Original Paper

Prognostic Significance of microRNA-7 and its Roles in the Regulation of Cisplatin Resistance in Lung Adenocarcinoma

Mao-Wei Cheng^{a,b} Ze-Tian Shen^c Gu-Yu Hu^a Li-Guo Luo^d

^aCollege of Command Information System, PLA University of Science and Technology, Nanjing,

^bDepartment of Internal Neurology, The People's Liberation Army 309 Hospital, Beijing, ^cDepartment of Radiation Oncology, ^dDepartment of Cardiothoracic Surgery, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, China

Key Words

miR-7 • Lung adenocarcinoma • Bcl-2 • Prognosis • Apoptosis • Chemosensitivity

Abstract

Background: Previously, microRNA (miR)-7 has been reported to function as a tumor suppressor in human cancers, but the correlations of miR-7 expression with prognosis and cisplatin (CDDP) resistance in lung adenocarcinoma (LA) are unclear. Here, our aim is to determine the prognostic significance of miR-7 and its roles in the regulation of CDDP resistance in LA. **Methods:** Quantitative real-time PCR (qRT-PCR) assay was performed to determine miR-7 expression in 108 paired of LA tissues and analyze its correlations with clinicopathological factors of patients. The patient survival data were collected retrospectively by Kaplan-Meier analyses, and multivariate analysis was performed using the Cox proportional hazards model to determine the prognostic significance of miR-7 expression. The effects of miR-7 expression on the chemosensitivity of LA cells to CDDP and its possible mechanisms were evaluated by MTT, flow cytometry, Western blot and luciferase assays. **Results:** It was observed that the relative expression level of miR-7 in LA tissues was significantly lower than that in the adjacent normal tissues and low miR-7 expression level was closely associated with poorer tumor differentiation, advanced pathological T-factor, higher incidence of lymph node metastasis and advanced p-TNM stage. Also, patients with low miR-7 expression showed a shorter overall survival than those with high miR-7 expression, and multivariate analysis indicated that status of miR-7 expression was an independent molecular biomarker for predicting the overall survival (OS) of LA patients. In addition, upregulation of miR-7 increases the sensitivity of LA cells to CDDP via induction of apoptosis by targeting Bcl-2. **Conclusions:** Our finding for the first time demonstrates that low miR-7 expression may be an independent poor prognostic factor and targeting miR-7 may be a potential strategy for the reversal of CDDP resistance in LA.

© 2017 The Author(s)
Published by S. Karger AG, Basel

M. Cheng and Z.-Tian Shen contributed equally to this work.

Gu-Yu Hu, Ph.D and Li-Guo Luo, M.D

College of Command Information System, PLA University of Science and Technology and Department of Cardiothoracic Surgery, Jinling Hospital, School of Medicine, Nanjing University, Nanjing 210002, Jiangsu Province (China)
E-Mail hugy_2017@126.com and luolg_nanjing@yeah.net

Introduction

Lung cancer is the most common cause of death due to cancer in both men and women around the world [1]. Non-small cell lung cancers (NSCLCs) accounts for 80 percent of lung cancers, and of these, roughly 50 percent are lung adenocarcinomas (LAs). Despite improvements in early diagnosis made possible by emerging technologies and newly developed chemo/targeted therapies that improve treatment responses, the 5-year overall survival for LA patients is still low and the recurrence rate is still high [2]. Lung cancer is multi-step process involving genetic and epigenetic alterations [3], so it is needed to need to elucidate the molecular mechanisms involved in LA development and to identify novel prognostic biomarkers and therapeutic targets for overcoming chemoresistance in LA.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules that play critical roles in the regulation of mRNA expression through degradation of transcripts and inhibition of translation by mainly binding to 3'-UTR of target messenger RNA (mRNA) [4]. It has been reported that miRNAs play critical roles in many human physiological and pathological processes including cancer, and the correlation of miRNA signatures with human diseases has been established [5]. Recently, the associations of dysregulated miRNAs with NSCLC development are also reported. By qRT-PCR analysis of miR-125a-5p, let-7e, miR-30a, miR-30e and miR-30e-3p expression in 70 paired tissue and serum samples from NSCLC patients, Zhu' et al showed that the lost expression of miR-125a-5p and let-7e was associated with shorter overall survival and let-7e was an independent prognostic factor for NSCLC patients [6]. The same research group also reported that four miRNAs (miR-93, miR-205, miR-221, and let-7e) may represent novel biomarkers for differential diagnosis and prognosis of certain NSCLC subtypes or be new targets of histology-specific treatments [7]. In addition, multiple miRNAs with aberrant expression have been identified as oncogenic or suppressive roles [8], and dysregulation of miRNAs is found to be correlated with chemo- or radioresistance of NSCLC cells [9]. Collectively, miRNAs may play diverse and critical roles in NSCLC progression and development. Recently, miR-7 has been reported to be encoded by three different genomic loci 9q21, 15q26, or 19q13, and the products of three different DNA sequences (termed as pri-miR-7-1, pri-miR-7-2, and pri-miR-7-3) can be processed into the same mature miR-7 sequence which comprising 23 nucleotides. In lung cancer, miR-7 has been reported to function as a tumor suppressor by targeting multiple oncogenes. Zhao' et al showed that miR-7 enhances cytotoxicity induced by gefitinib in NSCLC via inhibiting the EGFR and IGF1R signalling pathways [10]. Also, it was reported that miR-7 inhibits cell proliferation, migration and invasion in human non-small cell lung cancer cells by targeting FAK through ERK/MAPK signaling pathway [11]. However, the clinicopathological and prognostic significance of miR-7 in human LA and its roles in regulation of CDDP resistance of LA cells are unclear.

In the present study, reduced miR-7 was observed to be significantly correlated with poorer survival of LA patients. Upregulation of miR-7 enhances the CDDP chemosensitivity of LA cells by enhancing CDDP-induced apoptosis, and Bcl-2 was identified a direct target of miR-7.

Methods and Materials

Tissue specimens

A total of 108 paired of resected tumor and adjacent normal tissues from LA patients were collected from July 2006 to June 2009 in Jingling Hospital affiliated to Nanjing University or Nanjing Medical University Affiliated Hospital. No patients had received radiotherapy or chemotherapy before surgery. The clinical stage of these NSCLC patients was classified according to the sixth edition of the tumor-node metastases classification of the Union for International Cancer Control. The clinicopathological information of the patients is shown in Table 1. The tumors were frozen at -80°C in a guanidinium thiocyanate solution and RNA was extracted from the samples using a standard Trizol RNA extraction method (Invitrogen, CA,

USA). Written informed consent was obtained from the patient for the publication of this report and any accompanying images. For the use of these clinical materials for research aims, prior patient's consents and approval from the Chinese Medical Association Society of Medicine's Ethics Committee the Institutional Research Ethics Committee were obtained in accordance with the Helsinki Declaration.

Cell culture

The human LA cell line (SPC-A1) were routinely maintained in RPMI-1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin sodium, and 100 mg/ml of streptomycin sulfate at 37°C in a humidified air atmosphere containing 5% carbon dioxide.

Table 1. Association of miR-7 expression with clinicopathological factors of LA patients

Variables	Low miR-7 expression (n=63)		High miR-7 expression (n=45)		P-value
	N	%	N	%	
Gender					0.408
Male	40	63.4	32	62.8	
Female	23	36.6	13	37.2	
Age					0.939
≤60	15	29.3	11	28.6	
>60	48	70.7	34	71.4	
Smoking condition					0.309
Nonsmokers	33	53.7	28	51.4	
Smokers	30	46.2	17	48.6	
Tumor differentiation					0.006*
Well	16	9.8	23	37.1	
Moderate+poor	47	39.0	22	34.2	
Pathological T-factor					0.004*
T ₁₋₂	28	19.5	39	80.0	
T ₃₋₄	35	80.5	16	20.0	
Lymph node metastasis					0.037*
Absent	25	29.3	27	48.8	
Present	38	70.7	18	51.2	
p-TNM stage					0.015*
I-II	27	24.4	30	60.0	
III	36	75.6	15	40.0	

Quantitative real-time PCR (qRT-PCR) assay

Total RNA isolation from tissues or cells was performed using mirVana miRNA Isolation Kit (Applied Biosystems/Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA concentrations were measured using the SPECTR Amax microplate spectrophotometer (Molecular Devices Corp). Total miRNA from tissues was extracted by using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's instructions. cDNA was synthesized from 5 ng of total RNA by using the Taqman miRNA reverse transcription kit (Applied Biosystems, Foster City, CA), and the expression levels of miR-7 were quantified by using miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems). Additionally, qRT-PCR was performed to detect the relative level of Bcl-2 mRNA, using the Light-Cycler rapid thermal cycler system 2.0 (Roche Diagnostics Ltd, Burgess Hill, United Kingdom) with SYBR Green Master Mix (Toyobo). qRT-PCR was performed by using the Applied Biosystems 7500 Sequence Detection system. The expression of miR-7 was defined based on the threshold cycle (Ct), and relative expression levels were calculated as $2^{-((Ct \text{ of miR-7}) - (Ct \text{ of RNU6B}))}$ after normalization with reference to expression of RNU6B small nuclear RNA. The expression of Bcl-2 mRNA was normalized to GAPDH expression, using the $2^{-\Delta\Delta Ct}$ or $2^{-\Delta Ct}$ method as previously described [12].

Western blotting assay

Western blotting assay was performed as described previously [13]. In brief, the cells were harvested and lysed in RIPA buffer (Cell Signaling, Danvers, USA). The protein concentration determined by the bicinchoninic acid (BCA) protein reagent (Beyotime) Equal amounts of protein samples were run on the 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were probed with primary antibodies against human Bcl-2 and GAPDH (Santa Cruz, USA). After incubated with the secondary antibodies (HRP-conjugated goat anti-rabbit or goat anti-mouse IgG) and the signal bands were visualized by chemiluminescence using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, USA).

Transfections of oligonucleotides or plasmids

Cells were seeded in full growth medium. After 24h, medium was replaced with medium without antibiotics. All microRNA mimics and inhibitors were purchased from GenePharma (Shanghai, China). Short

hairpin RNA (shRNA) specifically targeting human Bcl-2 was designed to knockdown Bcl-2 expression (target sequence: sense 5'-AGTACATCCATTATAAGCT-3' and antisense 5'-AGCTTATAATGGATG TACT-3') and subcloned into pGPU6-GFP-shRNA vector, which is named pGPU6-GFP-shBcl-2. A scrambled vector with an imperfect sequence (pGPU6-GFP-shcontrol), which did not form a perfect hairpin structure, was used to develop the scrambled vector for use as a control. The plasmid vector (pcDNA3.1/Bcl-2) expressing open-reading frame of Bcl-2 was provided by Dr. Ming Sun. The cell transfection was performed using Lipofectamine™ 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

Cells were seeded in 96-well plate in 200 µl media containing 10% dialyzed fetal bovine serum at a density of 8000 cell. At the different time points after transfection or CDDP treatment, to evaluate the cell number, 20 µl of MTT (5 mg/mL) were added to each well and the plates were incubated at 37°C for 4 h. The MTT medium mixture was then removed and 100 µl of dimethyl sulfoxide (DMSO) was added to each well. Absorbance was measured at 490 nm using a multiwell spectrophotometer (Thermo Electron, Andover, USA).

Flow cytometric detection of apoptosis

Cells were harvested by trypsinization, washed in ice-cold phosphate-buffered saline (PBS) and fixed in 80% ice-cold ethanol in PBS. Before staining, cells were gently sedimented and resuspended in cold PBS. Bovine pancreatic RNase (Sigma-Aldrich) was added to a final concentration of 2 µg/mL, and cells were incubated at 37°C for 30 min, followed by incubation with 20 µg/mL propidium iodide (Sigma-Aldrich) for 20 min at room temperature. Cell samples were then analyzed by a FACSCalibur flow cytometer.

Luciferase reporter assay

The 3'-UTR of Bcl-2 mRNA containing the predicted miR-7 binding site was synthesized and cloned into the pGL3-basic vector (pGL3-Bcl-2/3'-UTR-wt). The mutant reporter plasmid (pGL3-Bcl-2/3'-UTR-mut) was constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. For luciferase reporter assay, SPC-A1 cells at logarithmic phase were seeded in 24-well plates. After 24 h, the cells were transiently cotransfected with 0.5 µg wt (pGL3-Bcl-2/3'-UTR-wt), or mutant reporter plasmid (pGL3-Bcl-2/3'-UTR-mut) and 1 µl miRNA mimic using lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions.

Statistical analysis

All statistical analyses were carried out using the SPSS 17.0 statistical software package (SPSS, Chicago, IL, USA). The experimental data were presented as the mean ± SD. The Chi-squared test was used to investigate the significance of miR-7 expression as correlated with clinicopathologic factors in NSCLC. Differences in patient survival were determined by the Kaplan-Meier method and log-rank test. A Cox proportional hazards regression analysis was used for multivariate analyses of prognostic values. A difference was considered statistically significant when $P < 0.05$.

Results

Correlations between miR-7 expression and clinicopathological factors of LA patients

The expression level of miR-7 in 30 pairs of LA tissues and adjacent normal tissues was detected by qRT-PCR, and its expression was normalized to RNU6B. As shown in Fig. 1A, the averaged expression level of miR-7 in LA tissues ($n=30$) was significantly lower than that in adjacent normal tissues ($n=30$; $P < 0.001$). To further analyze the association of miR-7 expression with clinicopathological factors of LA patients, we used the median expression level of miR-7 (3.44, a cutoff point) to divide all 108 patients into two groups: patients who express miR-7 at levels less than the cutoff value were assigned to the low expression group (mean expression value 2.04, $n=63$), and those with expression above the cutoff value

were assigned to the high expression group (mean expression value 4.86, $n=45$). It was observed that the relative expression level of miR-7 was significantly associated with tumor differentiation, pathological T-factor, status of lymph node metastasis and p-TNM stage of patients (Table 1; $P=0.006$, 0.004, 0.037 and 0.015, respectively). However, there were no significant difference between miR-7 expression and other clinical/pathological factors, including gender, age, smoking condition and histological type ($P=0.408$, 0.939, 0.309 and 0.346, respectively).

Expression of miR-7 is an independent biomarker for predicting the survival of LA patients

To further explore the correlation between miR-7 expression and OS of LA patients, Kaplan-Meier survival analysis was performed by comparing the patients with high and low miR-7 expression. As shown in Fig. 1B, patients with high miR-7 expression exhibited obviously higher OS rate than those with low miR-7 expression ($P=0.0145$). The five-year survival rate for patients with low miR-7 expression was 25.5%, compared with 42.3% for those patients with high miR-7 expression. Then, in a multivariate analysis based on the Cox proportional hazards regression model, we determined the independent predictive significance for miR-7 expression as well as relevant clinical and pathological parameters including gender, age, histological type, tumor differentiation, pathological stage, lymph node metastasis and clinical stage (Table 2). Multivariate analyses indicated that reduced miR-7 expression might be an independent biomarker for predicting the poor OS of LA patients ($P=0.015$; HR=3.238; 95% CI: 2.667-5.123), in addition to lymph node metastasis

Fig. 1. Expression of miR-7 in LA tissues and its correlation with prognosis of patients. (A) The relative levels of miR-7 expression in 30 paired of tumor and adjacent normal tissues from LA patients were detected by qRT-PCR. RNU6B was used as an internal control. T: LA tissues; N: adjacent normal tissues. (B) The Kaplan-Meier survival curve in relation to miR-7 expression level in 108 cases of LA patients. The 5-year OS of LA patients with low miR-7 expression was significantly lower than that of those with high miR-7 expression ($P=0.0145$). The P -value was calculated using the log-rank test between patients with high- and low-fold changes.

Table 2. Multivariate analyses for prognostic factors in LA patients. HR: hazard ratio; 95% CI: 95% confidence interval

Factor	P-value	HR	95% CI
Gender (Female vs Male)	0.545	1.234	0.563-1.717
Age (>60 vs ≤60)	0.378	0.982	0.425-1.066
Smoking condition (Non-smoker vs smoker)	0.601	1.451	0.912-2.118
Tumor differentiation (Well+Modertate vs Poor)	0.499	2.490	0.915-4.232
T-factor (T_{1+2} vs T_{3+4})	0.106	3.123	0.882-5.178
Lymph node metastasis (Absent vs Present)	0.007	2.396	1.563-3.424
p-TNM stage (I+II vs III)	0.026	1.689	1.187-2.382
miR-7 expression level (High vs Low)	0.015	3.238	2.667-5.123

($P=0.007$; HR=2.396; 95% CI: 1.563-3.424) and p-TNM stage ($P=0.026$; HR=1.689; 95% CI: 1.187-2.382).

Restoration of miR-7 significantly increases CDDP chemosensitivity in LA cells

To investigate the functions of miR-7, LA cells (SPC-A1) were transfected with miR-7/mimics or miR-7/inhibitor and qRT-PCR confirmed the upregulation of miR-7 in miR-7/mimics-transfected LA cells and downregulation of miR-7 in miR-7/inhibitor-transfected LA cells (Fig. 2A). Transfection of miR-7/mimics significantly reduced proliferation of LA cells, while transfection of miR-7/inhibitor increased proliferation of LA cells (Fig. 2B). Also, the effect of miR-7 expression on apoptosis was also determined by flow cytometry, and results indicated that upregulation of miR-7 enhanced apoptosis in LA cells (Fig. 2C). Although it has been reported that miR-7 sensitizes non-small cell lung cancer cells to paclitaxel, whether miR-7 regulates CDDP sensitivity of LA cells is unknown. Upregulation of miR-7 sensitized

Fig. 2. Effect of miR-7 expression on growth, apoptosis and CDDP sensitivity in LA cells. SPC-A1 cell line was transiently transfected with miR-7/mimics (or miR-NC/mimics) or miR-7/inhibitor (or miR-NC/inhibitor), respectively. (A) The expression levels of miR-7 in the transfected SPC-A1 cells were evaluated by qRT-PCR assay, and RNU6B was used as an internal loading control to normalize the results. (B) After transfection, every 24h, MTT assay was performed on the transfected SPC-A1 cells. (C) Flow cytometric detection of apoptosis in the miR-NC/mimics or miR-7/mimics-transfected SPC-A1 cells. (D) MTT analysis of growth in the miR-NC/mimics or miR-7/mimics-transfected SPC-A1 cells treated with different doses of CDDP. (E) Flow cytometric detection of apoptosis in the miR-NC/mimics or miR-7/mimics-transfected SPC-A1 cells with different doses of CDDP. The results are representative of three different experiments. Data are expressed as mean $\pm$ standard deviation. * $P<0.05$; ** $P<0.01$.

Fig. 3. Bcl-2 is a direct target mRNA of miR-7 in LA. (A) A putative miR-7-binding site exists in the 3'-UTR of Bcl-2 mRNA and seven point mutations were generated in the binding site. (B) Transfection of miR-7/mimics reduced the firefly luciferase activity of pGL3-Bcl-2/3'-UTR-wt (wild-type, wt) and such reduction was absent with mutations in the miR-7-binding site (mutant, mut). (C) qRT-PCR and Western blot detection of the expression of Bcl-2 mRNA and protein in the miR-NC/mimics or miR-7/mimics-transfected SPC-A1 cells. (D) qRT-PCR and Western blot detection of the expression of Bcl-2 mRNA and protein in the miR-NC/inhibitor or miR-7/inhibitor-transfected SPC-A1 cells. (E) The relative levels of Bcl-2 mRNA expression in 30 paired of tumor and adjacent normal tissues from LA patients were detected by qRT-PCR. (F) An inverse correlation between miR-7 and Bcl-2 mRNA expression by Pearson correlation analysis (Pearson's correlation: $r = -0.44$, $P < 0.001$). GAPDH was used as an internal loading control to normalize the results. The results are representative of three different experiments. Data are expressed as mean $\pm$ standard deviation. N.S., $P > 0.05$; * $P < 0.05$ and ** $P < 0.01$.

LA cells to CDDP by enhancing CDDP-induced apoptosis (Fig. 2D-E). Therefore, upregulation of miR-7 inhibits proliferation and enhances CDDP sensitivity of LA cells.

Bcl-2 is a direct target of miR-7 and inversely correlated with miR-7 in LA tissues

It has been demonstrated that miR-7 inhibits the growth of human NSCLC cells through targeting BCL-2 [14]. However, whether miR-7 targets Bcl-2 to regulate the sensitivity of LA cells to CDDP needs to be further elucidated. By the TargetScan and PicTar software, a potential targeting of the 3'-UTR of the human Bcl-2 gene by miR-7 (1368-1392nt) was predicted (Fig. 3A). To examine whether miR-7 directly binds to 3'-UTR of Bcl-2 mRNA, we performed luciferase reporter assay in SPC-A1 cells (Fig. 3B). The miR-7-transfected LA cells co-transfected with the wild-type Bcl-2/3'-UTR vector (pGL3-Bcl-2/3'-UTR-wt) showed the dramatically decreased activity of the luciferase ($P < 0.05$), whereas those co-transfected

with mutant Bcl-2/3'-UTR vector (pGL3-Bcl-2/3'-UTR-mut) showed no obvious changes in luciferase activity ($P > 0.05$). In addition, upregulation of miR-7 induced the decreased expression of Bcl-2 mRNA and protein in LA cells (Fig. 3C), whereas downregulation of miR-7 led to the increased expression of Bcl-2 mRNA and protein (Fig. 3D). Also, the expression level of Bcl-2 mRNA in LA tissues was significantly higher than that in the adjacent normal tissues from 30 LA patients (Fig. 3E; $P = 0.0025$). Finally, an inverse correlation between miR-7 and Bcl-2 mRNA expression in 30 LA tissues could be observed (Pearson's correlation $r = -0.44$, $P < 0.001$; Fig. 3E).

Bcl-2 is involved in miR-7-mediated CDDP resistance in LA cells

To further explore the roles of Bcl-2 in miR-7-mediated CDDP resistance in LA cells, we will employ small hairpin RNA (shRNA) to knockdown endogenous Bcl-2 in LA cells and analyze its effects on the CDDP sensitivity. As shown in Fig. 4A, compared with SPC-A1/shcontrol cells, the expression levels of Bcl-2 mRNA and protein in SPC-A1/shBcl-2 cells were significantly downregulated ($P < 0.05$). It was observed that silencing of Bcl-2 led to significant inhibition of proliferation and enhancement of apoptosis (Fig. 4B-C). Likewise, silencing of Bcl-2 significantly increased the sensitivity of SPC-A1 cells to CDDP by enhancing CDDP-induced apoptosis (Fig. 4D-E), suggesting that Bcl-2 knockdown mimics the effects of miR-7 upregulation on CDDP sensitivity in LA cells. Next, the Bcl-2 expression vector (pcDNA3.1/Bcl-2) was transfected into miR-7/mimics-transfected SPC-A1 cells to test whether restoration of Bcl-2 counteracted the effects of miR-7 upregulation on CDDP sensitivity. It could be found that reintroduction of Bcl-2 restored the inhibition of Bcl-2 mRNA and protein in SPC-A1 cells induced by transfection of miR-7/mimics (Fig. 4F). Meanwhile, reintroduction of Bcl-2 partially reversed miR-7 upregulation-mediated proliferation inhibition and apoptosis enhancement in SPC-A1 cells (Fig. 4G-H). Then, 48h after cotransfection with miR-7/mimics and pcDNA3.1 or pcDNA3.1/Bcl-2, SPC-A1 cells were treated with various doses of CDDP (1.0 and 5.0 μ M). Results showed that reintroduction of Bcl-2 significantly restored CDDP resistance in miR-7/mimics-transfected SPC-A1 cells by decreasing CDDP-induced apoptosis (Fig. 4I-J), suggesting that overexpression of Bcl-2 reverses the effects of miR-7 upregulation on CDDP sensitivity in LA cells.

Discussion

In the present study, we showed that miR-7 was significantly downregulated in LA tissues and reduced miR-7 expression was closely correlated with tumor development and poor prognosis of patients. Moreover, it was observed that restoration of miR-7 inhibited growth, induced apoptosis and enhanced the sensitivity of LA cells to chemotherapeutic agent (CDDP) by targeting Bcl-2. To the best of our knowledge, this is the first study about clinicopathological and prognostic significance of miR-7 in LA and to analyze its correlation with the sensitivity of LA cells to CDDP.

Recently, many published data have shown that miRNAs are involved in malignant transformation as either oncogenes or tumor suppressors and many cancer-related miRNAs have been identified functionally [15]. Therefore, identification of miRNA molecular profiles associated with the prognosis of patients with lung cancer may not only elucidate the underlying molecular mechanisms involved in the development or progression of the disease but also provide the opportunity to identify novel targets for the treatment of human lung cancer [16]. Some dysregulated miRNAs (miR-222, miR-183-3p, miR-146 and miR-155) in tumor tissues have been reported to be correlated with prognosis of lung cancer patients [17-20]. Also, miRNAs in body fluids are reported to be identified as biomarkers for the early detection and prognostic prediction of NSCLC. Hennessey et al showed that serum miRNAs (miR-15b and miR-27b) have the potential to be sensitive, cost-effective biomarkers for the early detection of NSCLC [21]. Of course, further testing in a Phase III biomarker study is necessary for validation of these results. Kaduthanam and his colleagues showed

Fig. 4. Bcl-2 is involved in miR-7-mediated CDDP resistance in LA cells. (A) qRT-PCR and Western blot detection of the expression of Bcl-2 mRNA and protein in the SPC-A1/shcontrol or SPC-A1/shBcl-2 cells. GAPDH was used as an internal loading control to normalize the results. (B) MTT analysis of growth in SPC-A1/shcontrol or SPC-A1/shBcl-2 cells. (C) Flow cytometric detection of apoptosis in SPC-A1/shcontrol or SPC-A1/shBcl-2 cells. (D) MTT analysis of growth in SPC-A1/shcontrol or SPC-A1/shBcl-2 cells treated with different doses of CDDP. (E) Flow cytometric detection of apoptosis in SPC-A1/shcontrol or SPC-A1/shBcl-2 cells with different doses of CDDP. (F) 48h after SPC-A1 cells were co-transfected with miR-7/mimics (or miR-NC/mimics) and pcDNA3.1/Bcl-2 (or pcDNA3.1/control), respectively, and qRT-PCR and Western blot assays were performed to detect the expression of Bcl-2 mRNA and protein. GAPDH was used as an internal loading control to normalize the results. (G) MTT analysis of growth in the co-transfected SPC-A1 cells at different time points. (H) 48h after co-transfection, flow cytometric detection of apoptosis in SPC-A1 cells. (I) 48h after co-transfection, SPC-A1 cells were treated with CDDP (1.0 and 5.0 μ M) and then, growth was evaluated by MTT assay. (J) 48h after co-transfection, SPC-A1 cells were treated with CDDP (1.0 and 5.0 μ M) and then, apoptosis was evaluated by flow cytometric assay. The results are representative of three different experiments. Data are expressed as mean $\pm$ standard deviation. *N.S.*, $P > 0.05$; * $P < 0.05$ and ** $P < 0.01$.

that serum miR-142-3p is associated with early relapse in operable lung adenocarcinoma patients [22]. Serum miR-19a expression was reported to correlate with worse prognosis of patients with NSCLC [23]. Importantly, miRNAs have been found to be associated with growth, apoptosis and chemo- or radioresistance of lung cancer cells. For example, Wang' et al showed that miR-451 functions as a tumor suppressor in human NSCLC by targeting ras-related protein 14 (RAB14) [24]. This research group also identified microRNA profiles in docetaxel-resistant human lung adenocarcinoma cells (SPC-A1) [25], and their further studies indicated that dysregulated miRNAs (miR-650, Let-7c, miR-200b, miR-100 and miR-

26a) play critical roles in formation of chemoresistant phenotype of lung adenocarcinoma cells [26-30]. Interestingly, overexpression of Let-7c could reverse epithelial-mesenchymal transition (EMT) and radioresistance in docetaxel-resistant lung adenocarcinoma cells. In addition, the roles of miRNAs in LA metastasis are also reported [31]. Some miRNAs were reported to promote LA metastasis [32,33], while other miRNAs were reported to inhibit LA metastasis [34,35]. These data clearly suggest that dysregulation of miRNAs is involved in tumorigenesis and development of LA.

MiR-7 is a 23 nucleotide miRNA whose expression is tightly regulated and restricted predominantly to the brain, spleen and pancreas. Recently, reduced levels of miR-7 have been linked to the development of cancer and metastasis [36]. Zhou' et al reported that miR-7 inhibits tumor metastasis and reverses epithelial-mesenchymal transition through AKT/ERK1/2 inactivation by targeting EGFR in epithelial ovarian cancer [37]. Zhang and his colleagues showed that microRNA-7 could arrest cell cycle in G1 phase by directly targeting CCNE1 in human hepatocellular carcinoma cells [38]. The tumor suppressive functions of miR-7 are also found in other human cancers, including cervical cancer, colorectal cancer, breast cancer, gastric cancer, tongue squamous cell carcinoma cells and glioblastoma [39-44]. The roles of miR-7 in formation of malignant phenotypes of lung cancer were also reported by several research groups. Xiong' et al showed that miR-7 inhibited the growth of human non-small cell lung cancer A549 cells through targeting BCL-2 [14]. The same research group also identified PA28gamma as a novel functional target of tumour suppressor microRNA-7 in non-small-cell lung cancer [45]. Xu and his colleagues reported that miR-7-regulated TLR9 signaling-enhanced growth and metastatic potential of human lung cancer cells by altering the phosphoinositide-3-kinase, regulatory subunit 3/Akt pathway [46]. Importantly, miR-7 was found to down-regulate EGFR mRNA and protein expression in cancer cell lines (lung cancer, breast cancer, and glioblastoma) via two of the three sites, inducing cell cycle arrest and cell death [47]. At the same time, miR-7 was also reported to increase radiosensitivity of human cancer cells with activated EGFR-associated signaling [48]. Also, liposomal delivery of MicroRNA-7-expressing plasmid overcomes epidermal growth factor receptor tyrosine kinase inhibitor-resistance in lung cancer cells [49]. Although the tumor suppressive roles of miR-7 in NSCLC are obviously reported, the clinicopathological and prognostic significance of miR-7 in human LA and its roles in regulation of CDDP resistance of LA cells are unclear. To further testify this issue, we first detected the expression of miR-7 in 30 paired of LA and the adjacent normal tissues, and showed that the relative expression level of miR-7 in LA tissues was significantly lower than that in the adjacent normal tissues. Next, the clinicopathological significance of miR-7 expression in LA were statistically analyzed, and it was observed that status of miR-7 expression was closely correlated with poorer tumor differentiation, advanced pathological T factor, higher incidence of lymph node metastasis and advanced p-TNM stage, suggesting that miR-7 may be associated with LA development. To further investigate the prognostic implication of miR-7 in LA, we performed Cox proportional hazards model, and results indicated that reduced miR-7 expression was an independent biomarker for predicting the poorer OS of LA patients. Thus, the positive linkage between reduced miR-7 and poor prognosis may not only be used for identifying LA patients with poorer prognosis but also for providing valuable clues to understand the possible mechanism of LA development. Adjuvant CDDP-based chemotherapy has become the standard therapy against resected NSCLC, but the resistance of CDDP is a major obstacle in the treatment of NSCLC. Here, we investigated the roles of miR-7 in CDDP resistance of LA cells and its possible mechanisms. Our data indicated that restoration of miR-7 significantly increased the CDDP sensitivity of LA cells by enhancing CDDP-induced apoptosis. Bcl-2 is the founding member of the Bcl-2 family of regulator proteins that regulate cell death (apoptosis). There are also a number of miRNAs reported to suppress Bcl-2 in various human cancers, including lung cancer [50]. Although other study have shown that miR-7 targets Bcl-2 to regulate growth of NSCLC cells [14], our work identified a novel role for Bcl-2 in miR-7-induced CDDP resistance, suggesting that miR-7 reverses CDDP resistance of LA cells, at least partially by targeting Bcl-2.

Collectively, our study has demonstrated that miR-7 is significantly reduced in LA and status of miR-7 expression may be an independent prognostic biomarker for LA patients. Meanwhile, restoration of miR-7 increases the sensitivity of LA cells to CDDP via enhancement of CDDP-induced apoptosis partially by targeting Bcl-2. Thus, targeting miR-7 might be a potential strategy to enhance chemosensitivity to CDDP treatment in LA.

Disclosure Statement

The authors declare that they have no competing interests.

References

- 1 Siegel R, Naishadham D, Jemal A: Cancer statistics, 2013. *CA Cancer J Clin* 2013;63:11-30.
- 2 Carbone DP, Felip E: Adjuvant therapy in non-small cell lung cancer: future treatment prospects and paradigms. *Clin Lung Cancer* 2011;12:261-271.
- 3 Tan Q, Cui J, Huang J, Ding Z, Lin H, Niu X, Li Z, Wang G, Luo Q, Lu S: Genomic Alteration During Metastasis of Lung Adenocarcinoma. *Cell Physiol Biochem* 2016;38:469-486.
- 4 Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281-297.
- 5 Stahlhut C, Slack FJ: MicroRNAs and the cancer phenotype: profiling, signatures and clinical implications. *Genome Med* 2013;5:111.
- 6 Zhu WY, Luo B, An JY, He JY, Chen DD, Xu LY, Huang YY, Liu XG, Le HB, Zhang YK: Differential Expression of miR-125a-5p and let-7e Predicts the Progression and Prognosis of Non-Small Cell Lung Cancer. *Cancer Invest* 2014;32:394-401.
- 7 Zhang YK, Zhu WY, He JY, Chen DD, Huang YY, Le HB, Liu XG: miRNAs expression profiling to distinguish lung squamous-cell carcinoma from adenocarcinoma subtypes. *J Cancer Res Clin Oncol* 2012;138:1641-1650.
- 8 Shenouda SK, Alahari SK: MicroRNA function in cancer: oncogene or a tumor suppressor? *Cancer Metastasis Rev* 2009;28:369-378.
- 9 Hummel R, Hussey DJ, Haier J: MicroRNAs: predictors and modifiers of chemo- and radiotherapy in different tumour types. *Eur J Cancer* 2010;46:298-311.
- 10 Zhao JG, Men WF, Tang J: MicroRNA-7 enhances cytotoxicity induced by gefitinib in non-small cell lung cancer via inhibiting the EGFR and IGF1R signalling pathways. *Contemp Oncol (Pozn)* 2015;19:201-206.
- 11 Cao Q, Mao ZD, Shi YJ, Chen Y, Sun Y, Zhang Q, Song L, Peng LP: MicroRNA-7 inhibits cell proliferation, migration and invasion in human non-small cell lung cancer cells by targeting FAK through ERK/MAPK signaling pathway. *Oncotarget* 2016;7:77468-77481.
- 12 Yang ZT, Li Z, Wang XG, Tan T, Yi F, Zhu H, Zhao JP, Zhou XF: Overexpression of Long Non-Coding RNA ZXF2 Promotes Lung Adenocarcinoma Progression Through c-Myc Pathway. *Cell Physiol Biochem* 2015;35:2360-2370.
- 13 Wang X, He X, Hu S, Sun A, Lu C: Involvement of Bim in Photofrin-mediated photodynamically induced apoptosis. *Cell Physiol Biochem*. 2015;35:1527-1536.
- 14 Xiong S, Zheng Y, Jiang P, Liu R, Liu X, Chu Y: MicroRNA-7 inhibits the growth of human non-small cell lung cancer A549 cells through targeting BCL-2. *Int J Biol Sci* 2011;7:805-814.
- 15 Jansson MD, Lund AH: microRNAs as tumor inhibitors, oncogenes, biomarkers for drug efficacy and outcome predictors in lung cancer (review). *Mol Med Rep* 2012;5:890-894.
- 16 Mao KP, Zhang WN, Liang XM, Ma Y: MicroRNA-222 Expression and Its Prognostic Potential in Non-Small Cell Lung Cancer. *Scientific World Journal* 2014;2014:908326.
- 17 Xu F, Zhang H, Su Y, Kong J, Yu H, Qian B: Up-regulation of microRNA-183-3p is a potent prognostic marker for lung adenocarcinoma of female non-smokers. *Clin Transl Oncol* 2014;16:980-985.
- 18 Li J, Yang H, Li Y, Liu Y, Chen S, Qi C, Zhang Q, Lan T, He X, Guan XY, Wang L.: microRNA-146 up-regulation predicts the prognosis of non-small cell lung cancer by miRNA in situ hybridization. *Exp Mol Pathol* 2014;96:195-199.

- 19 Xu TP, Zhu CH, Zhang J, Xia R, Wu FL, Han L, Shen H, Liu LX, Shu YQ.: MicroRNA-155 expression has prognostic value in patients with non-small cell lung cancer and digestive system carcinomas. *Asian Pac J Cancer Prev* 2013;14:7085-7090.
- 20 Hennessey PT1, Sanford T, Choudhary A, Mydlarz WW, Brown D, Adai AT, Ochs MF, Ahrendt SA, Mambo E, Califano JA: Serum microRNA biomarkers for detection of non-small cell lung cancer. *PLoS One* 2012;7:e32307.
- 21 Kaduthanam S1, Gade S, Meister M, Brase JC, Johannes M, Dienemann H, Warth A, Schnabel PA, Herth FJ, Sültmann H, Muley T and Kuner R. Serum miR-142-3p is associated with early relapse in operable lung adenocarcinoma patients. *Lung Cancer* 2013;80: 223-227.
- 22 Lin Q, Chen T, Lin Q, Lin G, Lin J, Chen G, Guo L: Serum miR-19a expression correlates with worse prognosis of patients with non-small cell lung cancer. *J Surg Oncol* 2013;107:767-771.
- 23 Wang R, Wang ZX, Yang JS, Pan X, De W, Chen LB: MicroRNA-451 functions as a tumor suppressor in human non-small cell lung cancer by targeting ras-related protein 14 (RAB14). *Oncogene* 2011;30:2644-2658.
- 24 Rui W, Bing F, Hai-Zhu S, Wei D, Long-Bang C: Identification of microRNA profiles in docetaxel-resistant human non-small cell lung carcinoma cells (SPC-A1). *J Cell Mol Med* 2010;14:206-214.
- 25 Huang JY, Cui SY, Chen YT, Song HZ, Huang GC, Feng B, Sun M, De W, Wang R, Chen LB. MicroRNA-650 was a prognostic factor in human lung adenocarcinoma and confers the docetaxel chemoresistance of lung adenocarcinoma cells via regulating Bcl-2/Bax expression. *PLoS One* 2013;8:e72615.
- 26 Cui SY, Huang JY, Chen YT, Song HZ, Feng B, Huang GC, Wang R, Chen LB, De W: Let-7c governs the acquisition of chemo- or radioresistance and epithelial-to-mesenchymal transition phenotypes in docetaxel-resistant lung adenocarcinoma. *Mol Cancer Res* 2013;11:699-713.
- 27 Feng B, Wang R, Song HZ, Chen LB: MicroRNA-200b reverses chemoresistance of docetaxel-resistant human lung adenocarcinoma cells by targeting E2F3. *Cancer* 2012;118:3365-3376.
- 28 Feng B, Wang R, Chen LB: MiR-100 resensitizes docetaxel-resistant human lung adenocarcinoma cells (SPC-A1) to docetaxel by targeting Plk1. *Cancer Lett* 2012;317:184-191.
- 29 Wang Y, Han R, Chen Z, Fu M, Kang J, Li K, Li L, Chen H, He Y: A transcriptional miRNA-gene network associated with lung adenocarcinoma metastasis based on the TCGA database. *Oncol Rep* 2016;35:2257-2269.
- 30 Chen J, Xu Y, Tao L, Pan Y, Zhang K, Wang R, Chen LB, Chu X: MiRNA-26a Contributes to the Acquisition of Malignant Behaviors of Docetaxel-Resistant Lung Adenocarcinoma Cells through Targeting EZH2. *Cell Physiol Biochem* 2017;41:583-597.
- 31 Long H, Wang Z, Chen J, Xiang T, Li Q, Diao X, Zhu B: microRNA-214 promotes epithelial-mesenchymal transition and metastasis in lung adenocarcinoma by targeting the suppressor-of-fused protein (Sufu). *Oncotarget* 2015;6:38705-38718.
- 32 Song Q, Xu Y, Yang C, Chen Z, Jia C, Chen J, Zhang Y, Lai P, Fan X, Zhou X, Lin J, Li M, Ma W, Luo S, Bai X: miR-483-5p promotes invasion and metastasis of lung adenocarcinoma by targeting RhoGDI1 and ALCAM. *Cancer Res* 2014;74:3031-3042.
- 33 Cai M, Wang Z, Zhang J, Zhou H, Jin L, Bai R, Weng Y: Adam17, a Target of Mir-326, Promotes Emt-Induced Cells Invasion in Lung Adenocarcinoma. *Cell Physiol Biochem* 2015;36:1175-1185.
- 34 Han HS, Son SM, Yun J, Jo YN, Lee OJ: MicroRNA-29a suppresses the growth, migration, and invasion of lung adenocarcinoma cells by targeting carcinoembryonic antigen-related cell adhesion molecule 6. *FEBS Lett* 2014;588:3744-3750.
- 35 Mou X, Liu S: MiR-485 inhibits metastasis and EMT of lung adenocarcinoma by targeting Flot2. *Biochem Biophys Res Commun* 2016;477:521-526.
- 36 Gu DN, Huang Q, Tian L: The molecular mechanisms and therapeutic potential of microRNA-7 in cancer. *Expert Opin Ther Targets* 2015;19:415-426.
- 37 Zhou X, Hu Y, Dai L, Wang Y, Zhou J, Wang W, Di W, Qiu L: MicroRNA-7 inhibits tumor metastasis and reverses epithelial-mesenchymal transition through AKT/ERK1/2 inactivation by targeting EGFR in epithelial ovarian cancer. *PLoS One* 2014;9:e96718.
- 38 Zhang X, Hu S, Zhang X, Wang L, Zhang X, Yan B, Zhao J, Yang A, Zhang R: MicroRNA-7 arrests cell cycle in G1 phase by directly targeting CCNE1 in human hepatocellular carcinoma cells. *Biochem Biophys Res Commun* 2014;443:1078-1084.
- 39 Liu S, Zhang P, Chen Z, Liu M, Li X, Tang H.: MicroRNA-7 downregulates XIAP expression to suppress cell growth and promote apoptosis in cervical cancer cells. *FEBS Lett* 2013;587:2247-2253.

- 40 Li Y, Li Y, Liu Y, Xie P, Li F, Li G: PAX6, a novel target of microRNA-7, promotes cellular proliferation and invasion in human colorectal cancer cells. *Dig Dis Sci* 2014;59:598-606.
- 41 Kong X, Li G, Yuan Y, He Y, Wu X, Zhang W, Wu Z, Chen T, Wu W, Lobie PE, Zhu T: MicroRNA-7 inhibits epithelial-to-mesenchymal transition and metastasis of breast cancer cells via targeting FAK expression. *PLoS One* 2012;7:e41523.
- 42 Zhao X, Dou W, He L, Liang S, Tie J, Liu C, Li T, Lu Y, Mo P, Shi Y, Wu K, Nie Y, Fan D: MicroRNA-7 functions as an anti-metastatic microRNA in gastric cancer by targeting insulin-like growth factor-1 receptor. *Oncogene* 2013;32:1363-1372.
- 43 Jiang L, Liu X, Chen Z, Jin Y, Heidbreder CE, Kolokythas A, Wang A, Dai Y, Zhou X: MicroRNA-7 targets IGF1R (insulin-like growth factor 1 receptor) in tongue squamous cell carcinoma cells. *Biochem J* 2010;432:199-205.
- 44 Liu Z, Jiang Z, Huang J, Huang S, Li Y, Yu S, Yu S, Liu X: miR-7 inhibits glioblastoma growth by simultaneously interfering with the PI3K/ATK and Raf/MEK/ERK pathways. *Int J Oncol* 2014;44:1571-1580.
- 45 Xiong S, Zheng Y, Jiang P, Liu R, Liu X, Qian J, Gu J, Chang L, Ge D, Chu Y: PA28gamma emerges as a novel functional target of tumour suppressor microRNA-7 in non-small-cell lung cancer. *Br J Cancer* 2014;110:353-362.
- 46 Xu L, Wen Z, Zhou Y, Liu Z, Li Q, Fei G, Luo J, Ren T: MicroRNA-7-regulated TLR9 signaling-enhanced growth and metastatic potential of human lung cancer cells by altering the phosphoinositide-3-kinase, regulatory subunit 3/Akt pathway. *Mol Biol Cell* 2013;24:42-55.
- 47 Webster RJ, Giles KM, Price KJ, Zhang PM, Mattick JS, Leedman PJ: Regulation of epidermal growth factor receptor signaling in human cancer cells by microRNA-7. *J Biol Chem* 2009;284:5731-5741.
- 48 Lee KM, Choi EJ, Kim IA: microRNA-7 increases radiosensitivity of human cancer cells with activated EGFR-associated signaling. *Radiother Oncol* 2011;101:171-176.
- 49 Rai K, Takigawa N, Ito S, Kashihara H, Ichihara E, Yasuda T, Shimizu K, Tanimoto M, Kiura K: Liposomal delivery of MicroRNA-7-expressing plasmid overcomes epidermal growth factor receptor tyrosine kinase inhibitor-resistance in lung cancer cells. *Mol Cancer Ther* 2011;10:1720-1727.
- 50 Huang P, Ye B, Yang Y, Shi J, Zhao H: MicroRNA-181 functions as a tumor suppressor in non-small cell lung cancer (NSCLC) by targeting Bcl-2. *Tumour Biol* 2015;36:3381-3387.