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miR-505 enhances doxorubicin-induced cytotoxicity in hepatocellular carcinoma through repressing the Akt pathway by directly targeting HMGB1

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ABSTRACT

Compelling evidence has suggested the relevance of miRNAs in resistance to chemotherapeutic agents in HCC. miR-505 was reported to be downregulated and function as a tumor suppressor in HCC cells by binding to high-mobility group box 1 (HMGB1). Whether miR-505/HMGB1 axis was involved in ADM cytotoxicity in HCC remains to be addressed. The aim of this study was to explore the effect of miR-505/HMGB1 axis on ADM cytotoxicity in HCC cells. MTT, flow cytometry analysis, and caspase-3 activity assays were conducted to assess ADM-induced cytotoxicity. The protein level of phosphorylation of histone H2 AX at Ser139 (γH2AX) was detected to evaluate DNA damage. The effects of miR-505 and HMGB1 on the protein kinase B (Akt) pathway were determined by examining the protein levels of phosphorylated Akt (p-Akt), Akt, phosphorylated glycogen synthase kinase-3β (p-GSK-3β), and GSK-3β. We found that HMGB1 knockdown and miR-505 overexpression exacerbated ADM-induced cell viability inhibition, enhanced ADM-induced apoptosis, and increased caspase-3 activity in ADM-treated HCC cells. However, HMGB1 overexpression reversed the effects of miR-505 on ADM-induced cytotoxicity in HCC cells. HMGB1 knockdown and miR-505 overexpression promoted ADM-induced DNA damage in HCC cells, which was abated by HMGB1 overexpression. On a molecular mechanism level, HMGB1 silencing and miR-505 overexpression inactivated the Akt pathway in HCC cells, while exogenous HMGB1 resisted miR-505-induced Akt pathway inactivation. In conclusion, miR-505 overexpression enhanced ADM-induced cytotoxicity in HCC cells, at least partly by targeting HMGB1 and inactivating the Akt pathway.

1. Introduction

Hepatocellular carcinoma (HCC), one of the most common malignancies, is the third leading cause of cancer-related death worldwide due to the late diagnosis and poor prognosis [1]. The poor prognosis is mainly attributable to high recurrence and the lack of treatment modalities [2]. Although surgical resection and liver transplantation have high rate of cure for early stage HCC patients, a large proportion of patients with HCC are diagnosed at advanced stages when surgical resection is no longer an option [3]. In these cases, systemic chemotherapy is considered as the most commonly used therapeutic strategy to prolong the survival time of HCC patients. Doxorubicin (ADM), an anthracycline based agent which induces cancer cell apoptosis by intercalating into their DNA to trigger a DNA damage response, has been widely used systemically or locally for the treatment of HCC [4]. However, the emergence of drug resistance and its mostly irreversible cardiotoxicity during long-term ADM chemotherapy severely block the successful management of HCC [5]. Given this, it is urgently needed to develop novel and efficient therapeutic approaches to overcome the ADM-resistance and improve the anti-tumor effects.

Recent studies on HCC chemoresistance have documented that aberrantly expressed non-coding genes, especially microRNAs (miRNAs), are closely implicated in HCC chemoresistance [6]. miRNAs are evolutionarily conversed, endogenous, single-stranded RNAs of 19–25 nucleotides, which induce either mRNA degradation or translational suppression *via* perfect or imperfect matching to the 3′-untranslated region (3′-UTR) of the target mRNA [7]. It has been suggested that miRNAs play significant roles in a wide range of

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physiological and pathological cellular processes [8]. Compelling evidence has suggested the relevance of miRNAs not only in carcinogenesis, but also in resistance to chemotherapeutic agents in various cancer types, including HCC [9]. For example, it was reported that miR-105/93-3p activated the Wnt/β-catenin signaling by downregulating SFRP1 and thereby promoted stemness, chemoresistance, and metastasis in triple negative breast cancer (TNBC), which may be utilized as a diagnostic biomarker for early- and late-stage TNBC [10]. miR-181a was discovered to provoke sorafenib resistance of HCC through downregulation of RASSF1 expression [11]. Hsa-let-7b enhanced anti-tumor effect of panobinostat by downregulation of HMGA2 in hepatocellular carcinoma HepG2 cells [12]. Among these human cancer-correlated miRNAs, miR-505 was documented to be abnormally expressed in several tumors, such as breast cancer [13] and bladder cancer [14], suggesting the involvement of miR-505 in the initiation and progression of tumor. We previously demonstrated that miR-505 was downregulated in HCC cells and restoring expression of miR-505 suppressed proliferation, invasion and epithelial-mesenchymal transition (EMT) in HCC cells by binding to the 3′-UTR of high-mobility group box 1 (HMGB1) [15]. HMGB1, a highly conserved chromatin-binding protein implicated in diverse biological processes [16], has been documented to promote sorafenib resistance in HCC cells *in vitro* and *in vivo* [17]. HMGB1 has also been reported to contribute to doxorubicin resistance in breast cancer and osteosarcoma cells [18,19]. Accordingly, we hypothesized whether miR-505/HMGB1 axis was involved in the regulation of ADM-induced cytotoxicity in HCC.

In the present study, we demonstrated that miR-505 overexpression enhanced ADM-induced cytotoxicity in HCC by suppressing cell viability, inducing apoptosis, and increasing caspase-3 activity through repressing the Akt pathway *via* targeting HMGB1, providing a novel insight into the regulation of ADM cytotoxicity in HCC.

2. Materials and methods

2.1. Cell culture and transfection

Human HCC cell lines MHC97 and HepG2 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO_2. miR-505 mimics (miR-505), negative control miRNA (Con-miR) were obtained from Biomics Biotechnologies Co., Ltd. (Nantong, China). HMGB1 siRNA (HMGB1-siR), control siRNA (Con-siR) were purchased from Cell Signaling (Beverly, MA, USA). PcDNA-HMGB1 (HMGB1) and pcDNA empty vector (Vector) were synthesized by GenaPharma (Shanghai, China). MHC97 and HepG2 cells were seeded into 24-well plates and incubated overnight. Then cells were transfected with HMGB1-siR, Con-siR, miR-505, Con-miR, miR-505 + HMGB1, or miR-505 + Vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.2. Western blot

Total protein of MHCC97 and HepG2 cells were extracted using ice-cold RIPA buffer (Beyotime, Nantong, China) with protease inhibitor PMSF (Sigma, St Louis, MO, USA). The protein lysates (20 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk for 2 h in PBS-Tween-20 (PBST) and then incubated overnight at 4 °C with the primary antibodies including phosphorylation of histone H2AX at Ser139 (γH2AX) (Cell Signaling Technologies, Danvers, MA, USA), phosphorylated Akt (p-Akt; Ser473, Cell Signaling Technologies), Akt (Cell Signaling Technologies), phosphorylated glycogen synthase kinase-3β (p-GSK-3β; Ser9, Cell Signaling Technologies), GSK-3β (Cell Signaling Technologies), or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with horseradish peroxidase (HRP)-conjugated immunoglobulin G (Santa Cruz Biotechnology) for 1 h at room temperature. The protein bands were visualized using the typhoon scanner (Amersham Biosciences, Piscataway, NJ, USA) and quantified with quantity one (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Cell viability assay

3 -(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to assess cell viability. Briefly, MHCC97 and HepG2 cells were seeded in 96-well plates at a density of 1×10^4 cells/well in 100 μL of medium and incubated overnight to allow attachment. Then, cells were treated with the indicated concentrations of ADM (0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, or 2.25 μM) (Sigma). After an incubation period of 48 h at 37 °C, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated for an additional 4 h. After the supernatant was removed, 150 μL of dimethyl sulfoxide (DMSO) was added to dissolve the insoluble formazan precipitates. Cell viability was assessed by measuring the absorbance at a wavelength of 490 nm with a microplate reader (Bio-Rad Laboratories). After transfection, MHCC97 and HepG2 cells were seeded into 96-well plates and incubated with 0.5 and 0.75 μM ADM for 48 h, respectively. Subsequently, cell viability was detected *via* MTT assay as above.

2.4. Flow cytometry analysis

Cell apoptosis was detected using Annexin V/fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kits (Beyotime). Briefly, approximately 5×10^5 transfected MHCC97 and HepG2 cells were seeded into 6-well plates and exposed to 0.5 and 0.75 μM ADM for 48 h, respectively. Subsequently, cells were harvested, washing with PBS three times, and resuspended in 100 μL binding buffer. Cells were then double stained with 5 μL of Annexin V/FITC and 5 μL of PI for 15 min at room temperature in the dark. The apoptotic cells were analyzed using a FACScan flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

2.5. Caspase-3 activity assay

The activity of caspase-3 was determined using a caspase-3 activity kit (Beyotime). Briefly, the transfected MHCC97 and HepG2 cells were seeded at a density of 3.0×10^5 cells/well and treated with 0.5 and 0.75 mM ADM for 48 h, respectively. Subsequently, cells were harvested, washed with PBS, and resuspended in the cold lysis buffer for 30 min. After centrifugation, the suspernatants (10 μL) were collected and then mixed with 80 μL reaction buffers containing 10 μL of caspase-3 substrate (Ac-DEVD-pNA, 2 mM) and incubated at 37 °C for 2 h in the dark. The caspase-3 activity was determined by detecting the absorbance at 405 nm using a microplate reader (Bio-Rad Laboratories).

2.6. Statistical analysis

Data are presented as mean ± standard deviation (SD). Comparisons between two or more groups were carried out using Student's *t*-test or one-way analysis of variance (ANOVA) using SPSS 19.0 software (IBM Corporation, Armonk, NY, USA). $P < 0.05$ was considered as statistically significant.