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Upregulated miR-130a increases drug resistance by regulating RUNX3 and Wnt signaling in cisplatin-treated HCC cell

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ABSTRACT

Cisplatin is one of the commonly used chemotherapeutic drugs for the treatment of patients with advanced liver cancer. However, acquisition of cisplatin resistance is common in patients with hepatocellular carcinoma (HCC), and the underlying mechanism of such resistance is not fully understood. In the study, we found that miR-130a levels were significantly increased in HCC patients treated with cisplatin-based chemotherapy. miR-130a levels were also higher in cisplatin-resistant Huh7 cells than in Huh7 cells. Overexpression of miR-130a contributed to cisplatin resistance in Huh7 cell, whereas knockdown of miR-130a overcame cisplatin resistance in cisplatin-resistant Huh7 cell. We further demonstrated that upregulated miR-130a directly inhibited expression of tumor suppressor gene RUNX3, which resulted in activation of Wnt/β-catenin signaling and increased drug resistance. These data suggest that miR-130a/RUNX3/Wnt 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 role 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.

Cisplatin is an extensively used as a chemotherapeutic agent for the treatment of HCC. A major problem with cisplatin treatment of HCC is the development of cisplatin chemoresistance. Oncogenic activation of the Wnt/β-catenin signaling pathway is common in HCC [4,5]. Colnot et al. found that liver-targeted disruption of Apc in mice activates Wnt/β-catenin signaling and leads to hepatocellular carcinomas [5]. Gosepath et al. further demonstrated that acquired cisplatin resistance in the head–neck cancer cell line is associated with decreased DKK1 expression and can partially be reversed by overexpression of DKK1 [6]. Epigenetic silencing of secreted frizzled-related protein (SFRP) is related to malignant phenotype and chemoresistance of ovarian cancer through Wnt signaling pathway [7].

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 [8–10]. Our data demonstrated that miR-199a-5p levels are significantly reduced in HCC patients treated with cisplatin-based chemotherapy. Forced expression of miR-199a-5p promoted cisplatin-induced inhibition of cell proliferation. We further found that downregulated miR-199a-5p increases drug resistance by activating autophagy in HCC cell [11]. 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 [12]. 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. showed that miR-34a overexpression results in cell cycle arrest and growth inhibition and attenuated chemoresistance to the camptothecin by targeting *SIRT1* gene [13]. miR-130a was upregulated after cisplatin treatment in cancer cell lines, and it may target molecular pathways involved in cell survival after chemotherapy [14]. However, the molecular mechanisms underlying this process are not well understood.

Based on these findings, we tested whether and how miR-130a represents a novel pathway regulating chemoresistance. We found

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that miR-130a levels were significantly increased in HCC patients treated with cisplatin-based chemotherapy. Upregulated miR-130a inhibited RUNX3 expression, which resulted in activation of Wnt/β-catenin signaling and sequent cisplatin resistance.

2. Materials and methods

2.1. Samples and cell lines

A total of 25 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 62, range 48–77). 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. Huh7 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). The cisplatin-resistant subline Huh7-R was established by continuous exposure to increasing concentrations of cisplatin over a time period of 12 months, as reported previously [15].

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-130a-special prime. The specific stem–loop RT primers for miR-130a were designed as previously described [16]. 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 reference for miR-130a. 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 Huh7-R 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-130a (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-130a or microRNA control (miRcontrol), 50 ng of either pGL3-RUNX3-3′-UTR-WT or pGL3-RUNX3-3′-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 β-catenin, Lamin A, RUNX3 and β-actin expression was performed as previously described [11]. The anti-β-catenin primary antibodies were purchased from Cell Signaling Technology (Beverly, MA). The anti-Lamin A primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RUNX3 and β-actin primary antibodies were purchased from Sigma (MO, USA).

2.6. RNAi

RUNX3 RNAi was performed as described previously [17]. shRNAs targeting RUNX3 (sh1, 5′-GCCCAGAGAAGATGAGTCTAT-3′; sh2, 5′-AAGCAGCTATGAATCCATTGT-3′; sh3, 5′-TCAGTAGTGGGTACCAATCTT-3′) and control shRNA were obtained from Genepharm (Genepharm Group, Shanghai, China). pcDNA-RUNX3 or pcDNA-DKK1 was constructed to overexpress RUNX3 or DKK1 by introducing a BamHI–EcoRI fragment containing the RUNX3 or DKK1 precursor into the same sites in pcDNA3.1 .

2.7. Statistical analysis

Statistical comparison between two groups was performed using unpaired *t*-test. All of the groups were compared using one-way analysis of variance (ANOVA), followed by Tukey post hoc test where appropriate. The difference was deemed statistically significant at $p < 0.05$. All data were represented as

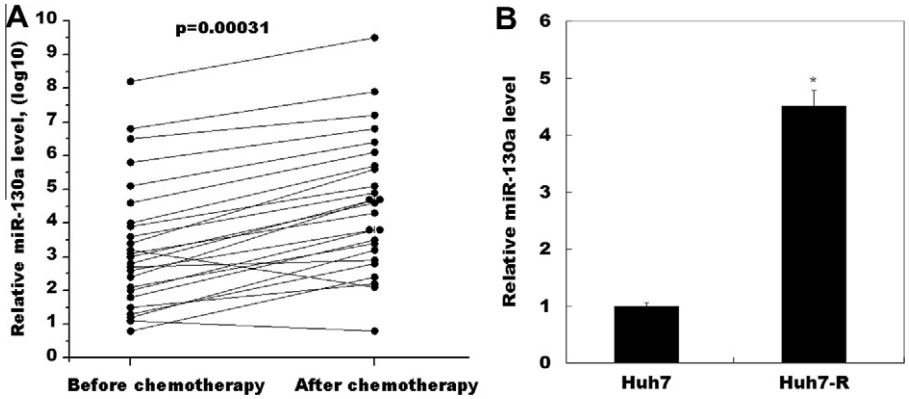

Fig. 1. Cisplatin treatment results in upregulation of miR-130a in vivo and in vitro. (A) The analysis of the miR-130a expression level was performed in blood samples with advanced HCC ($n = 25$) after cisplatin-based combination chemotherapy. Total RNA was extracted and subjected to real-time RT-PCR to analyze the expression level of miR-130a 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) The cisplatin-resistant subline Huh7-R was established by continuous exposure to increasing concentrations of cisplatin over a time period of 12 months, and miR-130a levels were analyzed by real-time PCR. Relative miR-130a levels were calculated with respect to the control. $^*p < 0.05$.

mean ± standard deviation from at least three separate experiments.

3. Results

3.1. Cisplatin treatment results in upregulation of miR-130a 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 chemotherapy. We found that miR-130a expression levels were significantly increased in HCC patients after chemotherapy (Fig. 1A). To further determine whether miR-130a levels might be correlated with cisplatin treatment, we established a cisplatin-resistant hepatoma cell line (Huh7-R) by continuous exposure of Huh7 cells to cisplatin. Consistent with above results, miR-130a levels was higher in Huh7-R cells compared with Huh7 cells (Fig. 1B). These data indicate that upregulation of miR-130a may be related to cisplatin resistance.

3.2. Forced expression of miR-130a decreases cisplatin-induced inhibition of cell proliferation

To study the role of miR-130a in cisplatin-induced inhibition of cell proliferation, the human hepatoma cell lines were treated with cisplatin plus miR- miR-130a and cell proliferation was assayed. We found that miR-130a expression levels were increased after miR-130a mimics treatment (Fig. 2A). Cisplatin treatment markedly inhibited Huh7 cell proliferation compared with PBS control, and forced expression of miR-130a decreased cisplatin-induced cell proliferation inhibition in Huh7 cells (Fig. 2B). Cisplatin treatment induced modest inhibition of Huh7-R cell proliferation, whereas knockdown of miR-130a markedly increased cisplatin-induced inhibition (Fig. 2C). These data suggest that upregulated miR-130a resists cisplatin-induced inhibition of cell proliferation.

3.3. miR-130a inhibits RUNX3 and activates Wnt signaling

In order to further identify the role of miR-130a in regulating cell survival, we searched for target genes of miR-130a using TargetScan5.2 (http://www.targetscan.org/). miR-130a targets runt-related transcription factor 3 (RUNX3) gene. We therefore constructed luciferase reporter vector containing 3′-UTR of RUNX3 (pGL3-RUNX3-3′-UTR-WT). The reporter assay showed that miR-130a overexpression was able to markedly suppress luciferase expression of pGL3-RUNX3-3′-UTR-WT in HEK293 cells (Fig. 3A and B), and mutation of 4 nt in the miR-130a target sequence led to complete abrogation of the suppressive effect (Fig. 4B). Moreover, we observed that ectopic expression of miR-130a decreased the RUNX3 protein content in Huh7 cells (Fig. 3C).

Previous studies showed that Wnt signaling is activated in HCC cells and cisplatin resistance in the head–neck cancer cell line is associated with activation of Wnt signaling [6]. Therefore, we assayed whether Wnt signaling is activated in Huh7-R. Fig. 3D showed that total β-catenin expression was higher in Huh7-R cells compared with Huh7 cells, and β-catenin in Huh7 cells was mainly in the cytoplasmic fraction. Little nuclear accumulation of β-catenin was observed in Huh7 cells, but nuclear β-catenin was very obvious in Huh7-R cells (Fig. 3D, left). Axin2 is the standard marker to assess β-catenin activation. Real-time PCR analysis showed that Axin2 mRNA levels were also significantly increased in Huh7-R cells (Fig. 3D, right). These results indicated that miR-130a inhibits RUNX3 and activates Wnt signaling.

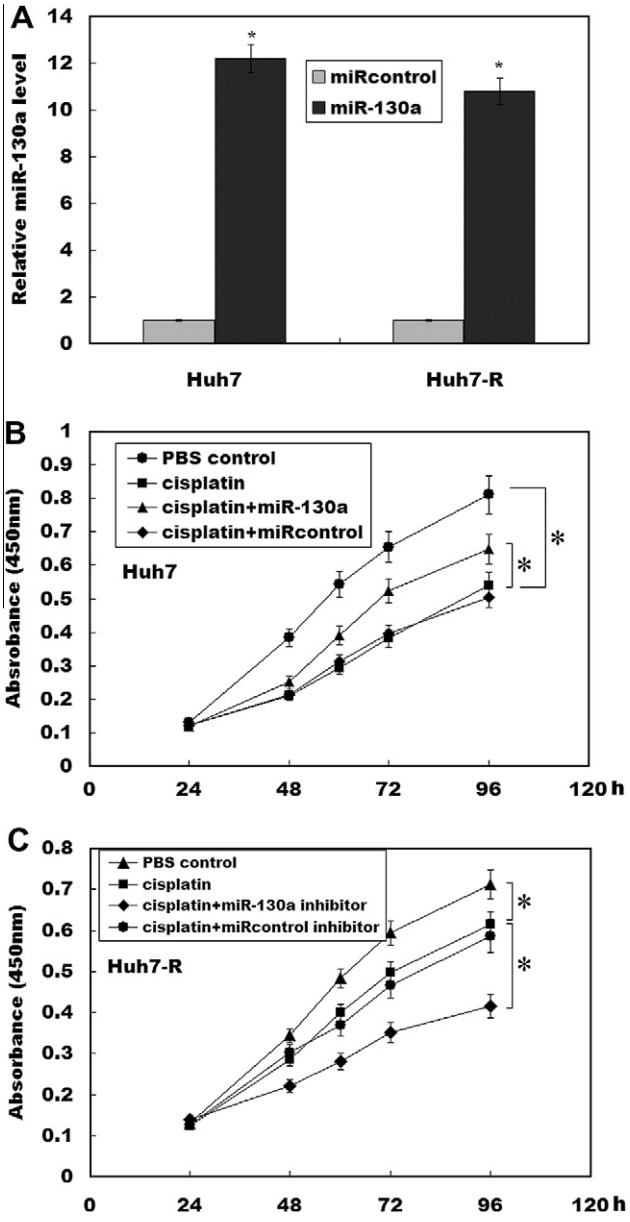

Fig. 2. Forced expression of miR-130a decreases cisplatin-induced inhibition of cell proliferation. (A) Huh7 or Huh7-R cells were treated with mature miR-130a and miR-130a expression level was assayed by real-time PCR. The miRcontrol (UUCUCCGAACGUGUCACGUTT) is negative control. (B) Huh7 cells were treated with cisplatin or cisplatin plus miR-130a (CAGUGCAAUGUUAAAAGGGCAU), 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) Huh7-R cells were treated with cisplatin or cisplatin plus miR-130a inhibitor (2′-*O*-methyl-AUGCCCUUUUAACAUUGCACUG), and at the indicated time points, cell proliferation was assayed by using CCK-8 according to the manufacturer's protocol. The results show data from at least three independent experiments, expressed as the mean ± SD. $^{*}p < 0.05$.

3.4. Cisplatin-induced upregulation of miR-130a increases cisplatin resistance by inhibiting RUNX3 and activating Wnt signaling

We then investigated how miR-130a increases drug resistance of hepatoma cells to cisplatin treatment. Huh7 cells were transiently transfected with the Wnt signaling reporter TOPFlash or the negative control FOPFlash, along with RUNX3-siRNA or siRNA control. In cells transfected with the control, treatment with Wnt3a was unable to markedly increase TOPFlash activity. How-

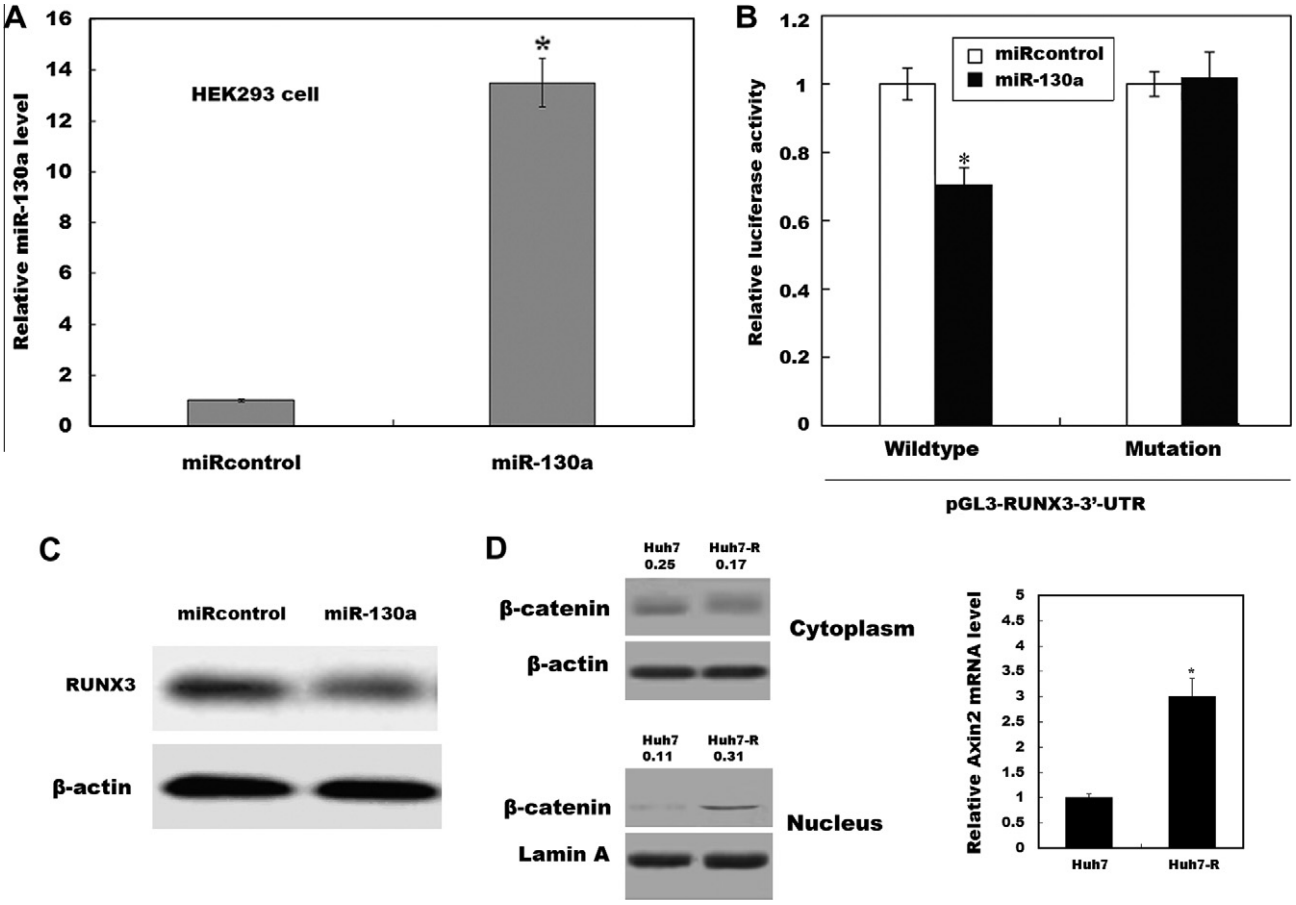

Fig. 3. miR-130a inhibits RUNX3 and activates Wnt signaling. (A) HEK293 cells were treated with mature miR-130a and miR-130a expression level was assayed by real-time PCR. (B) The 3′UTR reporter assay was carried out in HEK293 cells overexpressed miR-130a. pGL3-RUNX3-3′-UTR-WT or pGL3-RUNX3-3′-UTR-Mutation 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 RUNX3 protein level using antibodies against RUNX3 in Huh7 cells. (D) Western blot analysis of cytoplasmic or nuclear β-catenin in Huh7 cell or Huh7-R cells. β-Actin and Lamin A were used as internal controls (left). The analysis of the Axin2 mRNA level was performed in Huh7 cell or Huh7-R cells. Total RNA was extracted and subjected to real-time RT-PCR to analyze the expression level of Axin2 (right). β-Actin was used as a reference.

ever, Wnt3a caused a significant increase in TOPFlash activity in cells treated with RUNX3-siRNA, indicating that Wnt signaling was activated by RUNX3 inhibition (Fig. 4A). These data suggest that miR-130a can activate Wnt signaling, at least in part, by inhibiting RUNX3. More important, RUNX3 overexpression impaired miR-130a upregulation-induced cell proliferation, and inhibition of Wnt signaling also decreased the role of miR-130a in drug resistance (Fig. 4B). These results confirmed that upregulated miR-130a increases drug resistance by regulating RUNX3 and Wnt signaling in cisplatin-treated HCC cell.

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 [18]. 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 and metastasis of cancer [19]. 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.

Much effort has been exerted in analyzing the role of miRNAs in the development of drug resistance in a variety of malignancies. Several researches have shown the abnormal expressions of miRNAs in chemoresistant cancer cells. The molecular targets and mechanisms of chemosensitivity and chemoresistance are also elucidated. In a previous study, we demonstrated 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. 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 [20].

Oncogenic activation of the Wnt/β-catenin signaling pathway is common in HCC. Recent research demonstrated that acquired cisplatin resistance in the head–neck cancer cell line is also associated with decreased DKK1 expression and can partially be reversed by overexpression of DKK1 [6]. Kendziorra et al. demonstrated that silencing of Wnt transcription factor TCF4 caused a significant sensitization of colorectal cancer cells to chemotherapy and radiotherapy. This effect was restricted to tumor cells with high TCF reporter activity [21]. Based on these findings, we tested whether abnormal activation of Wnt signaling mediated by miRNAs represents a

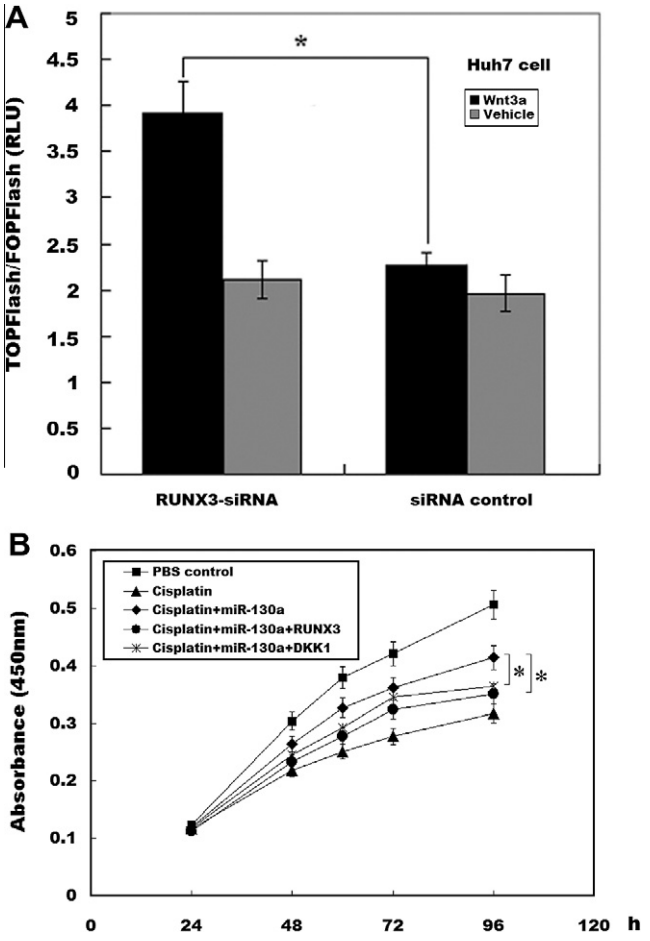

Fig. 4. Cisplatin-induced upregulation of miR-130a increases drug resistance by inhibiting RUNX3 and activating Wnt signaling. (A) Luciferase activity of TOPFlash/FOPFlash in Huh7 cell treated with RUNX3-siRNA or siRNA control. $^{*}p < 0.05$. (B) Huh7 cells were treated with miR-130a or miR-130a plus RUNX3 or DKK1, and at the indicated time points, cell proliferation was assayed by using CCK-8 according to the manufacturer's protocol. The results show data from at least three independent experiments, expressed as the mean ± SD. $^{*}p < 0.05$.

novel pathway regulating chemoresistance in the study. We found that miR-130a expression levels were significantly increased in HCC patients treated with cisplatin-based chemotherapy. miR-130a levels were also higher in cisplatin-resistant Huh7 cells (Huh7-R) than in Huh7 cells. Forced expression of miR-130a increased cisplatin resistance in Huh7 cell, whereas miR-130a downregulation overcame cisplatin resistance in Huh7-R cell. Nuclear accumulation of β-catenin and Axin2 mRNA levels was higher in cisplatin-resistant Huh7 cell, indicating that Wnt signaling was activated in Huh7-R cell. We further demonstrated that upregulated miR-130a directly inhibited RUNX3 expression, which resulted in activation of Wnt/β-catenin signaling and increased cisplatin resistance.

5. Conclusion

These data demonstrated that miR-130a/RUNX3/Wnt signaling represents a novel pathway regulating chemoresistance, thus offering a new target for chemotherapy of HCC.

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