the number of colonies was counted.

2.6. Tumor xenografts

All protocols involving animal experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai, China. The 6-week-old BALB/c-nude mice were randomly divided into two groups ($n = 5$ per group) and Saos2 cells over-expressing miR-490-3p or negative control (2×10^6) were injected subcutaneously into the scapula of each mouse. Tumor volume was determined every week using an external caliper and calculated using the equation $(L \times W^2)/2$. The mice were sacrificed 6 weeks after inoculation and the tumors were excised and weighed.

2.7. Cell cycle and cell apoptosis analysis

Osteosarcoma cells were transfected with the indicated oligos or plasmids in six-well plates. After transfection for 48 h, the cells were collected and washed with PBS three times. For cell cycle analysis, the collected cells were fixed with 70% ethanol overnight at −20 °C, and subsequently treated with RNaseA (Sigma–Aldrich) for 30 min at 37 °C, following which they were stained with 50 μg/ml propidium iodide (PI) (Sigma–Aldrich) for another 10 min. After incubation, the cells were subjected to flow cytometry analysis using a FACSCalibur (BD Biosciences, Bedford, MA, USA). Apoptosis was detected by Annexin V-FITC (BD Biosciences) and PI (BD Biosciences) staining according to the manufacturer's instructions, followed by flow cytometry analysis.

2.8. Western blotting

The cells were lysed in RIPA buffer (Thermo Scientific, Rockford, IL, USA) containing complete protease inhibitors. The protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Western blotting was performed as a standard protocol. In brief, 40 μg total proteins were separated by 10% SDS–PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk and then incubated with primary antibody overnight at 4 °C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized using an enhanced chemiluminescence kit (Millipore). GAPDH was normalized as a loading control. The antibodies used in this study were as follows. Rabbit monoclonal antibodies against p21 (#2947), cleaved Caspase 9(c-Casp, #7237), cleaved PARP (c-PARP, #9541), HMGA2 (#8179) and GAPDH were obtained from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-cyclin D1 (sc-753) was obtained from Santa