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Cisplatin-induced downregulation of miR-199a-5p increases drug resistance by activating autophagy in HCC cell

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Systemic chemotherapy plays an important role in the treatment of patients with advanced liver cancer. However, chemoresistance to cisplatin is a major limitation of cisplatin-based chemotherapy in the clinic, and the underlying mechanism of such resistance is not fully understood. In the study, we found that miR-199a-5p levels were significantly reduced in HCC patients treated with cisplatin-based chemotherapy. Cisplatin treatment also resulted in decreased miR-199a-5p levels in human HCC cell lines. Forced expression of miR-199a-5p promoted cisplatin-induced inhibition of cell proliferation. Cisplatin treatment activated autophagy in Huh7 and HepG2 cells, which increased cell proliferation. We further demonstrated that downregulated miR-199a-5p enhanced autophagy activation by targeting autophagy-associated gene 7 (ATG7). More important, autophagy inhibition abrogated miR-199a-5p downregulation-induced cell proliferation. These data demonstrated that miR-199a-5p/autophagy signaling represents a novel pathway regulating chemoresistance, thus offering a new target for chemotherapy of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and has an increasing incidence in the East Asia and western countries [1,2]. Systematic chemotherapy plays a crucial roles in HCC treatment especially for patients with advanced HCC [3]. However, the advances in chemotherapy for the purpose of HCC treatment have been limited because the underlying mechanisms causing chemoresistance are not known. Revealing the cellular and molecular mechanism for the development of chemoresistance is indispensable for developing effective chemotherapeutic agents.

MicroRNAs (miRNAs) are a group of noncoding RNAs that have been highly conserved during evolution and have emerged recently as potent regulators of gene expression, cell proliferation, apoptosis and tumorigenesis [4–6]. Recently, some miRNAs have been reported to be involved in drug-resistance. miR-221/222 inhibition has been shown to sensitize MDA-MB-468 cells to tamoxifen-induced cell growth arrest and apoptosis [7]. Zhou et al. found that miR-125b is upregulated in Taxol-resistant cells, causing a marked inhibition of Taxol-induced cytotoxicity and apoptosis and a subsequent increase in the resistance to Taxol in breast cancer cells [8]. They further demonstrated that the pro-apoptotic Bak1 is a direct target of miR-125b, and downregulation of Bak1 suppresses Taxol-induced apoptosis and leads to an increased resistance to Taxol. Fujita et al. demonstrated that miR-34a overexpression results in cell cycle arrest and growth inhibition and attenuated chemoresistance to the camptothecin by targeting *SIRT1* gene [9]. miR-199a-5p was also deregulated after cisplatin treatment in cancer cell lines, and it may target molecular pathways involved in cell survival after chemotherapy [10]. However, the molecular mechanisms underlying this process are not well understood.

Autophagy is a lysosome-mediated intracellular catabolic process by which cells remove their damaged organelles and long-lived proteins for the maintenance of cellular homeostasis [11]. Some researchers showed that autophagy is involved in the regulation of chemoresistance. Yoon et al. found that autophagy contributes to the sustained survival of breast cancer cells through DNA repair regulated by ATM-mediated activation of DNA-PKcs and PARP-1 [12]. In leukemia cells, upregulated autophagy by endogenous high mobility group box-1 (HMGB1) promotes chemoresistance [13]. O'Donovan et al. demonstrated that autophagic response to chemotherapy is a survival mechanism that promotes chemoresistance and recovery, and that autophagy inhibition has the potential to improve chemotherapeutic regimes [14].

Based on these findings, we tested whether miR-199a-5p/autophagy pathway represents a novel pathway regulating chemoresistance. We found that miR-199a-5p levels were significantly decreased in HCC patients treated with cisplatin-based chemotherapy. Downregulated miR-199a-5p enhanced autophagy activation

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by targeting ATG7. We further demonstrated that cisplatin-induced downregulation of miR-199a-5p increased drug resistance by activating autophagy in HCC cell.

2. Materials and methods

2.1. Samples and cell lines

A total of 21 unresectable/metastatic HCC patients were included in the study, and all the patients received cisplatin-based combination chemotherapy between 04/2010 and 12/2011 (median age 61, range 48–75). Blood samples were obtained with informed consent from Renji hospital affiliated to School of Medicine Shanghai Jiaotong University. The protocols used in the study were approved by the Hospital's Protection of Human Subjects Committee. Hepatoma cells (Huh7 cells, HepG2 cells) and HEK 293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's modified essential medium (Gibco, Carlsbad, CA,) with 10% fetal bovine serum (FBS; Gibco).

2.2. Real-time polymerase chain reaction (PCR)

Total RNA was extracted from blood samples and hepatoma cells by using Trizol reagent (Invitrogen, Carlsbad, CA), and the reverse transcription (RT) reactions were carried out using miR-199a-5p-special prime. The specific stem–loop RT primers for miR-199a-5p were designed as previously described [15]. Real-time PCR was performed using a standard protocol from the SYBR Green PCR kit (Toyobo, Osaka, Japan) on a Rotor-Gene RG-3000A (Corbett Life Science, Sydney, NSW). U6 was used as references for miR-199a-5p. ΔCt values were normalized to U6 levels. Each sample was analyzed in triplicate.

2.3. Cell proliferation assay

Cell proliferation assays were performed by using Cell Counting Kit-8 (Dojindo, Japan). Huh7 cells or HepG2 cells were plated in 24-well plates in triplicate at approximately 2×10^5 cells per well and cultured in the growth medium. Cells were then treated with cisplatin (Sigma, 1 μg/ml) or cisplatin plus miR-199a-5p (50 nM), and the numbers of cells per well were measured by the absorbance (450 nm) of reduced WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-isulfophenyl)-2H-tetrazolium, monosodium salt) at the indicated time points.

2.4. Luciferase reporter assay

HEK293 cells (2×10^5 cells/well) were plated in a 24-well plate and cotransfected with 20 nM of either miR-199a-5p or microRNA control (miRcontrol), 50 ng of either pGL3-ATG7-3′-UTR-WT or pGL3-ATG7-3′-UTR-Mutation, and 5 ng of pRL-TK (Promega, Madison, WI) by using Lipofectamine™ 2000. The pRL-TK vector was cotransfected as an internal control to correct the differences in both transfection and harvest efficiencies. HEK293 cells were collected 48 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega).

2.5. Western blot analysis

Western blot analysis to assess LC3-I, LC3-II, p62, ATG7 and β-actin expression was performed as previously described [16]. The anti-LC3-I primary antibodies and anti-LC3-II primary antibodies were purchased from Cell Signaling Technology (Beverly, MA). The anti- p62/ATG7 primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). β-actin primary antibodies were purchased from Sigma (MO, USA).

2.6. Fluorescence microscopy analysis

HepG2 cells were treated with cisplatin or cisplatin plus miR-199a-5p and fixed with 3% paraformaldehyde, and subjected to immunocytochemistry as previously described [17]. Samples were examined under a fluorescence laser scanning confocal FV1000 microscope (Olympus).

2.7. Statistical analysis

Data are expressed as mean ± standard deviation (SD) from at least three separate experiments. The differences between groups were analyzed using Student's *t* test. Differences were deemed statistically significant at $p < 0.05$.

3. Results

3.1. Cisplatin treatment results in downregulation of miR-199a-5p in vivo and in vitro

To investigate underlying mechanism for the resistance of hepatoma cells to cisplatin treatment, we focused on identifying the changes in miRNAs expression after cisplatin-based combination

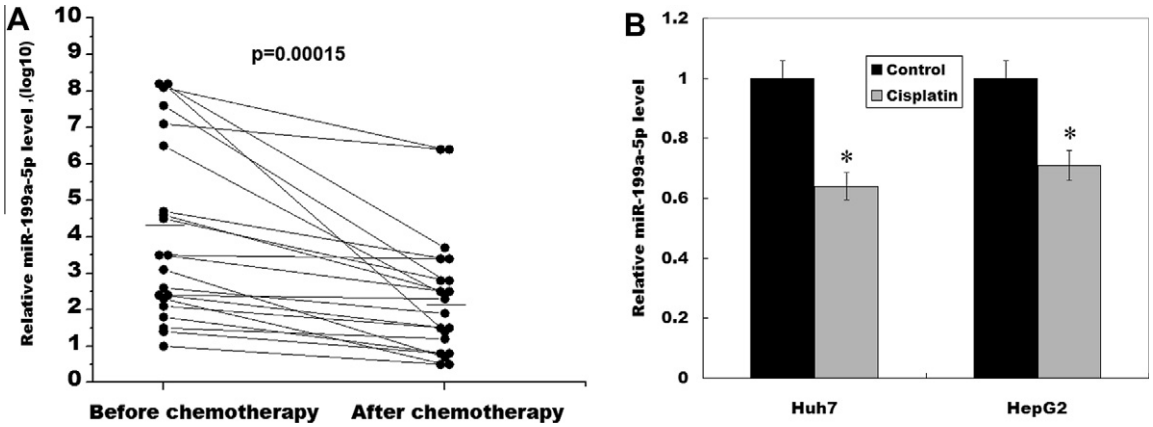

Fig. 1. miR-199a-5p levels were significantly decreased after cisplatin treatment. (A) The analysis of the miR-199a-5p expression level was performed in blood samples with advanced HCC ($n = 21$) after cisplatin-based combination chemotherapy. Total RNA was extracted and subjected to real-time RT-PCR to analyze the expression level of miR-199a-5p in each sample. U6 was used as a reference for miRNAs. Relative expression was calculated with respect to a tissue. The results were expressed as Log10 ($2^{-\Delta\Delta Ct}$). (B) Huh7 or HepG2 cells were treated with cisplatin, and miR-199a-5p levels were analyzed by real-time PCR. Relative miR-199a-5p levels were calculated with respect to the control. $^*p < 0.05$.

chemotherapy. We found that miR-199a-5p expression levels were significantly decreased in HCC patients after chemotherapy (Fig. 1A). To further determine whether miR-199a-5p levels might be correlated with chemotherapeutic response, Huh7 cells and HepG2 cells were treated with cisplatin for 48 h and miR-199a-5p levels were assayed. Consistent with above results, cisplatin treatment also resulted in decreased miR-199a-5p levels *in vitro* (Fig. 1B). These data indicate that downregulation of miR-199a-5p may be related to cisplatin-based combination chemotherapy.

3.2. Forced expression of miR-199a-5p increases cisplatin-induced inhibition of cell proliferation

To study the role of miR-199a-5p in cisplatin-induced inhibition of cell proliferation, the human hepatoma cell lines were treated with cisplatin plus miR-199a-5p and cell proliferation was assayed. We found that miR-199a-5p expression levels were increased after miR-199a-5p mimics treatment (Fig. 2A). Cisplatin treatment markedly inhibited Huh7 cell proliferation compared with PBS control, and forced expression of miR-199a-5p enhanced cisplatin-induced cell proliferation inhibition (Fig. 2B). Similarly, miR-199a-5p overexpression also increased cisplatin-induced inhibition of HepG2 cell proliferation (Fig. 2C). These data suggest that downregulated miR-199a-5p resists cisplatin-induced inhibition of cell proliferation.

3.3. miR-199a-5p inhibits cisplatin-induced activation of autophagy

To explore the mechanisms of miR-199a-5p in regulating cell proliferation, we then investigated whether miR-199a-5p regulates cell proliferation-related signals. Autophagy is a lysosome-mediated intracellular catabolic process. Recent research shows that autophagy is involved in the regulation of cell proliferation and chemoresistance [12–14]. The ratio of LC3-II to LC3-I has been shown to be a reliable indicator of autophagy. Fig. 3A showed that cisplatin treatment induced an increase in the ratio of LC3-II/LC3-I. The ubiquitin-binding protein SQSTM1 (p62) is an autophagy substrate, which is efficiently degraded by autophagy [18]. We examined p62 protein levels following cisplatin treatment, and a progressive degradation of p62 was found in Huh7 cells (Fig. 3A). Similarly, cisplatin treatment also induced autophagy activation in HepG2 cells (Fig. 3B). Fluorescence microscopy analysis further demonstrated that forced expression of miR-199a-5p markedly inhibited cisplatin-induced autophagic in HepG2 cells (Fig. 3C).

3.4. Cisplatin-induced downregulation of miR-199a-5p increases cell proliferation by activating autophagy

In order to further identify the role of miR-199a-5p in regulating autophagy activation, we searched for target genes of miR-199a-5p using miRBase (http://www.mirbase.org/) and TargetScan5.2 (http://www.targetscan.org/). miR-199a-5p targets autophagy-associated gene 7 (ATG7) gene. We therefore constructed luciferase reporter vector containing 3′-UTR of ATG7 (pGL3-ATG7-3′-UTR-WT). The reporter assay showed that miR-199a-5p overexpression was able to markedly suppress luciferase expression of pGL3-ATG7-3′-UTR-WT (Fig. 4A), and mutation of 5 nt in the miR-199a-5p target sequence led to complete abrogation of the suppressive effect (Fig. 4A). Moreover, we observed that ectopic expression of miR-199a-5p decreased the ATG7 protein content in Huh7 cells (Fig. 4B). More important, autohpagy inhibition impaired miR-199a-5p downregulation-induced cell proliferation (Fig. 4C). These results confirmed that cisplatin-induced downregulation of miR-199a-5p increases drug resistance by activating autophagy in HCC cell.

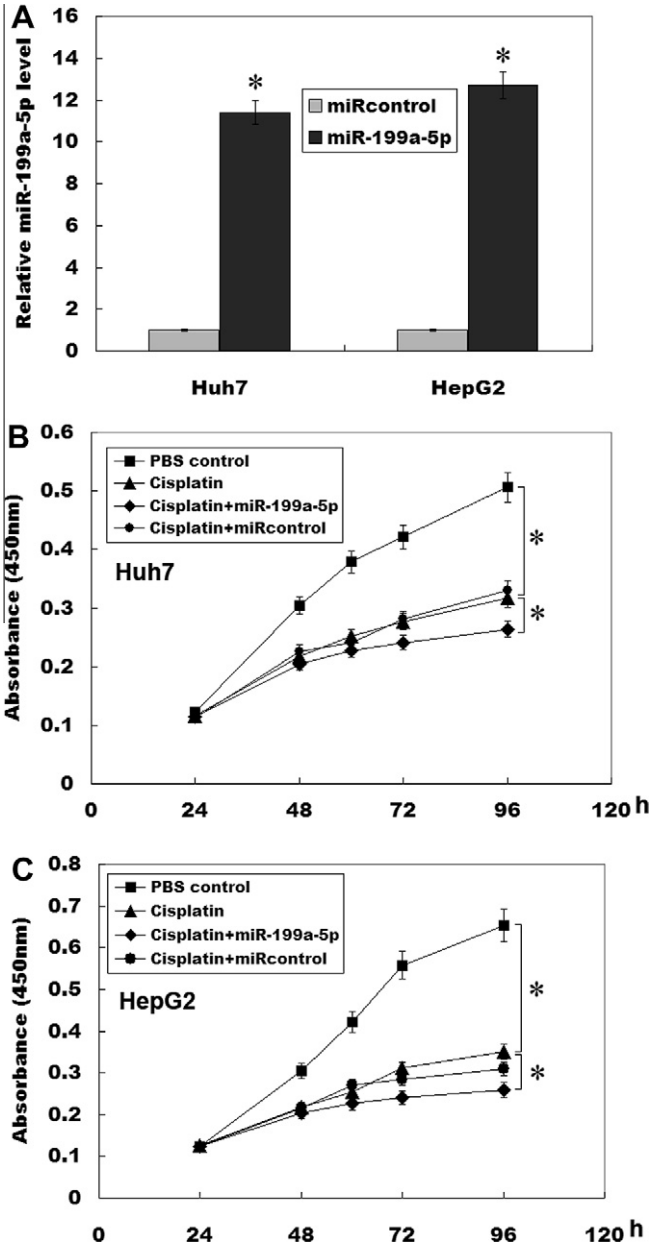

Fig. 2. Forced expression of miR-199a-5p increases cisplatin-induced inhibition of cell proliferation. (A) Huh7 or HepG2 cells were treated with mature miR-199a-5p and miR-199a-5p expression level was assayed by real-time PCR. The miRcontrol (UUCUCCGAACGUGUCACGUTT) is negative control. (B) Huh7 cells were treated with cisplatin or cisplatin plus miR-199a-5p, and at the indicated time points, cell proliferation was assayed by using CCK-8 according to the manufacture's protocol. The results show data from at least three independent experiments, expressed as the mean ± SD. $^*p < 0.05$. (C) HepG2 cells were treated with cisplatin or cisplatin plus miR-199a-5p, and at the indicated time points, cell proliferation was assayed by using CCK-8 according to the manufacture's protocol. The results show data from at least three independent experiments, expressed as the mean ± SD. $^*p < 0.05$.

4. Discussion

HCC is one of the most fatal diseases all over the world, and its incidence is increasing in many countries including China. Besides surgical treatments, systematic chemotherapy, play an important role in HCC treatment especially for patients with advanced HCC [19]. Cisplatin is a common therapeutic agent used for chemotherapy in HCC patients. However, despite a rapid shrinkage in tumor mass following chemotherapeutic cycles, the resistance of cancer cells to cisplatin frequently results in the subsequent recurrence

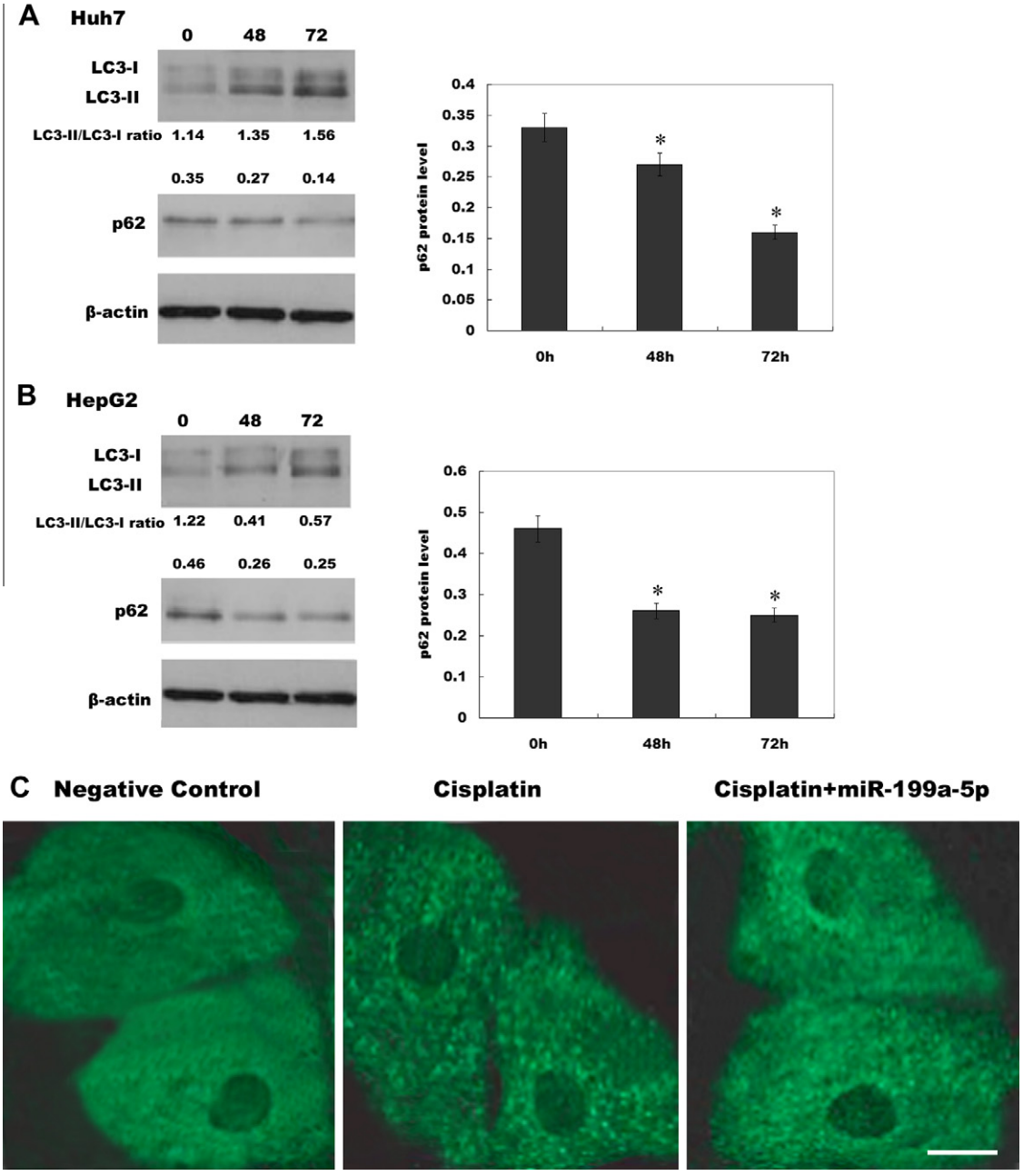

Fig. 3. miR-199a-5p inhibits cisplatin-induced activation of autophagy. Huh7 (A) or HepG2 (B) cells were treated with cisplatin, and Western blot analysis of LC3-I, LC3-II and p62 proteins was performed at 0, 48 and 72 h (left). We showed quantification of LC3-II/LC3-I ratio and p62 (right, $n = 3$). $^{*}p < 0.05$. (C) Immunofluorescence of LC3-II in HepG2 cells 48 h after cisplatin or cisplatin plus miR-199a-5p treatment. Scale bar = 50 μm.

and metastasis of cancer [20]. Currently the mechanisms involved in cancer cell chemoresistance are still largely unknown. Therefore, it is an urgent need to identify novel pathways in HCC patients resistant to chemotherapy.

Autophagy is a highly conserved survival response to growth limiting conditions such as hypoxia, nutritional deprivation and reactive oxygen species (ROS), in which cellular components are sequestered, degraded and released for recycling [21]. Autophagy activation may be a necessary homeostatic process which removes damaged organelles and recycles macromolecules, thus protecting against cancer [22]. Recently, several researches showed that autophagy is also implicated in promoting chemoresistance of cancer cells so as to attenuate therapeutic efficacy of chemotherapy. O'Donovan et al. demonstrated that autophagy inhibition by knockdown of Beclin 1 and ATG7 significantly increases the effect of 5-FU and decreases the recovery of drug-treated cells [14]. Pharmacological inhibitors of autophagy also improve chemotherapeutic effect. Their observations suggest that autophagic response to chemotherapy is a survival mechanism that promotes chemoresistance and that selective inhibition of autophagy regulators has the potential to improve chemotherapeutic regimes. The autophagy activation also contributes to the sustained survival of breast cancer cells through DNA repair regulated by ATM-mediated activation of DNA-PKcs and PARP-1 [12]. Here we found that cisplatin treatment induced autophagy activation in hepatoma cells, which in-

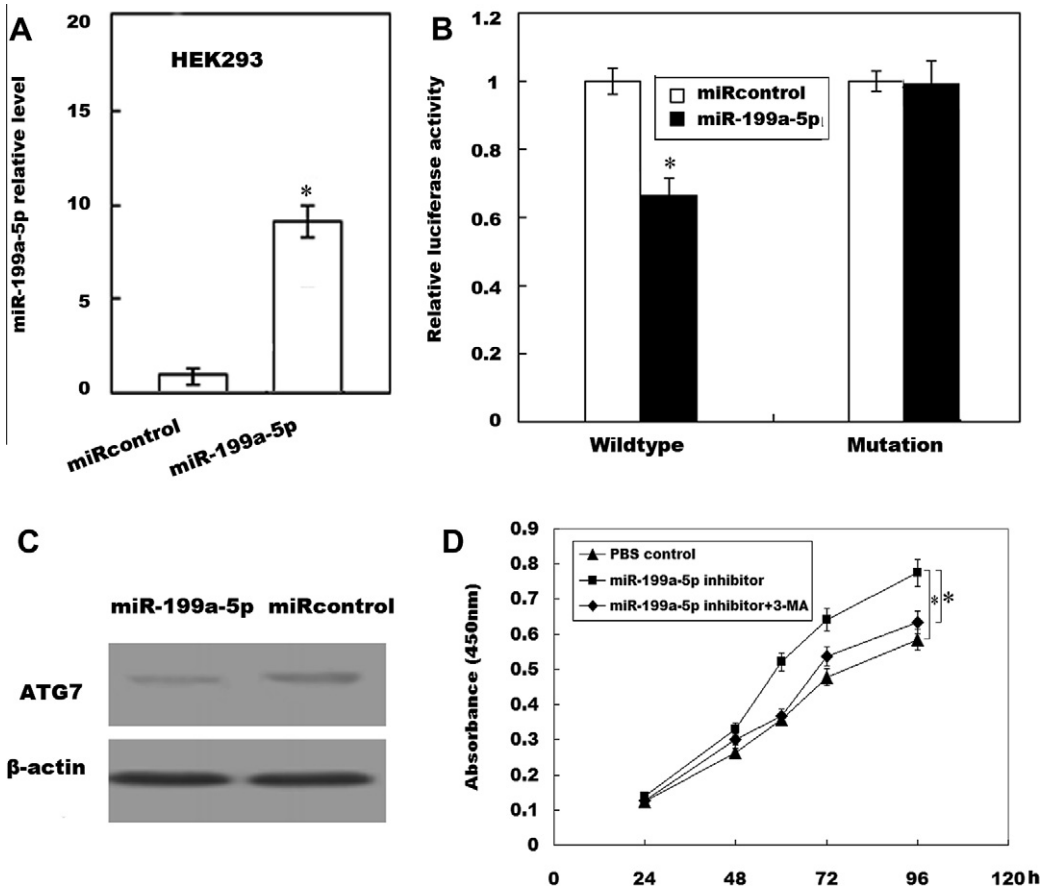

Fig. 4. Cisplatin-induced downregulation of miR-199a-5p increases cell proliferation by activating autophagy. (A) HEK293 cells were treated with mature miR-199a-5p and miR-199a-5p expression level was assayed by real-time PCR. (B) The 3′UTR reporter assay was carried out in HEK293 cells overexpressed miR-199a-5p. pGL3-ATG7-3′-UTR-WT or pGL3-ATG7-3′-UTR-Mutation was co-transfected with pRL-TK using Lipofectamine 2000. Luciferase assays were performed 48 h after transfection using the Dual-Luciferase Reporter Assay System. $^{*}p < 0.05$. (C) Western blot analysis for endogenous ATG7 protein level using antibodies against ATG7 in Huh7 cells. (D) Huh7 cells were treated with miR-199a-5p inhibitor or miR-199a-5p inhibitor plus 3-MA, and at the indicated time points, cell proliferation was assayed by using CCK-8 according to the manufacture's protocol. The results show data from at least three independent experiments, expressed as the mean ± SD. $^{*}p < 0.05$.

creased cell proliferation. Then, revealing the underlying mechanism for activating autophagy in chemotherapy is indispensable for developing effective chemotherapeutic agents.

In the study, we found that miR-199a-5p levels were significantly decreased in HCC patients treated with cisplatin and in hepatoma cells treated with cisplatin. Forced expression of miR-199a-5p promoted cisplatin-induced inhibition of cell proliferation. We further demonstrated that miR-199a-5p downregulation increased autophagy activation by targeting ATG7. More important, autophagy inhibition abrogated miR-199a-5p downregulation-induced cell proliferation. These data suggest that cisplatin-induced downregulation of miR-199a-5p increased drug resistance by activating autophagy in HCC cells. High mobility group box-1 (HMGB1) plays an important role in regulating autophagy response. Yang et al. demonstrated that HMGB1 is abundantly expressed in various kinds of both leukemia and non-blood cancer cell-lines, and its expression is positively correlated with clinical status in childhood leukemia. Further investigation found that HMGB1 is an intrinsic activator of autophagy in leukemia cells and it enhances leukemia cell chemoresistance through the PI3 K/Akt/mTORC1/autophagy pathway [13]. Conclusion, our data demonstrated that cisplatin-induced downregulation of miR-199a-5p contributes to drug resistance by activating autophagy in HCC cell.

Conflict of interest

None declared

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