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ZEB1 mediates doxorubicin (Dox) resistance and mesenchymal characteristics of hepatocarcinoma cells

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ABSTRACT

The acquired chemoresistance during long term chemotherapy is one of the most important factors to limit the application of Doxorubicin (Dox) on clinical treatment of hepatocellular carcinoma (HCC) patients. Our present study found that Dox resistant HCC (HCC/Dox) cells had greater capability of *in vitro* migration and invasion compared to their parental cells. HCC/Dox cells exhibited mesenchymal characteristics, which was evidenced by the up regulation of fibronectin, vimentin while down regulation of *E*-Cadherin. Zeb1, one powerful epithelial mesenchymal transition related transcription factor (EMT-TF), was markedly upregulated in HCC/Dox cells. Targeted inhibition of Zeb1 *via* siRNA can suppress the cell migration and re-sensitized cells to Dox treatment. The upregulation of Zeb1 in HCC/Dox cells was due to the increasing protein and mRNA stability of Zeb1. In HCC/Dox cells, the down regulation of SIAH1 mediated the upregulation of protein stability of Zeb1, while decreased levels of miR-3129-5p was responsible for the increasing mRNA stability of Zeb1. Collectively, our data suggested that SIAH1 and miR-3129-5p induced upregulation of Zeb1 mediated the Dox resistance of HCC cells. Targeted inhibition of Zeb1 might be helpful to overcome of Dox resistance of HCC

1. Introduction

As the third leading cause of cancer-related deaths, hepatocellular carcinoma (HCC) has persistently increasing rates of both incidence and mortality (Siegel et al., 2016). The systemic chemotherapy is the major treatment approach for patients diagnosed when the disease has reached a stage beyond curative surgery (Raoul et al., 2018). Doxorubicin (Dox), one anthracycline-based agent, has been widely used as the first-line chemotherapy agent for transarterial chemoembolization (TACE) of HCC (Desai et al., 2017). Dox can intercalate base pairs of DNA strands, inhibit the DNA/RNA synthesis, and then induce apoptosis of tumor cells (Minotti et al., 2004). Unfortunately, the acquired chemoresistance during long term chemotherapy will lead to limited therapy efficiency and a poor prognosis (Zhu, 2006). The investigation about the mechanisms responsible for Dox resistance will be great helpful for drug development and improvement of prognosis.

Epithelial–mesenchymal transition (EMT) is defined as the process that epithelial cells lose the apical-basal polarity and cell–cell adhesion and get mesenchymal characteristics (Thiery et al., 2009). The roles of EMT in cancer drug resistance has been increasingly recognized due to cancer cells undergoing EMT will strength the drug resistance (Thiery, 2003). Further, signals involved in the progression of EMT can also promote the drug resistance. For examples, the EMT related transcription factors (EMT-TFs) Twist and Snail can link Hedgehog signals to acquire chemoresistance (Kong et al., 2015). The Dox resistance can also induce EMT of colorectal cancer (CRC) cells *via* upregulation of transforming growth factor β (TGF-β) (Li et al., 2015). Targeted inhibition of EMT-TF such as Snail can enhance the sensitivity of Dox in HCC cells (Dai et al., 2016). In mantle cell lymphoma, Zeb1 can promote tumor growth and determine differential response to chemotherapy (Sanchez-Tillo et al., 2014). Further, it has been reported that promoters of ATP binding cassette transporters (ABC transporters) have the binding sites of EMT-TFs. Over expression of Twist, Snail, and Foxc2 can increase the promoter activities of ABC transporters (Saxena et al., 2011).

Epigenetic signals such as micro-RNA can regulate the progression of Dox resistance *via* multiple downstream pathways(Li and Yang, 2014). For examples, miR-451 can regulate the expression of multidrug resistance 1 gene (MDR1) to regulate the Dox resistance of MCF-7 cells. Similarly, miR-21 can modulate chemosensitivity of breast cancer cells to Dox *via* targeting PTEN (Wang et al., 2011). As to HCC, miR-233 can down regulate the expression of ABCB1 to modulate multidrug resistance (Yang et al., 2013). Our present study suggested that Dox resistant HCC cells exhibited EMT characteristics with higher capability of migration and invasion. Upregulation of EMT-TF Zeb1 was essential for the Dox resistance and EMT progression.

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2. Materials and methods

2.1. Cells culture and establish of Dox resistant HCC cells

Human HCC cell line HepG2 and Huh7 were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco modified Eagle medium (DMEM)/F12 (HyClone, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) at 37 °C with 5% CO_2. The Dox resistant HCC cells (HCC/Dox) were generated according to the published studies (Jin et al., 2017). Briefly, HepG2 or Huh7 cells were exposed to medium containing stepwise increasing concentration of Dox at a range of 0.5–25 μM over a period of 10 months. After generation, the Dox resistant cells were maintained in medium containing 2 μM Dox and named as HepG2/Dox and Huh7/Dox, respectively. All cell lines have been authenticated by the use of short tandem repeat method by the Shanghai Institute of Cell Biology.

2.2. Chemicals and reagents

Doxorubicin hydrochloride (Dox), actinomycin-d (Act-D), cyclohexane (CHX), and proteasome inhibitor MG-132 were purchased from Sigma Aldrich (St Louis, MO, USA). The BCA protein assay kit was obtained from Invitrogen (Rockville, MD, USA). siRNA negative control (si-NC), or siRNAs for Zeb1 and SIAH1 were obtained from GenePharma (Suzhou, Jiangsu, China). The miR-3129-5p mimic was synthesized by Guangzhou Ribobio Company (Guangzhou, China). All primary antibodies and the according secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

2.3. Wound healing assay

Cells were seeded in 6-well plates and grown to 90% confluence. After scratched by use of a 200 μl sterilized pipet tip, cells were washed twice with PBS to remove nonadherent cells. The wound healing was recorded by use of a microscope (Nikon TS100; Nikon, Tokyo, Japan). The relative *in vitro* migration was assessed by using Image-Pro Plus 6.0 software (IPP; Media Cybernetics; Acton, MA) and calculated by the formula: Relative motility = (initial distance-a time-point distance)/ initial distance × 100%.

2.4. In vitro invasion assay

The 6.5 mm diameter polycarbonate filters (8 μm pore) of the transwell chambers (Corning; NY, USA) pre-coated with matrigel solution (1:5 diluted with FBS-free DMEM) was used to evaluate the *in vitro* invasion ability of cancer cells. Cells (1×10^4) in 100 μl serum-free DMEM media were seeded in membrane insert, while 600 μl of 10% FBS in DMEM was added to the bottom chamber. The invaded cells were fixed with 100% methanol and stained with hematoxylin. Cell number was counted under the microscope (Nikon ECLPSE 80i system; ×200).

2.5. Western blot analysis

After lysis, the protein concentration was measured by use of the BCA protein assay kit (Invitrogen, Rockville, MD, USA). Then 20 μg protein per lane was loaded and separated by 8%–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred onto nitrocellulose membranes and incubated with primary antibodies (1:1000) overnight at 4 °C. After washed three times by use of Tris-buffered saline containing 0.1% Tween, membranes were incubated with infrared-labeled secondary antibodies (1: 10,000) and measured by LI-COR Odyssey Imaging System (LI-COR Biosciences). GAPDH was used as loading control for analysis. The density of the protein band was quantitated using Quantity One software (Bio-Rad).

2.6. Cell proliferation assay

Cell Counting kit-8 reagent (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to analyze the relative cell proliferation rate according to the instructions of manufacture. Briefly, cells were seeded into 96-well plates and treated as indicated. At the end of experiment, 10 μl CCK-8 was added and cells were further incubated for 2 h at room temperature. The absorbance was detected at a wavelength of 450 nm. Each experiment was processed in triplicate.

2.7. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNAs were extracted by use of TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The purified RNA was further treated with DNase (Promega#M6101) to eliminate genomic DNA according to the instructions. cDNA was reverse transcribed from 1000 ng RNA using the PrimeScript™ RT Reagent kit (Takara Bio, Inc., Otsu, Japan). The RT-qPCR was conducted by using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) and a ROX Plus Reagent kit (Takara Bio, Inc.). GAPDH (mRNA) or U6 (miRNA) was used as loading controls for normalization. The sequences of the primers used were as follows: Zeb1 (spanning the 1st and 2nd exon) forward primer, 5′- GATGATGAATG CGAGTCAGATGC -3′; and reverse primer, 5′- ACAGCAGTGTCTTGTTG TTGT -3′; pre-Zeb1 (in the 1st intron) forward primer, 5′- GGCATACA CCTACTCAACTACGG -3′; and reverse primer, 5′- TGGGCGGTGTAGA ATCAGAGT C -3′; GAPDH forward primer, 5′- GTCTCCTCTGACTTCA ACAGC-3′; and reverse primer, 5′- ACCACCCTGTTGCTGTAGCCAA -3′; U6 forward primer, 5′- GGAACGATACAGAGAAGATTAGC -3′; and reverse primer, 5′- TGGAACGCTTCACGAATTTGCG -3′; SIAH1 forward primer, 5′- TCTTCCTGGTGCTGTTGACTGG -3′; and reverse primer, 5′- CGATTGCGAAGAACTGCTGGTG -3′; FBXO45 forward primer, 5′- AGA GGCTCTGCGCACGGACAT -3′; and reverse primer, 5′- TGTAGACATTC CTGGAGCAGTCA -3′; ATM forward primer, 5′- AGAGACAGGGTTGCC ATTG -3′; and reverse primer, 5′- TGCAGAAAGAGTTCCAGCTT -3′. The mRNA and miRNA expression levels were analyzed using the $2^{-\Delta\Delta Cq}$ method.

2.8. Dual-luciferase reporter assay

The 3'-UTR containing miR-3129-5p targeting sequence was insert to the pGL3 basic plasmid to generate pGL3-WT-*ZEB1*–3′-UTR (wild-type) and analyzed with the Dual-Luciferase Reporter Assay system (Promega, Wisconsin, USA) according to the instructions of manufacturer. To confirm the direct binding between miR-3129-5p and 3'-UTR of ZEB1, the targeting sequence of miR-3129-5p in 3′-UTR of *ZEB1* was mutant to generate pGL3-Mut-*ZEB1*–3′-UTR (wild-type). The firefly luciferase activity was normalized to the Renilla luciferase activity.

2.9. Statistical analysis

The data were presented as mean ± standard error of the mean. All data were analyzed by use of the SPSS13.0 software package (SPSS Inc., Chicago, IL, USA). The comparison between two groups was analyzed by *t*-test (two-sided). $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. The cell motility of parental and Dox resistant HCC cells

The cell motility of parental and Dox resistant HCC cells was evaluated by use of wound healing and invasion assay. Our data showed that the *in vitro* migration capability of HepG2/Dox (Fig. 1A) and Huh7/Dox (Fig. 1B) cells were significantly greater than that of their corresponding parental cells. Transwell analysis confirmed that the *in*

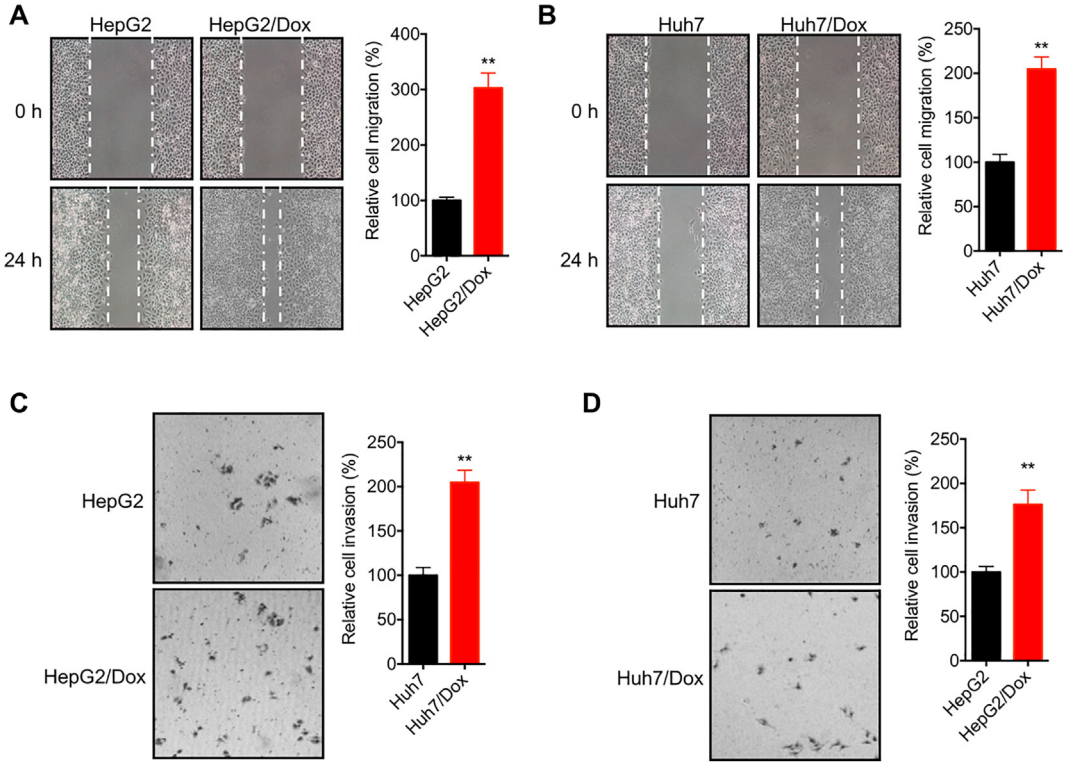

Fig. 1. The cell motility of parental and Dox resistant HCC cells. The *in vitro* migration of HepG2/Dox (A) and Huh7/Dox (B) cells and their corresponding parental cells analyzed by wound healing assay were recorded (left) and statistically analyzed (right); The *in vitro* invasion of HepG2/Dox (A) and Huh7/Dox (B) cells and their corresponding parental cells analyzed by transwell chamber for 24 h were recorded (left) and statistically analyzed (right). Data were presented as means ± SD of three independent experiments. **p < 0.01 as compared with the control group.

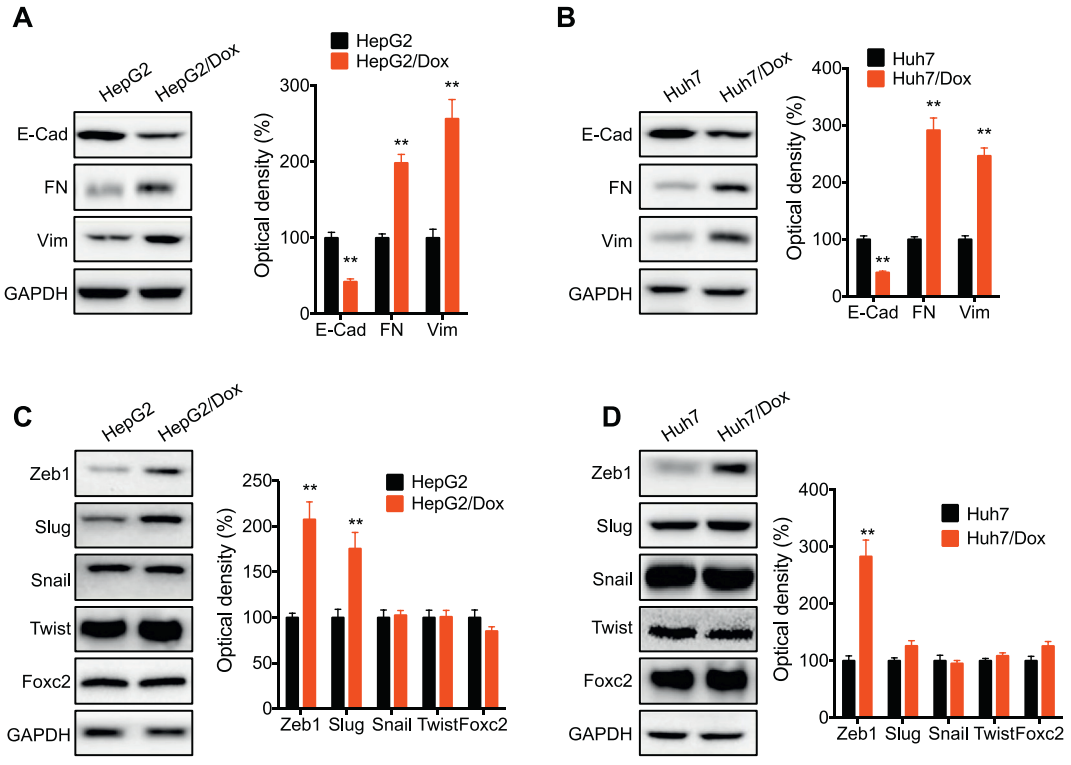

Fig. 2. HCC/Dox cells exhibited EMT characteristics. The EMT markers E-Cad, FN, and Vim in HepG2/Dox (A) and Huh7/Dox (B) cells and their corresponding parental cells analyzed by western blot analysis were recorded (left) and statistically analyzed (right); The EM-TFs Zeb1, Slug, Snail, Twist and Foxc2 in HepG2/Dox (C) and Huh7/Dox (D) cells and their corresponding parental cells analyzed by western blot analysis were recorded (left) and statistically analyzed (right). Data were presented as means ± SD of three independent experiments. ** p < 0.01 as compared with the control group.

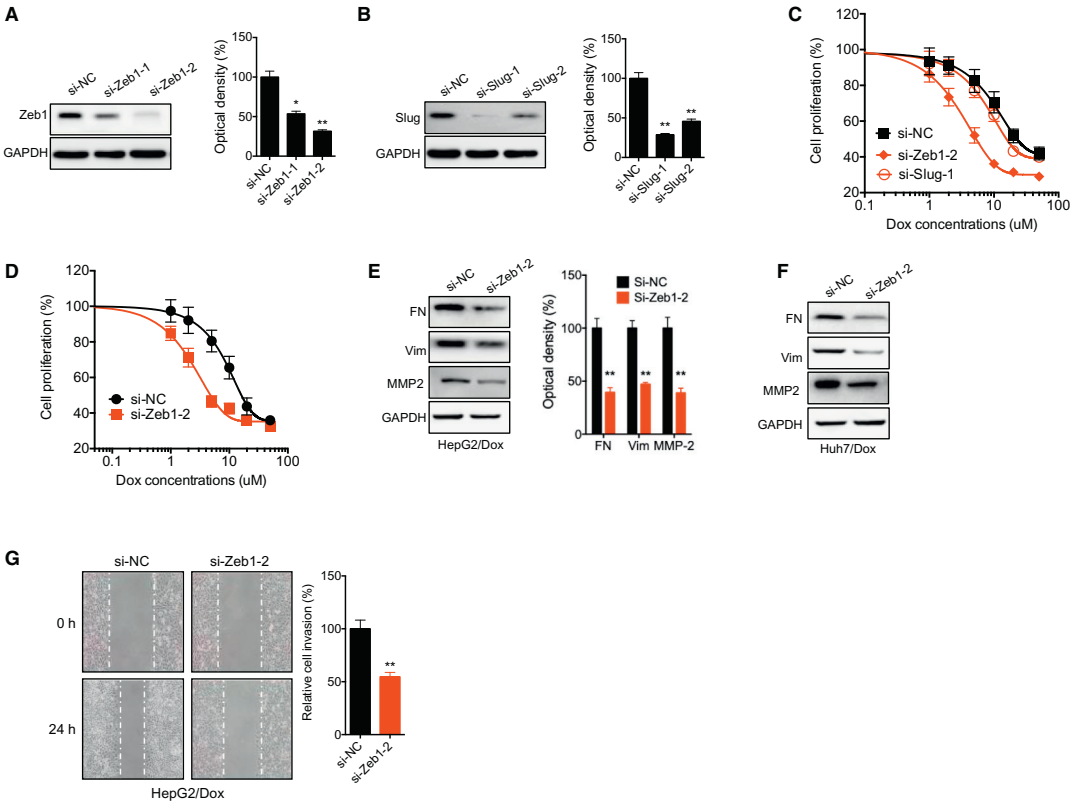

Fig. 3. Zeb1 was involved in EMT and drug resistance of HCC/Dox cells. HepG2/Dox cells were transfected with si-NC, si-Zeb1 (A), or si-Slug (B) for 24 h, the expression of Zeb1 and Slug analyzed by western blot analysis were recorded (left) and statistically analyzed (right), respectively; HepG2/Dox (C) or Huh7/Dox (D) cells were transfected with si-NC, si-Zeb1, or si-Slug for 12 h, and further treated with increasing concentrations of Dox for 48 h, the cell proliferation was detected by CCK-8 kit; HepG2/Dox (E) or Huh7/Dox (F) cells were transfected with si-NC or si-Zeb1 for 24 h, the expression of FN, Vim and MMP-2 analyzed by western blot analysis were recorded (left) and statistically analyzed (right); (G) The *in vitro* migration of HepG2/Dox cells transfected with si-NC or si-Zeb1 for 24 h was analyzed by wound healing assay (left) and statistically analyzed (right). Data were presented as means ± SD of three independent experiments. *$p < 0.05$ as compared with the control group, **$p < 0.01$ as compared with the control group.

vitro invasion ability of HepG2/Dox and Huh7/Dox cells were significantly increased as compared to their control cells (Fig. 1C). These data suggested that the Dox resistant HCC cells showed increased cell motility as compared to their parental cells.

3.2. HCC/Dox cells exhibited EMT characteristics

The increased cell motility suggested that HCC/Dox cells may have EMT properties compared with that of their parental cells. We then checked the expression of EMT related markers. The results showed that the expression of *E*-Cad decreased while the expression of fibronectin (FN) and vimentin (vim) increased in HepG2/Dox cells as compared to HepG2 cells (Fig. 2A). Consistently, this was also observed in Huh7/Dox cells as compared to Huh7 cells (Fig. 2B). We further checked the expression of EMT-TF between HCC/Dox and HCC cells. Increased expression of Slug and Zeb1 has been observed in HepG2/Dox cells as compared to that in HepG2 cells (Fig. 2C). While only increased Zeb1 was observed in Huh7/Dox cells as compared to Huh7 cells (Fig. 2D). These results indicated that HCC/Dox cells exhibited EMT characteristics.

3.3. Zeb1 was involved in EMT and drug resistance of HCC/Dox cells

We then knocked down the expression of Zeb1 (Fig. 3A) and Slug (Fig. 3B) in HepG2/Dox cells by use of their corresponding siRNAs. Our data showed that si-Zeb1, while not si-Slug, can significantly sensitize HepG2/Dox cells to Dox treatment (Fig. 3C). Further, si-Zeb1 can also sensitize the Huh7/Dox cells to Dox treatment (Fig. 3D). Western blot analysis showed that si-Zeb1 can decrease the expression of FN, Vim, and MMP-2 in both HepG2/Dox (E) and Huh7/Dox (F) cells. Wound healing assay showed that si-Zeb1 can suppress the *in vitro* migration of HepG2/Dox cells (Fig. 3G). These data indicated that Zeb1 was involved in EMT and drug resistance of HCC/Dox cells.

3.4. The protein stability and mRNA levels of Zeb1 were upregulated in HCC/Dox cells

We further investigated the potential mechanisms responsible for the upregulation of Zeb1 in HCC/Dox cells. Our data showed that the mature mRNA (Fig. 4A), while not the precursor-mRNA (Fig. 4B), was significantly increased in both HepG2/Dox and Huh7/Dox cells as compared to their corresponding parental cells. Further, we found that mRNA stability of *ZEB1* in HepG2/Dox cells were significantly greater than that in HepG2 cells (Fig. 4C). Similarly, the mRNA stability of *ZEB1* in Huh7/Dox cells were significantly greater than that in Huh7 cells (Fig. 4D). We further checked the protein stability of Zeb1 in HepG2/Dox and HepG2 cells by treated cells with CHX, the translation inhibitor. Western blot analysis showed that the half-life of Zeb1 protein in HepG2/Dox cells was significantly greater than that in HepG2 cells (Fig. 4E). The half-life of Zeb1 protein in Huh7/Dox cells were also significantly greater than that in Huh7 cells (data not shown). These data suggested that the protein stability and mRNA levels of Zeb1 were upregulated in HCC/Dox cells.

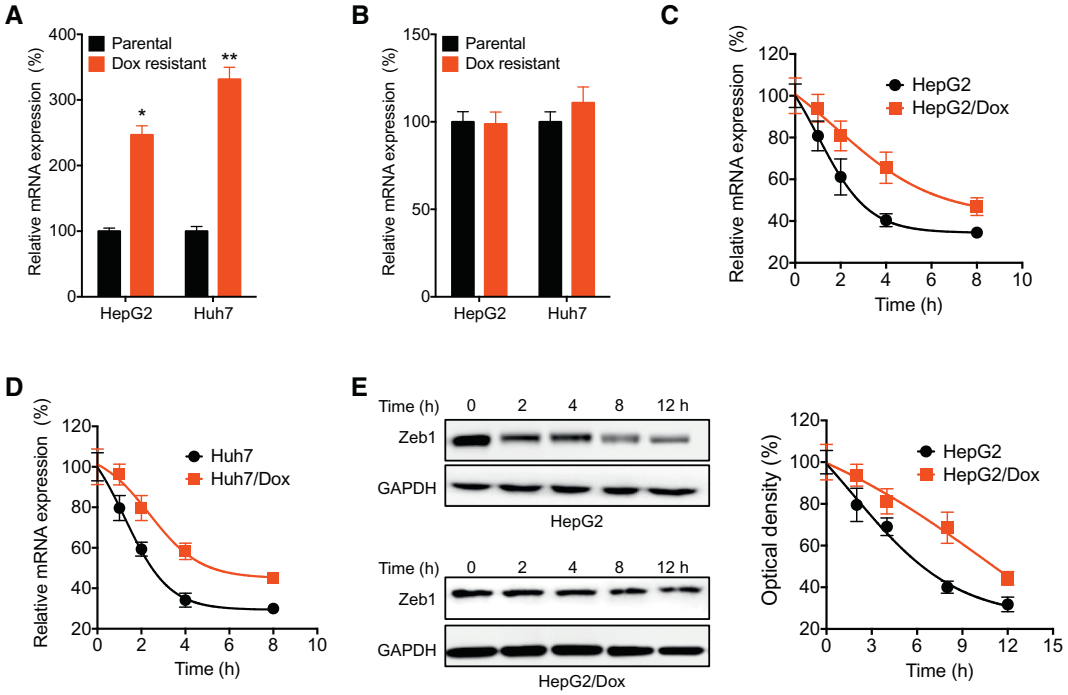

Fig. 4. The protein stability and mRNA levels of Zeb1 increased in HCC/Dox cells. The mature (A) or precursor (B) mRNA of *ZEB1* in HepG2/Dox or Huh7/Dox cells were detected by qRT-PCR, normalized to GAPDH, and compared to their parental cells; Both HepG2/Dox and HepG2 cells (C), or Huh7 and Huh7/Dox (D), were treated with Act-D for the indicated time periods, the mature mRNA of *ZEB1* was measured by qRT-PCR; (E) Both HepG2/Dox and HepG2 cells were treated with CHX for the indicated time periods, the expression of Zeb1 protein analyzed by western blot analysis were recorded (left) and statistically analyzed (right). Data were presented as means ± SD of three independent experiments. * $p < 0.05$ as compared with the control group, ** $p < 0.01$ as compared with the control group.

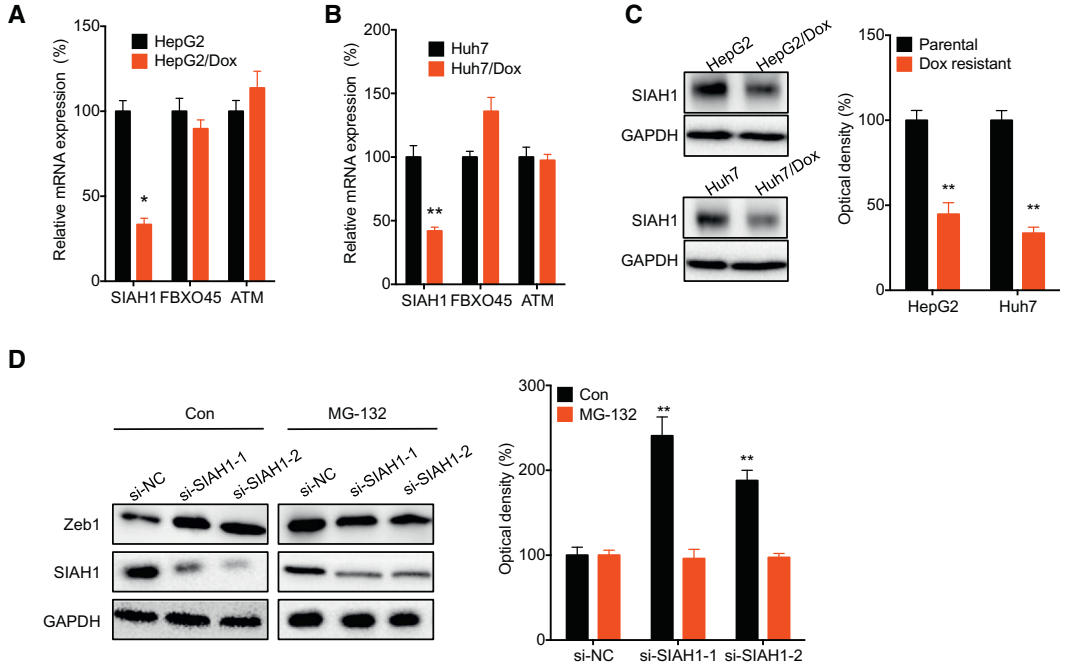

Fig. 5. SIAH1 mediated the upregulation of protein stability of Zeb1 in HCC/Dox cells. The mRNA of SIAH1, FBXO45, ATM in HepG2/Dox (A) or Huh7/Dox (B) cells were detected by qRT-PCR and compared to their parental cells; (C) The protein expression of SIAH1 in HepG2/Dox or Huh7/Dox cells and their parental cells analyzed by western blot analysis were recorded (left) and statistically analyzed (right); (D) HepG2 cells transfected with si-NC or si-SIAH1-1/-2 were further treated with or without MG-132, the protein expression of Zeb1 and SIAH1 analyzed by western blot analysis were recorded (left) and statistically analyzed (right). Data were presented as means ± SD of three independent experiments. *$p < 0.05$ as compared with the control group, ** $p < 0.01$ as compared with the control group.

3.5. SIAH1 mediated the upregulation of protein stability of Zeb1 in HCC/Dox cells

It has been revealed that SIAH1, FBXO45, and ATM can regulate the protein stability of Zeb1 in cancer cells (Zhang et al., 2014; Abshire et al., 2016). We found that mRNA of SIAH1, while not FBXO45 or ATM, was decreased in HepG2/Dox (Fig. 5A) and Huh7/Dox (Fig. 5B) cells as compared to that in their parental cells. Western blot analysis

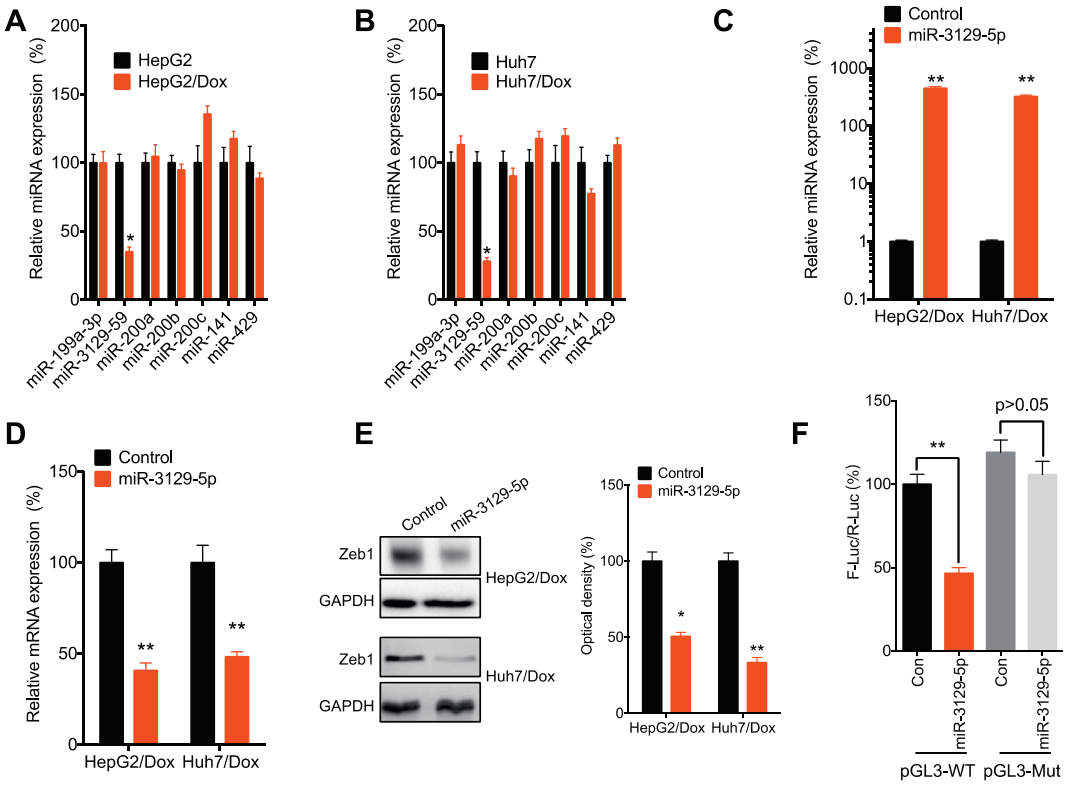

Fig. 6. MiR-3129-5p regulated the expression of *ZEB1* mRNA in HCC/Dox cells. The miRNA expression of miR-199a-3p, miR-3129-5p, miR-200a/b/c, miR-141, and miR-429 in HepG2/Dox (A) or Huh7/Dox (B) cells were detected by qRT-PCR and compared to that in their parental cells; (C) The over expression of miR-3129-5p was checked by qRT-PCR; HepG2/Dox or Huh7/Dox cells were transfected with control or miR-3129-5p mimic for 24 h, the mRNA (D, normalized to GAPDH) or protein (E) expression of Zeb1 was measured by qRT-PCR or western blot analysis, respectively; (F) HepG2/Dox cells were transfected with miR-3129-5p mimic and pGL3-WT-*ZEB1*-3′UTR or pGL3-Mut-*ZEB1*-3′UTR for 24 h. pRL-TK was used for normalization of transfection efficiency. Data were presented as means ± SD of three independent experiments. *p < 0.05 as compared with the control group, **p < 0.01 as compared with the control group.

confirmed that the down regulation of SIAH1 protein in in HepG2/Dox and Huh7/Dox cells as compared to that in their parental cells (Fig. 5C). We then knocked down the expression of SIAH1 in HepG2 cells (Fig. 5D). Our data showed that the knockdown of SIAH1 can significantly increase the expression of Zeb1 in HepG2 cells, however, si-SIAH1 induced up regulation of Zeb1 was attenuated in the presence of MG-132 (Fig. 5D). All these data suggested decreased SIAH1 mediated the upregulation of protein stability of Zeb1 in HCC/Dox cells.

3.6. miR-3129-5p regulated the expression of ZEB1 mRNA in HCC/Dox cells

Our results suggested that the mRNA of Zeb1 in HCC/Dox cells was more stable than that in parental cells. *In silico* analysis (http://www.targetscan.org/) suggested that miR-199a-3p and miR-3129-5p are conserved miRNAs which can regulate the mRNA of Zeb1. It has been reported that miR200 family such as miR-200a can regulate the expression of Zeb1 in cancer cells (Gregory et al., 2008). qRT-PCR showed that the expression of miR-3129-5p, while not miR-199a-3p, miR-200a/b/c, miR-141, or miR-429, was decreased in HepG2/Dox (Fig. 6A) and Huh7/Dox (Fig. 6B) cells as compared to that in their parental cells. Further, over expression of miR-3129-5p (Fig. 6C) can decrease the mRNA (Fig. 6D) and protein (Fig. 6E) of Zeb1 in both HepG2/Dox and Huh7/Dox cells. HepG2/Dox cells were co-transfected with miR-3129-5p and pGL3-WT-*ZEB1*–3′UTR or pGL3-Mut-*ZEB1*–3′UTR (mutant the binding site ACUACUG to AGGAUGC). Luciferase assay showed that miR-3129-5p can significant decrease the luciferase activity of pGL3-WT-*ZEB1*–3′UTR while had no effect on that of pGL3-Mut-*ZEB1*–3′UTR (Fig. 6F). These data suggested that miR-3129-5p directly regulated the Zeb1 mRNA in HCC/Dox cells.

4. Discussion

The EMT and drug resistance have been linked together since EMT can contribute to drug resistance during chemotherapy (Shibue and Weinberg, 2017). Our present study showed that the Dox resistant HCC cells had greater capability of *in vitro* migration and invasion and exhibited EMT characteristics. The upregulation of EMT-TF Zeb1 was involved in this process duo to knockdown of Zeb1 can sensitize HCC cells to Dox and suppress the *in vitro* migration. Mechanistical studies suggested that the upregulation of Zeb1 in HCC cells was due to 1) SIAH1 increased protein stability of Zeb1, and 2) miR-3129-5p suppressed mRNA stability of Zeb1 in HCC cells. All these results suggested that targeted inhibition of Zeb1 might be helpful to overcome the Dox resistance of HCC cells.

As one powerful EMT-TF, Zeb1 can mediate EMT and chemo-resistance of various cancer cells (Meidhof et al., 2015; Hashimoto et al., 2016; Zhang et al., 2015). Our present study showed that the *in vitro* migration and invasion capability, and mesenchymal markers of HCC/Dox were significantly increased compared to that in their corresponding parental cells. While knockdown of Zeb1 can attenuate these characteristics. Zeb1 can induce EMT *via* repression of epithelial genes such as E-cadherin (Lim and Thiery, 2012). The mechanisms underlying Zeb1 mediated chemo-resistance seems to be more complex. Previous studies indicated that Zeb1 can regulate the radioresistance of cancer cells through an ATM − ZEB1 − CHK1 signaling axis (Zhang et al., 2014). Further, only Zeb1, but not Snail or Twist, conferred radioresistance on MCF7 cells even without inducing EMT (Zhang et al., 2014). It indicated that ZEB1, but not EMT itself, can induce radioresistance of cancer cells. It has been indicated that ZEB1 associated drug resistance in cancer cells can be reversed by the class I HDAC

inhibitor (Meidhof et al., 2015). Over expression of ZEB1 has been observed in human cancers to promote the cancer progression and development (Zhang et al., 2015). Our data suggested that the drug resistant patients are likely to benefit from targeted inhibition of ZEB1 and ZEB1-targeting agents have the potential to be used as tumor chemosensitizers (Zhang et al., 2018).

Our present study revealed that SIAH1 decreased protein stability and miR-3129-5p suppressed mRNA stability are two major reasons for the upregulation of Zeb1 in HCC/Dox cells. The expression of Zeb1 can be regulated by several post-transcriptional and post-translational factors (Exposito-Villen et al., 2018). As a member of E3 ubiquitin ligase family, SIAH1 can trigger the degradation of ZEB1 in cancer cells (Chen et al., 2015). Our present study revealed that SIAH1 was down regulated in HCC/Dox cells. Knockdown of SIAH1 in HepG2 cells can increase the expression of Zeb1, while this effect was abolished in the presence of MG-132. It confirmed that SIAH1 can decrease the protein stability of Zeb1 in HCC cells. Other factors such as CSN5 (Zhang et al., 2017) and USP51 (Zhou et al., 2017) can also regulate the protein stability of Zeb1. Their roles in Zeb1 mediated Dox resistance of HCC cells need further studies. Further, miR-31295p was found to trigger the decay of Zeb1 mRNA in HCC cells. Several miRNAs such as miR-204 (Wu et al., 2017), miR-200c (Kurata et al., 2018), miR-200a (Li et al., 2016), and miR-205 (Niu et al., 2015) can also directly target Zeb1 to regulate its expression. Interestingly, Dox treatment can increase the expression of miR-200c and then down regulate the expression of Zeb1 in human cardiac mesenchymal progenitor cells (Beji et al., 2017). However, in our present study we found that Dox treatment had no effect on the expression of Zeb1 in HepG2 cells (data not shown). It might be due to the variation of cell lines and also need further studies. It has been reported that miR200 family inhibited ovarian cancer cell invasiveness and metastasis by downregulating MMP3, possibly through ZEB1/pSMAD3 (Sun et al., 2014). Consistently, our present study revealed that knockdown of Zeb1 can inhibit the expression of MMP2 in HepG2/Dox and Huh7/Dox cells.

In summary, our present study showed that HCC/Dox cells exhibited mesenchymal characteristics and great capability of cell migration and invasion. SIAH1 and miR-3129 mediated upregulation of Zeb1 was responsible for the aggressive phenotypes of HCC/Dox cells. Therefor targeted inhibition of Zeb1 might be helpful for clinical overcome the Dox resistance.

Conflict of interest

The authors declare no conflict of interest.

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