European Review for Medical and Pharmacological Sciences 2016; 20: 1080-1086

MicroRNA-138 regulates chemoresistance in human non-small cell lung cancer via epithelial mesenchymal transition

Z. JIN, L. GUAN, Y. SONG, G.-M. XIANG, S.-X. CHEN, B. GAO

Institute of Respiratory Disease, China Three Gorges University, Yichang Central People's Hospital, China

Zhu Jin, *Li Guan* and *Yaya Song* equally contribute to this study

Abstract. – OBJECTIVE: Down-regulation of miR-138 is observed in a variety of cancers, which suggests that miR-138 may be involved in cancer pathogenesis. Our current work aimed to evaluate the effects of miR-138 in adriamycin (ADM)-resistant human NSCLC cells.

MATERIALS AND METHODS: Cell proliferation was determined by MTT assay. Real-time PCR and western blot were performed to detect the mRNA and protein expression levels. The target of miR-138 was validated by luciferase activity assay.

RESULTS: Compared with the chemosensitive parental cells, miR-138 was remarkably decreased in A549/ADM and NCI-H23/ADM cells. Ectopic expression of miR-138 sensitized chemoresistant tumor cells to ADM administration. In addition, the epithelial-mesenchymal transition (EMT) related markers E-cadherin or vimentin was up-regulated or down-regulated upon the overexpression of miR-138 in NSCLC cells. Further studies identified zinc finger E-box-binding homeobox 2 (ZEB2) as the target of miR-138 and up-regulation of miR-138 suppressed the mRNA and protein expression of ZEB2. Notably, luciferase reporter assay confirmed that ZEB2 was a direct target of miR-138.

CONCLUSIONS: Our study demonstrates that miR-138 sensitizes NSCLC cells to ADM via EMT, suggesting that miR-138 might be a potential therapeutic target for drug-resistant NSCLC patients.

Key Words:
Non-small cell lung cancer, miR-138, Drug resistance, Epithelial mesenchymal transition.

Introduction

Lung cancer remains as one of the leading causes of cancer-related deaths worldwide, in which 80% of lung cancers are non-small cell lung cancer (NSCLC) with poor therapeutic efficacy when diagnosed[1]. In addition to surgical resection, chemotherapy has become one of the important adjuvant therapies for lung cancer. However, resistance to therapeutics is a great challenge in NSCLC treatment, which has drawn much attention in recent years[2,3]. Thus, it is urgent to develop new therapeutic strategies to overcome the drug resistance of NSCLC.

Emerging evidence indicates that a wide range of genetic and epigenetic alterations are involved in the pathogenesis of NSCLC[4]. MicroRNAs (miRNAs) are a class of 18 to 25 nucleotides single-stranded non-coding RNA, and can transcriptionally regulate genes expression[5]. In recent year, it has been demonstrated that miRNAs play diverse roles in tumor cell behaviors, including cell growth, differentiation, migration, invasion, apoptosis and drug resistance[6]. Several studies[7-9] reported that miR-138 was down-regulated in breast cancer, colorectal cancer, and head and neck squamous cell carcinomas. Documented biological function of miR-138 includes induction of apoptosis, and inhibition of tumor growth, invasion and metastasis[10-12]. A recent study reported[13] the regulatory role of miR-138-5p on drug resistance in NSCLC through targeting G protein-coupled receptor. However, to date, the role of miR-138 on NSCLC cells chemoresistance has not been fully elucidated. In the current study, we aimed to evaluate the relevance of miR-138 in drug resistance of NSCLC and further explore the underlying molecular mechanism.

Materials and Methods

Cell Culture

The human NSCLC cell lines including A549 and NCI-H23 were purchased from the ATCC (Manassas, VA, USA). ADM was purchased

Corresponding Author: Baoan Gao, MD; e-mail: 222xiaozhao@163.com

from Sigma-Aldrich (St. Louis, MO, USA). Multidrug resistant human NSCLC cell lines, A549/adriamycin (ADM) and NCI-H23/ADM were established by treating A549/WT and NCI-H23/WT cells with stepwise increasing concentrations of ADM and removing the non-resistant dead cells. All cells were maintained at 37°C in 5% CO_2 incubator and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere containing 5% CO_2 at 37°C.

Cell Proliferation Assay

Cell proliferation was measured by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. To be brief, cultured NSCLC cells were seeded into 96-well plates at the density of 4×10^4 (cells/well). Then 10 ml of 5 mg/ml MTT was added and incubated in dark at 37°C for another 2 h. The absorbance was determined with the wavelength of 490 nm.

RNA Extraction, Real-Time PCR

NSCLC cells were seeded on to 12-well plates and total RNAs were isolated by TRIzol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. Total RNA was used to perform reverse transcription by One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Dalian, China). Real-time PCR was performed with the SYBR green Premix Ex Taq II (Takara) with StepOne Plus Real-Time PCR System (Applied Biosystems, Carlsband, CA, USA) with U6 or gapdh used as the endogenous control.

Luciferase Activity Assay

Luciferase reporters were generated based on the firefly luciferase expressing vector pMIR-REPORT (Ambion, Walthan, MA, USA). Cells at the density of 5×10^4 cells per well were seeded in 24-well plates the day before transfection. Luciferase reporter (500 ng), 50 pmol (miRNA-138 mimic or control) and 40 ng of pRL-TK were added in each well. Cells were collected 48 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western Blot

NSCLC cells were lysed and protein concentration was quantified with Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). Protein samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then incubated with anti-E-cadherin, anti-vimentin and anti-zinc finger E-box-binding homeobox 2 (ZEB2) (Cell Signaling Technology, Danvers, MA, USA) antibodies at 4°C overnight. The membranes were washed three times with TBST and then incubated with the appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. Protein expression was detected by chemiluminescence (GE Healthcare, Piscataway, NJ, USA).

Statistical Analysis

Each experiment was performed in triplicate, and repeated at least three times. All the data were presented as means ± SD and treated for statistics analysis by SPSS program. Comparison between groups was made using ANOVA and statistically significant difference was defined as $p < 0.05$.

Results

miR-138 is Down-Regulated in Chemoresistant NSCLC Cells

Firstly, NSCLC cells the ADM sensitivity of A549 and NCI-H23 cells and showed that A549/ADM cells were less sensitive to ADM treatment compared with their parental cells (Figure 1A). Similarly, we also found that NCI-H23/ADM cells became more resistant to ADM compared with NCI-H23 cells (Figure 1B). To evaluate the biological relevance of miR-138 in drug resistance, we measured the expression of miR-138 in ADM sensitive/resistant NSCLC cells. Data from real-time PCR showed that miR-138 was significantly down-regulated in A549/ADM and NCI-H23/ADM cells compared with their parental cells (Figure 1C and D). These results suggested that miR-138 might play a critical role in the drug resistance of NSCLC cells.

miR-138 is Involved in the Chemoresistance of NSCLC Cells

Next miR-138 mimic was transfected into A549/ADM and NCI-H23/ADM cells to confirm whether miR-138 was involved in the ADM sensitivity in NSCLC cells. With transfection of miR-138, real-time PCR showed that A549/ADM cells transfected with miR-138 mimic exhibited increased miR-138 levels compared to those trans-

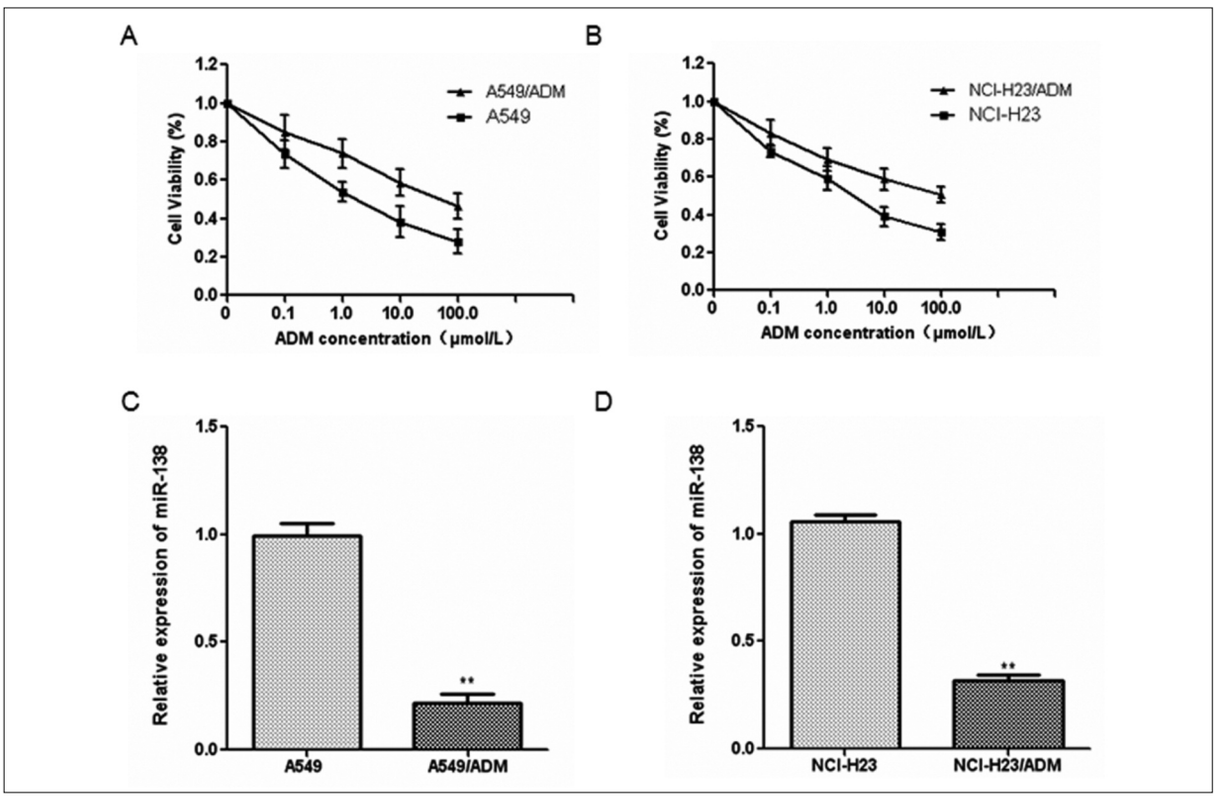

Figure 1. Down-regulation of miR-138 in chemoresistant NSCLC cells. ADM cytotoxicity on A549 and A549/ADM ***(A)***, NCI-H23 and NCI-H23/ADM ***(B)*** cells was assessed by MTT assay. Real-time PCR was performed to measure the expression of miR-138 in A549 and A549/ADM ***(C)***, NCI-H23 and NCI-H23/ADM ***(D)*** cells. $^{**}p < 0.01$.

fected with negative controls (Figure 2A). As a result, ectopic expression of miR-138 remarkably enhanced the ADM sensitivity in A549/ADM cells transfected with miR-138 mimic (Figure 2B). In addition, transfection of miR-138 mimic into NCI-H23/ADM cells also augmented the expression of miR-138 and, consequently, suppressed cells proliferation (Figure 2C and D). Taken together, these data indicated that up-regulation of miR-138 contributed to the chemotherapeutics sensitivity in NSCLC cells.

Effects of miR-138 on NSCLC Cell Epithelial-Mesenchymal Transition (EMT) Program

The involvement of EMT has been reported in many vital cellular processes in malignant tumors, and thus we determined the association between miR-138 and EMT. Results showed that ectopic expression of miR-138 in A549/ADM cells led to up-regulation of E-cadherin and down-regulation of vimentin at the mRNA levels (Figure 3A and B). Moreover, Western blot analysis revealed that the epithelial marker E-cadherin was significantly up-regulated, and the mesenchymal marker vimentin was down-regulated in miR-138 mimic transfected NSCLC cells (Figure 3C and D). These results suggested that miR-138 functioned as a potential EMT-suppressive miRNAs.

Up-regulation of miR-138 Inhibited ZEB2 Expression in NSCLC Cells

Further, we searched the potential targets of miR-138 by performing computational predictions, and found 3'UTR of ZEB2 containing the conserved putative miRNA-138 binding sites. A549/ADM cells were then transfected with miR-138 mimic and negative control. Data from real-time PCR showed that ectopic expression of miR-138 inhibited the mRNA expression of ZEB2 in A549/ADM cells (Figure 4A). Consistently, the protein expression of ZEB2 was also reduced in miR-138-transfected tumor cells (Figure 4B). Also, a marked reduction in luciferase activity was observed in A549/ADM cells after

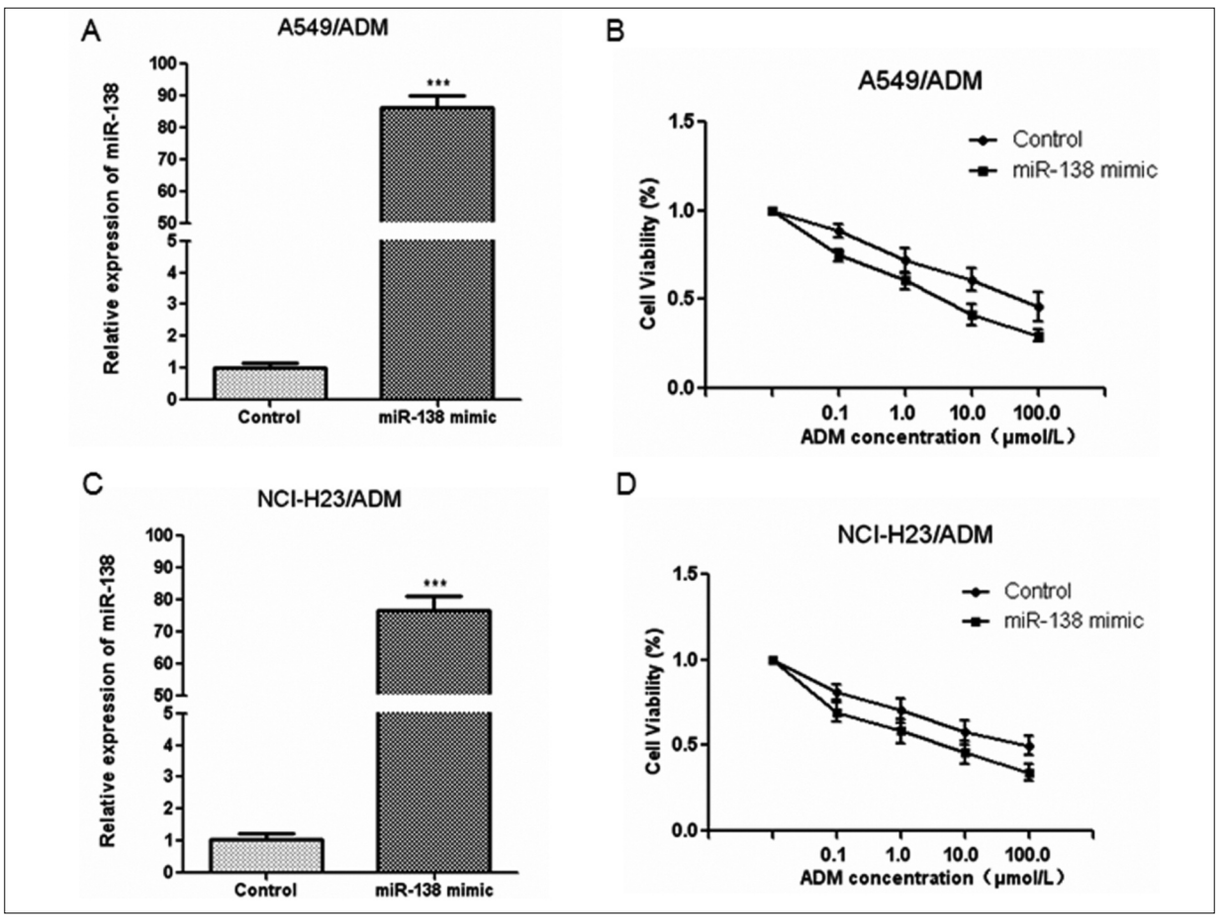

Figure 2. miR-138 was involved in chemoresistance of NSCLC cells. A549/ADM ***(A)*** and NCI-H23/ADM cells ***(C)*** were transfected with miR-138 mimic or negative control, and up-regulation of miR-138 was confirmed by real-time PCR. ***(B*** and ***D)*** Cell viability was evaluated using MTT assay after transfection with miR-138 mimic. ***$p < 0.001$.

transfection with miR-138 mimic (Figure 4C). These results indicated that ZEB2 was a direct target of miR-138 in NSCLC cells.

Discussion

ADM-based chemotherapy serves as one of the standard protocols for malignancies treatment, but resistance to ADM usually results in failure in clinical practice[14]. In recent years, increasing evidence suggest that miRNAs are important regulators of chemoresistance in a wide range of cancers[15]. Therefore, the revelation of the biological roles of miRNAs may contribute to find novel therapeutic targets in NSCLC treatment. In the current work, we demonstrated that ectopic expression of miR-138 sensitized NSCLC cells to ADM via reversing EMT program.

Previous studies[16-18] have suggested that miR-138 is down-regulated in several cancers such as leukemia, esophageal squamous cell carcinoma, anaplastic thyroid cancer. Documented biological roles of miR-138 include inhibition of proliferation, induction of apoptosis, and sensitization to chemotherapeutics in cancer cells[10,16,19]. In addition, multiple miR-138-targeted genes and signaling pathways have been identified including RhoC, FAK, Src, ROCK2, and Erk1/2 which are critical modulators in cellular behaviors[20,21]. Our study indicated that miR-138 was down-regulated in chemoresistant NSCLCL cells and its up-regulation inhibited tumor cell growth, implying that miR-138 functioned as a tumor-suppressive miRNA in NSCLC.

EMT refers to the complicated progress in which tumor cell loses epithelial properties and gains mesenchymal morphology with capacity

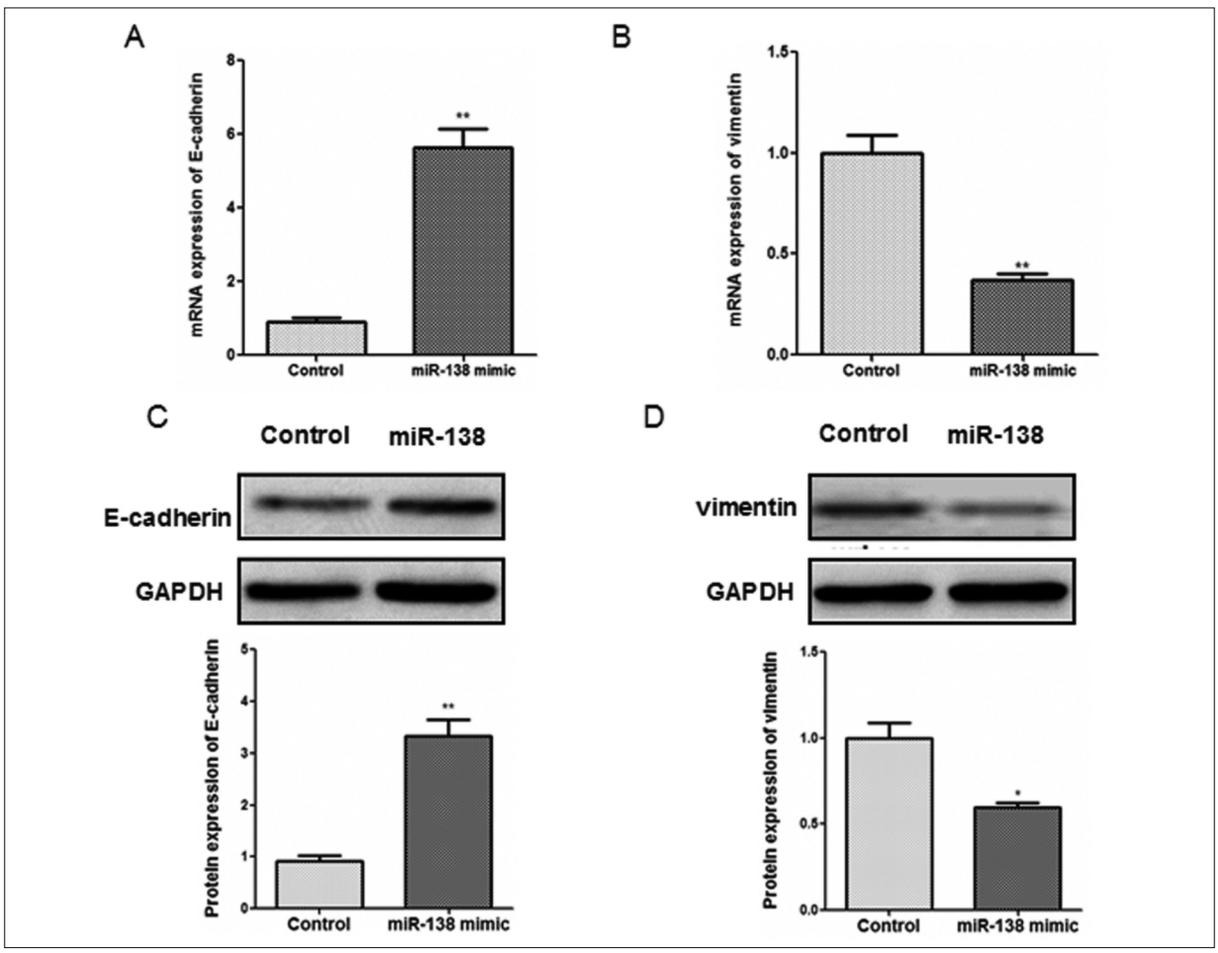

Figure 3. miR-138 regulated EMT in NSCLC cells. The mRNA expression of EMT-related biomarkers including E-cadherin ***(A)*** and vimentin ***(B)*** in A549/ADM cells were measured by real-time PCR. Western blot was used to measure the protein levels of E-cadherin ***(C)*** and vimentin ***(D)*** in A549/ADM cells transfected with miR-138 mimic or negative control. $*p < 0.05$, $**p < 0.01$.

for metastasis[22]. Previous studies[23] have shown that EMT is involved in stem cell behaviors, wound healing, and development. Emerging evidence suggest that EMT also plays diverse roles in cancer cell growth, metastasis, invasion, and resistance to chemotherapeutics[24]. An investigation[25] of squamous cell carcinoma has revealed that miR-138 serves as a multi-functional molecular regulator and plays critical roles in EMT program. In this current work, we found that up-regulation of miR-138 promoted the epithelial marker E-cadherin and suppressed the mesenchymal marker vimentin both at the mRNA and protein levels, suggesting that miR-138 could serve as an EMT-suppressive miRNA in NSCLC cells.

ZEB2, a member of the zinc finger E-box-binding homeobox family, was closely associated with carcinogenesis, progression and response to chemotherapy of cancer[26]. Functioning as an EMT inducer and E-cadherin repressor, inhibition of ZEB2 by several miRNAs could suppress the migration, invasion and chemoresistance of tumor cells[25]. For instance, miR-132 inhibits the migratory and invasive abilities in lung cancer cells through down-regulation of ZEB2[27]. Another study[28] demonstrates that miR-335 inhibited cell migration and invasion *in vitro* and lung metastasis *in vivo* through targeting ZEB2, suggesting that miR-335 may be a new potential therapeutic target for colorectal cancer. Consistent with these previous study, our study also showed that ectopic expression of miR-138 decreased ZEB2 expression and inhibited the luciferase activity in chemoresistant tumor cells, suggesting that miR-138 could regulate EMT, at least partly, through targeting ZEB2 in NSCLC cells.

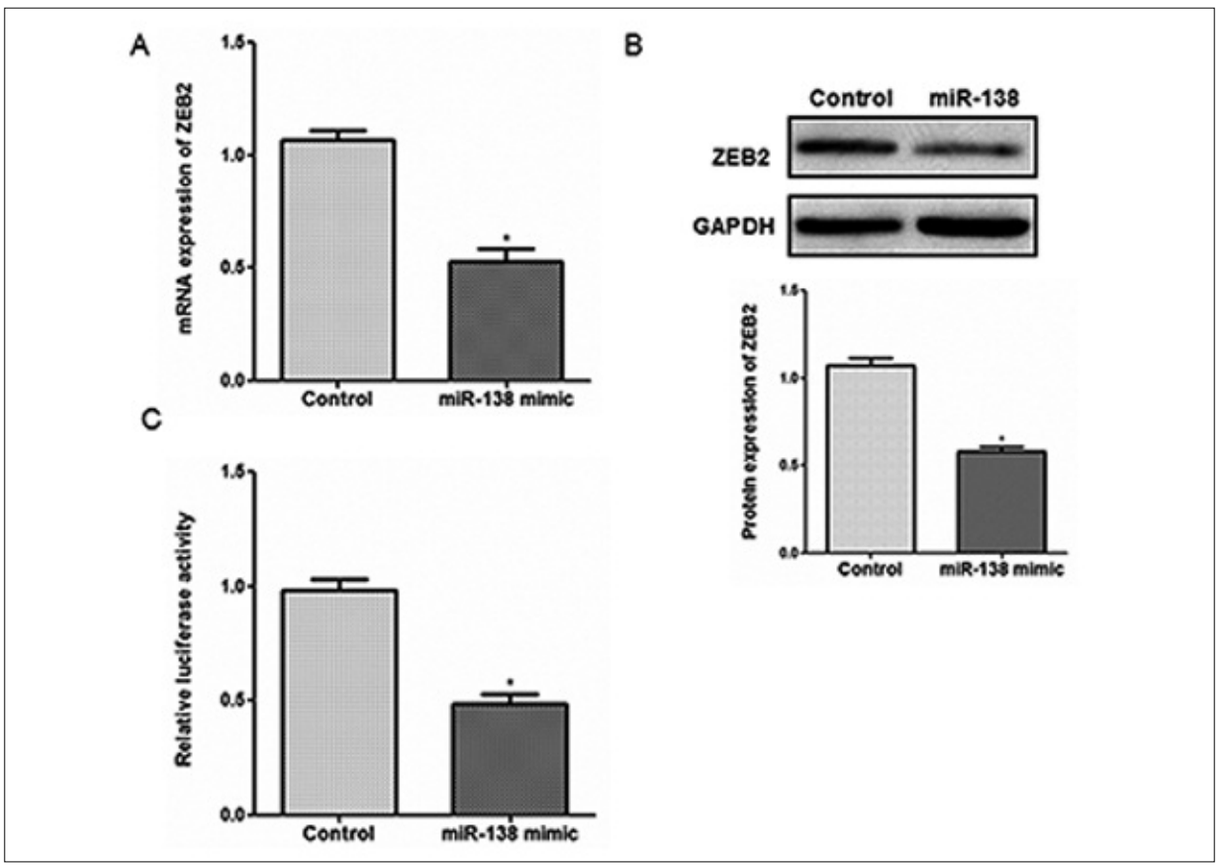

Figure 4. ZEB2 was a target of miR-138 in NSCLC cells. The mRNA ***(A)*** and protein ***(B)*** levels of ZEB2 in A549/ADM cells were determined by real-time PCR and western blot, respectively. Luciferase reporter assay was performed to measure the relative luciferase activity in miR-138-transfected A549/ADM cells ***(C)***. *$p < 0.05$.

Conclusions

Our work demonstrates that miR-138 sensitizes NSCLC cells to ADM through regulation of EMT regulator ZEB2. These findings provide new insight into the mechanism responsible for the chemoresistance in human NSCLC and imply that miR-138 may serve as a potential therapeutic candidate in drug-resistant NSCLC patients.

Acknowledgements

Expression and significance of peroxisome proliferator-activated receptor γ (PPARγ) in pulmonary artery of rats with hypoxic pulmonary hypertension and the impact of bosentan for it. (Natural Science Foundation of Hubei Province. 2014CFC1037). The study of the mechanism for glucocorticoid effecting to hypoxic pulmonary hypertension. (Natural Science Foundation of Hubei Province. 2012FFB06301).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015; 65: 87-108.
2) Koch J, Hau J, Jensen HE, Rieneck K. Cancer resistance as an acquired and inheritable trait. Anticancer Res 2014; 34: 6315-6325.
3) Brown R, Curry E, Magnani L, Wilhelm-Benartzi CS, Borley J. Poised epigenetic states and acquired drug resistance in cancer. Nat Rev Cancer 2014; 14: 747-753.
4) Ryan BM, Robles AI, Harris CC. Genetic variation in microRNA networks: the implications for cancer research. Nat Rev Cancer 2010; 10: 389-402.

5) Pritchard CC, Cheng HH, Tewari M. MicroRNA profiling: approaches and considerations. Nat Rev Genet 2012; 13: 358-369.

6) Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med 2012; 4: 143-159.

7) Long L, Huang G, Zhu H, Guo Y, Liu Y, Huo J. Down-regulation of miR-138 promotes colorectal cancer metastasis via directly targeting TWIST2. J Transl Med 2013; 11: 275.

8) Bockhorn J, Prat A, Chang YF, Liu X, Huang S, Shang M, Nwachukwu C, Gomez-Vega MJ, Harrell JC, Olopade OI, Perou CM, Liu H. Differentiation and loss of malignant character of spontaneous pulmonary metastases in patient-derived breast cancer models. Cancer Res 2014; 74: 7406-7417.

9) Manikandan M, Deva Magendhra Rao AK, Rajkumar KS, Rajaraman R, Munirajan AK. Altered levels of miR-21, miR-125b-2*, miR-138, miR-155, miR-184, and miR-205 in oral squamous cell carcinoma and association with clinicopathological characteristics. J Oral Pathol Med 2014.

10) Zhang H, Zhang H, Zhao M, Lv Z, Zhang X, Qin X, Wang H, Wang S, Su J, Lv X, Liu H, Du W, Zhou W, Chen X, Fei K. MiR-138 inhibits tumor growth through repression of EZH2 in non-small cell lung cancer. Cell Physiol Biochem 2013; 31: 56-65.

11) Xiong H, Luo T, He W, Xi D, Lu H, Li M, Liu J, Guo Z. Up-regulation of miR-138 inhibits hypoxia-induced cardiomyocyte apoptosis via down-regulating lipocalin-2 expression. Exp Biol Med (Maywood) 2016; 241: 25-30.

12) Golubovskaya VM, Sumbler B, Ho B, Yemma M, Cance WG. MiR-138 and MiR-135 directly target focal adhesion kinase, inhibit cell invasion, and increase sensitivity to chemotherapy in cancer cells. Anticancer Agents Med Chem 2014; 14: 18-28.

13) Gao Y, Fan X, Li W, Ping W, Deng Y, Fu X. miR-138-5p reverses gefitinib resistance in non-small cell lung cancer cells via negatively regulating G protein-coupled receptor 124. Biochem Biophys Res Commun 2014; 446: 179-186.

14) Vatsyayan R, Chaudhary P, Lelsani PC, Singhal P, Awasthi YC, Awasthi S, Singhal SS. Role of RLIP76 in doxorubicin resistance in lung cancer. Int J Oncol 2009; 34: 1505-1511.

15) Gong Z, Yang J, Li J, Yang L, Le Y, Wang S, Lin HK. Novel insights into the role of microRNA in lung cancer resistance to treatment and targeted therapy. Curr Cancer Drug Targets 2014; 14: 241-258.

16) Zhao X, Yang L, Hu J, Ruan J. miR-138 might reverse multidrug resistance of leukemia cells. Leuk Res 2010; 34: 1078-1082.

17) Gong H, Song L, Lin C, Liu A, Lin X, Wu J, Li M, Li J. Downregulation of miR-138 sustains NF-kappaB activation and promotes lipid raft formation in esophageal squamous cell carcinoma. Clin Cancer Res 2013; 19: 1083-1093.

18) Mitomo S, Maesawa C, Ogasawara S, Iwaya T, Shibazaki M, Yashima-Abo A, Kotani K, Oikawa H, Sakurai E, Izutsu N, Kato K, Komatsu H, Ikeda K, Wakabayashi G, Masuda T. Downregulation of miR-138 is associated with overexpression of human telomerase reverse transcriptase protein in human anaplastic thyroid carcinoma cell lines. Cancer Sci 2008; 99: 280-286.

19) Liu X, Lv XB, Wang XP, Sang Y, Xu S, Hu K, Wu M, Liang Y, Liu P, Tang J, Lu WH, Feng QS, Chen LZ, Qian CN, Bei JX, Kang T, Zeng YX. MiR-138 suppressed nasopharyngeal carcinoma growth and tumorigenesis by targeting the CCND1 oncogene. Cell Cycle 2012; 11: 2495-2506.

20) Islam M, Datta J, Lang JC, Teknos TN. Down regulation of RhoC by microRNA-138 results in de-activation of FAK, Src and Erk1/2 signaling pathway in head and neck squamous cell carcinoma. Oral Oncol 2014; 50: 448-456.

21) Jiang L, Liu X, Kolokythas A, Yu J, Wang A, Heidbreder CE, Shi F, Zhou X. Downregulation of the Rho GTPase signaling pathway is involved in the microRNA-138-mediated inhibition of cell migration and invasion in tongue squamous cell carcinoma. Int J Cancer 2010; 127: 505-512.

22) Zhang L, Jiao M, Wu K, Li L, Zhu G, Wang X, He D, Wu D. TNF-alpha induced epithelial mesenchymal transition increases stemness properties in renal cell carcinoma cells. Int J Clin Exp Med 2014; 7: 4951-4958.

23) Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 2014; 15: 178-196.

24) Wang Z. Targeting epithelial-to-mesenchymal transition for cancer therapy. Curr Pharm Des 2015; 21: 1239.

25) Liu X, Wang C, Chen Z, Jin Y, Wang Y, Kolokythas A, Dai Y, Zhou X. MicroRNA-138 suppresses epithelial-mesenchymal transition in squamous cell carcinoma cell lines. Biochem J 2011; 440: 23-31.

26) Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer 2007; 7: 415-428.

27) You J, Li Y, Fang N, Liu B, Zu L, Chang R, Li X, Zhou Q. MiR-132 suppresses the migration and invasion of lung cancer cells via targeting the EMT regulator ZEB2. PLoS One 2014; 9: e91827.

28) Sun Z, Zhang Z, Liu Z, Qiu B, Liu K, Dong G. MicroRNA-335 inhibits invasion and metastasis of colorectal cancer by targeting ZEB2. Med Oncol 2014; 31: 982.