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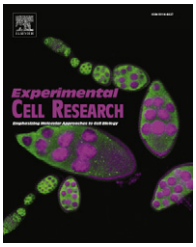

Research Article

Downregulation of miR-210 expression inhibits proliferation, induces apoptosis and enhances radiosensitivity in hypoxic human hepatoma cells in vitro

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ABSTRACT

Hypoxia is a common feature of solid tumors and an important contributor to tumor radioresistance. miR-210 is the most consistently and robustly induced microRNA under hypoxia in different types of tumor cells and normal cells. In the present study, to explore the feasibility of miR-210 as an effective therapeutic target, lentiviral-mediated anti-sense miR-210 gene transfer technique was employed to downregulate miR-210 expression in hypoxic human hepatoma SMMC-7721, HepG2 and HuH7 cells, and phenotypic changes of which were analyzed. Hypoxia led to an increased hypoxia inducible factor-1α (HIF-1α) and miR-210 expression and cell arrest in the G_0/G_1 phase in all cell lines. miR-210 downregulation significantly suppressed cell viability, induced cell arrest in the G_0/G_1 phase, increased apoptotic rate and enhanced radiosensitivity in hypoxic human hepatoma cells. Moreover, apoptosis-inducing factor, mitochondrion-associated, 3 (AIFM3) was identified as a direct target gene of miR-210. AIFM3 downregulation by siRNA attenuated radiation induced apoptosis in miR-210 downregulated hypoxic human hepatoma cells. Taken together, these data suggest that miR-210 might be a potential therapeutic target and specific inhibition of miR-210 expression in combination with radiotherapy might be expected to exert strong anti-tumor effect on hypoxic human hepatoma cells.

Introduction

Owing to an imbalance in oxygen supply and demand, hypoxia is a common feature of pathological conditions such as tissue ischemia and inflammation, as well as solid tumors, including hepatocellular carcinoma (HCC). Hypoxia is an important contributor to tumor radioresistance [1,2]. Oxygen can chemically modify the radiation-induced DNA damage and produce adducts that are difficult to repair by cells. Hypoxia can abolish oxygen effect and lead to decreased radiosensitivity [3]. In addition, hypoxia may also modulate tumor radiosensitivity through biological effects [4]. Signaling pathways stimulated by hypoxia are commonly activated in tumors. Hypoxia inducible factor-1 (HIF-1) is one of the key mediators of hypoxia signaling pathways [5]. HIF-1 is a heterodimeric transcription factor consisting of two subunits, HIF-1α and HIF-1β. HIF-1β is constitutively expressed while HIF-1α is rapidly degraded

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in normoxic conditions and can be stabilized by hypoxia [6,7]. Therefore, hypoxia can induce HIF-1 expression, which modulates more than 100 genes involved in regulating important processes such as angiogenesis, metabolism, proliferation and apoptosis [8].

Recently, some microRNAs (miRs) have been found to be regulated by hypoxia [9,10]. miRs are a family of small (20 to 22 nucleotide in length) noncoding RNAs that regulate gene expression by sequence-selective targeting of mRNAs, inducing translational repression or mRNA degradation, depending on the degree of complementarities between miRs and the target sequences [11]. miRs have emerged as important regulators in developmental, physiological and pathological settings including cell growth, differentiation, apoptosis, metabolism and tumorigenesis [12]. Among these hypoxia-induced miRs, miR-210 is the most consistently and robustly induced miRNA under hypoxia in different types of tumor cells and normal cells [13,14]. Several miR-210 targets which influence cell proliferation, ATP metabolism, and angiogenesis have been identified such as E2F3, MYC antagonist (MNT), ephrin-A3 (EFNA3) and iron-sulfur cluster scaffold protein (ISCU) [15–20]. Based on the functions of these targets, we hypothesize that miR-210 might be a logical novel target to overcome hypoxia-induced radioresistance in cancer.

In the present study, human hepatoma cells with stable integration of the anti-sense miR-210 were generated through lentiviral-mediated gene transfer, which followed by exposure to hypoxia. Cell proliferation, apoptosis and radiosensitivity were detected to explore the effect of miR-210 downregulation on biological function of hypoxic human hepatoma cells and its mechanism.

Materials and methods

Plasmid construction

MiR-210 anti-sense (5′-TCAGCCGCTGTCACACGCACAG-3′) or scramble (5′-TTCTCCGAACGTGTCACGTTTC-3′) oligos (GenePharma Co. Shanghai, China) and the target vector pGLV-H1-GFP (GenePharma Co. Shanghai, China) were cut with BamHI and EcoRI. After gel purification, the vector and insert were ligated overnight using T4 DNA Ligase, resulting in pGLV-anti-210-GFP and pGLV-scr-GFP, and sequenced.

Generation of stable cell lines

Cells with stable integration of the anti-sense miR-210 or scramble sequence were generated through lentiviral-mediated gene transfer [21]. To generate the respective viruses, 293 T cells were transfected with the lentiviral vector, pGLV-anti-210-GFP or pGLV-scr-GFP, and the packaging plasmid PG-P1-VSVG, PG-P2-REV and PG-P3-RRE according to standard protocols. The target human hepatocarcinoma cells SMMC-7721, HepG2 and HuH7 were infected with both of the viruses (encoding either anti-sense miR-210 or scramble sequence) and selected using puromycin. Clonal cell populations carrying anti-sense miR-210 or scramble sequence were obtained by limiting dilution of 100–300 cells in three 96-well plates. After 4 weeks, single clones were analyzed for positive GFP signals. The positive clones were expanded for additional testing.

Cell line and cell culture

The 293 T, p53 wild-type human hepatocarcinoma cell lines SMMC-7721 and HepG2 and p53 mutant human hepatocarcinoma cell line HuH7 were purchased from the Type Culture Collection of the Chinese Academy of Sciences and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin, in a 37 °C incubator in a 5% CO_2 humidified atmosphere. Cells treated with hypoxia were exposed to a steady flow of low-oxygen gas mixture (1% O2, 5% CO2, 94% N2) in a modular incubator chamber (MiniGalaxy, RSBiotech, Irvine, Scotland).

Real-time Reverse transcription-Polymerase Chain Reaction (RT-PCR) analysis of HIF-1 α mRNA, AIFM3 mRNA and miR-210 expression

Total cellular RNA was isolated using Trizol reagent (Sangon Inc. Shanghai, China) according to the manufacturer's protocol. For HIF-1α and AIFM3 mRNA expression analysis total RNA was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and analyzed using the SYBR Green PCR Master Mix (Applied Biosystems) on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) using standard conditions. Fold changes in HIF-1α and AIFM3 mRNA expression were quantified with the $2^{-\Delta\Delta CT}$ relative quantification method using β-actin as house keeping control. The primer sequences for HIF-1α were F-ATCGCGGGGACCGATT and R-CGACGTTCAGAACTTATCTTTTTCTT. The primer sequences for AIFM3 were F-GGGAGCCATCCACACTGGTC and R-TCTTGCCAAACATGGCGGTC. The primer sequences for β-actin were F-GATCATTGCTCCTCCTGAGC and R-TGTGGACTTGGGAGAGGACT. For miR-210 expression analysis total RNA was transcribed using TaqMan microRNA reverse transcription kit (Applied Biosystems) according to manufacturer's instructions. miR-210 expression was assessed by real-time PCR according to the TaqMan MicroRNA Assay protocol (Applied Biosystems). The 20 μL reactions were incubated in a 96-well optical plate at 95 °C for 3 min, followed by 40 cycles of 95 °C for 12 s, and 58 °C for 30 s. Fold changes in miR-210 expression between treatments and controls were determined by the $2^{-\Delta\Delta CT}$ method, normalizing the results to U6 RNA expression level.

Cell viability assay

Cell viability was measured by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Sigma, St. Louis, MO, USA) after 24, 48 and 72 h normoxic or hypoxic culture. Cells were seeded into 96-well culture plates at 4.0×10^3 per well. After 24, 48 and 72 h normoxic or hypoxic culture, 200 μL MTT (5 mg/mL) was added to each well, and the cells were incubated at 37 °C for additional 4 h. After incubation, MTT-containing medium was removed and 150 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals. Optical densities (OD) were determined on a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 490 nm. Cell viability was calculated as: OD value/cell number, and the cell viability of control cells after 24 h normoxic culture was taken as 100%.

Flow cytometric analysis of cell cycle and apoptosis

Cells were harvested and fixed overnight with 70% ethanol at 4 °C, followed by resuspension in 500 μL of PBS. After addition of 10 μL