MiR-224 promotes the chemoresistance of human lung adenocarcinoma cells to cisplatin via regulating G$_1$/S transition and apoptosis by targeting p21$^{WAF1/CIP1}$

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Background: Increasing evidence has shown that microRNAs (miRNAs) can serve as oncogenes and tumour suppressors to participate in tumour development. However, the roles of miRNAs in chemoresistance of human lung adenocarcinoma (LA) remain largely undefined.

Methods: On the basis of miRNA microarray data, miR-224 was identified as the most upregulated miRNA in cisplatin (DDP; *cis*-diamminedichloroplatinum II)-resistant A549 cells compared with parental A549 cells. The aim of our study was to investigate the roles of miR-224 in the formation of DDP-resistant phenotype of LA cells and its possible molecular mechanisms.

Results: Here we showed that miR-224 could promote the *in vitro* and *in vivo* DDP resistance of LA cells via regulating G$_1$/S cell cycle transition and apoptosis. p21$^{WAF1/CIP1}$, a potent cyclin-dependent kinase inhibitor, was identified as the direct and functional target gene of miR-224. Overexpression of p21$^{WAF1/CIP1}$ could phenocopy the effect of miR-224 downregulation and silencing of p21$^{WAF1/CIP1}$ could partially reverse the effect of miR-224 downregulation on DDP resistance of DDP-resistant LA cells. In addition, miR-224 could affect the G$_1$/S transition of cell cycle and apoptosis in LA cells through the p21$^{WAF1/CIP1}$-pRb pathway and the intrinsic mitochondrial death pathway. Furthermore, miR-224 was found to be downregulated in DDP-responding LA tissues, and its expression was inversely correlated with p21$^{WAF1/CIP1}$. Multivariate analyses indicated that the status of miR-224 might be an independent prognostic factor for predicting the survival of LA patients.

Conclusions: Our findings shed novel light on the roles of miR-224/p21$^{WAF1/CIP1}$ signalling in the DDP resistance of LA cells, and targeting it will be a potential strategic approach for reversing the DDP resistance in human LAs.

Lung cancer, one of the most common malignancies around the world, is the leading cause of cancer death worldwide (Jemal *et al*, 2011). In China, about 300 000 new lung cancer patients and more than 250 000 deaths from the disease are predicted each year (She *et al*, 2013). Lung adenocarcinoma (LA) is the most common type of lung cancer and accounts for 30–35% of primary lung tumours. Currently, the most effective therapy for LA is complete lung resection. However, many patients with LA have advanced stage IIIB or IV disease when first diagnosed in clinic. Systemic chemotherapy still forms the mainstay of treatment for patients with advanced LA. Cisplatin (DDP; *cis*-diamminedichloroplatinum II)-based chemotherapy is the mainstay of first-line therapy. The majority of patients with advanced LA initially respond to DDP treatment; however, development of drug resistance is expected

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resulting in disease progression (Rosell *et al*, 2002). Therefore, a better understanding of the molecular mechanisms involved in DDP resistance will help to predict the DDP resistance in advance and to improve the efficacy of therapeutic intervention.

microRNAs (miRNAs) are a large class of endogenous tiny noncoding RNAs, 21–22 nucleotides in length that regulate about 30% of human gene expression (Ke *et al*, 2003). miRNAs can function post-transcriptionally through imperfect base pairing with specific sequences in the 3′ untranslated regions (UTRs) of target mRNAs, leading to transcript degradation or translational inhibition (Valencia-Sanchez *et al*, 2006; Ying *et al*, 2008). Increasing evidence has shown that miRNAs have critical roles in the control of various human biological processes, such as development, angiogenesis, apoptosis and differentiation (Amiel *et al*, 2012; Roy and Sen, 2012; Liwak *et al*, 2012; Hinton *et al*, 2012). Dysregulation of specific miRNAs contributes to a variety of diseases, most notably the development and progression of cancer, including lung cancer (Qi and Mu, 2012). Presently, increasing researches have shown the existence and importance of miRNAs in the evolution of anticancer drug resistance and miRNAs expression profiling can be correlated with the development of drug resistance, suggesting that the miRNAs-mediated form of drug resistance adds another molecular mechanism of multidrug resistance (Wu and Xiao, 2009; Haenisch and Cascorbi, 2012). A couple of recent studies have reported the role of miRNAs in modulating LA chemoresistance. Feng *et al* (2012) showed that miR-200b could reverse chemoresistance of docetaxel-resistant human LA cells by targeting E2F3. Meanwhile, this group also reported that miR-100 could resensitise docetaxel-resistant human LA cells to docetaxel by targeting plk1 (Feng *et al*, 2012). Xiang *et al* (2013) showed that miR-98 could re-sensitise cisplatin-resistant human LA cells by upregulation of HMGA2. Zhang *et al* (2012) reported that miR-513a-3p could sensitise human lung adenocarcinoma cells to cisplatin by targeting GSTP1. These studies provided initial clues for miRNAs in regulating LA chemoresistance. In our previous study, we reported that upregulation of miR-451 could inhibit growth, promote apoptosis and increase DDP sensitivity in non-small cell lung cancer cells by targeting RAB14 (Wang *et al*, 2011; Bian *et al*, 2011). To further investigate the roles of miRNAs in the acquired DDP resistance of LA cells, we established a DDP-resistant LA subclone (A549/DDP) from the DDP-nonresistant cell line (A549). By miRNA microarray analysis, miR-224 was found to be significantly upregulated in DDP-resistant LA cell line. Previous reports have shown that miR-224 is upregulated and may function as an oncogene in a variety of human cancers, including colorectal cancer, cervical cancer, hepatocellular cancer (HCC) and breast cancer. To date, there have been no published data regarding the roles of miR-224 in the formation of DDP-resistant phenotype in LA cells.

In the present study, we demonstrated that miR-224 could promote DDP resistance of LA cells via regulating G_1/S cell cycle transition and apoptosis by targeting p21$^{WAF1/CIP1}$ and predicting poor response of LA patients to DDP-based chemotherapy. Thus, this report identifies novel signalling pathways and molecules as potential therapeutic targets for the treatment of DDP-resistant human LAs.

MATERIALS AND METHODS

Cell culture. The human LA cell line (A549) was purchased from Shanghai Institute of Cell Biology (Shanghai, China). The DDP-resistant LA cell line (A549/DDP) was established and preserved in our lab. The DDP-resistant A549 cell line was selected by continuous exposure to increasing concentrations of DDP. DDP was added into exponentially growing cultures of A549 cells at a concentration of $0.2\,\mu g\,ml^{-1}$ and allowed to remain in the culture until cell growth resumed. The cultures were then split and treated again with progressively higher concentrations of DDP. Over the course of selection, the DDP concentration was increased to $1.0\,\mu g\,ml^{-1}$. The resulting subline was designated as A549/DDP. The DDP-resistant LA cell line (SPC-A1/DDP) and parental SPC-A1 cell line were provided by the Department of Molecular Biology and Biochemistry (Nanjing Medical University). All cell lines were cultured in RPMI 1640 (GIBCO-BRL, Rockville, MD, USA) medium supplemented with 10% fetal bovine serum, $100\,U\,ml^{-1}$ penicillin and $100\,\mu g\,ml^{-1}$ streptomycin in humidified air at $37\,^{\circ}C$ with 5% CO_2.

Patients and tissue samples. A total of 64 tumour tissues were collected from advanced LA patients who received DDP-based chemotherapy at the First or Second Affiliated Hospital of Nanjing Medical University during April 2007 and November 2009. A more detailed description of the patients are described in Supplementary Materials and Methods.

miRNA microarray assay. Standard procedures for miRNA microarray are described in Supplementary Materials and Methods.

Quantitative real-time PCR assay. Standard procedures for quantitative real-time PCR (qRT-PCR) assay are described in Supplementary Materials and Methods.

Transfection of siRNA, miRNA mimics or inhibitors, and plasmids. MiR-224 or miR-663 inhibitors (anti-miR-224 or anti-miR-663), miR-663 mimics and their negative control oligonucleotides (anti-miR-NC or miR-NC mimics) were obtained from Ambion Inc. (Austin, TX, USA). siRNA/p21 (sc-29428) (Santa Cruz Biotechnology, Santa Clara, CA, USA) or siRNA/control was purchased from Santa Cruz Inc. (Santa Clara, CA, USA). The open reading frame of p21 that was generated by PCR was then inserted into the pcDNA 3.1 expression vector, which was named pcDNA/p21. The recombinant vector was confirmed by the digestion analysis of restriction endonuclease and DNA sequencing. For ectopic expression of miR-224, the pGCMV/miR-224 or pGCMV/miR-NC vectors were purchased from GenePharm (Shanghai, China). The transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions provided by the manufacturer. The cells were transfected with those recombinant DNA vectors containing a G418 selection marker and were selected by G418 (Sigma, St Louis, MO, USA) at $500\,mg\,ml^{-1}$ for 4 weeks Then, single clones were obtained and maintained in G418 with concentration of $200\,mg\,ml^{-1}$.

Western blot assay. Standard procedures for western blot assay are described in Supplementary Materials and Methods.

***In vitro* chemosensitivity assay.** The *in vitro* chemosensitivity assay was determined by MTT assay. Standard procedures are described in Supplementary Materials and Methods.

Colony formation assay. Standard procedures are described in Supplementary Materials and Methods.

***In vivo* chemosensitivity assay.** The male athymic BALB/c nude mice aged 5 weeks were maintained under specific pathogen-free conditions and manipulated according to protocols approved by the Shanghai Medical Experimental Animal Care Commission. Standard procedures are described in Supplementary Materials and Methods.

Immunohistochemistry. Standard procedures for immunohistochemistry are described in Supplementary Materials and Methods.

Luciferase reporter assay. Standard procedures are described in Supplementary Materials and Methods.

Flow cytometric analysis of cell cycle and apoptosis. Standard procedures are described in Supplementary Materials and Methods.

Protein kinase assay. Cdk1/cdc2 kinase activity was analysed using a commercially available kit (Cdk1/cdc2 Kinase Assay Kit; Catalog #17–137; Upstate Biotechnology, Lake Placid, NY, USA) as per the manufacturer's instructions. $[r(\gamma)\text{-}32P]$-incorporated histone H1 was separated by 12% SDS–PAGE and visualised by exposure of the dried gels to X-ray film at $-80\,^{\circ}C$ for 3 h.

Statistical analysis. All experimental data were expressed as the mean ± s.e.m. The significance of differences of clinical data according to miR-224 expression was determined by Student's t-test. The Mann–Whitney U-test was employed to compare tumour sizes between two groups. The survival probabilities were determined using the Kaplan–Meier analysis, and the significance of difference was analysed by the log-rank test. Cox proportional hazards modeling of factors potentially related to survival was performed to identify factors that might have a significant influence on survival. All analyses were performed with SPSS 16.0 (SPSS Inc, Chicago, IL, USA) for Windows. The significance level was set at $P < 0.05$.

RESULTS

Establishment and characterisation of DDP-resistant LA cell line (A549/DDP). To assess the acquired resistance of LA cells to DDP, we established a DDP-resistant LA subclone (A549/DDP) from the DDP-nonresistant cell line (A549). When A549 and A549/DDP cells were treated with different concentrations of DDP for 24 h, the cell growth was detected by MTT assay. DDP could inhibit the growth of both cell lines, but parental A549 cells showed more sensitivity to DDP treatment than A549/DDP cells (Supplementary Figure S1A). As shown in Supplementary Figure S1B, the IC$_{50}$ value of DDP in A549/DDP cells $(33.13 \pm 2.24\,\mu g\,ml^{-1})$ was significantly higher than that to parental A549 cells $(13.66 \pm 1.32\,\mu g\,ml^{-1};\ P < 0.01)$. Also, the doubling time of A549/DDP cell line was similar to that of A549 cell line (data not shown). On the basis of the MTT findings, flow cytometry was performed to determine whether cell apoptosis accumulated for the chemoresistance of A549/DDP cells after DDP exposure. Without DDP treatment, apoptosis showed no difference between A549/DDP and A549 cells. In contrast, at 24 h after DDP $(2.0\,\mu g\,ml^{-1})$, the apoptosis of A549 cells was significantly higher than that of A549/DDP cells $(P < 0.05;$ Supplementary Figure S1C). Next, flow cytometry was used to detect cell cycle of both cell lines (Supplementary Figure S1D). The percentage of G_0/G_1 phase in A549/DDP cells was significantly lower than that in A549 cells $(P < 0.05)$ and the percentage of S phase in A549/DDP cells was significantly higher than that in parental cells $(P < 0.05)$, but the percentage of G_2/M showed no difference between two cell lines $(P > 0.01)$. These results indicate that A549/DDP cells are much more chemoresistant than its parental A549 cells. Increasing evidence has shown that epithelial to mesenchymal transition (EMT) has vital roles in the resistance of tumour cells to most conventional therapeutics. Then, we explore whether their A549/DDP cells exhibit the characteristics of mesenchymal cells by determining the expression of epithelial adhesion markers (E-cadherin and N-cadherin) and mesenchymal markers (vimentin). Western blotting showed that the relative expression of E-cadherin protein in A549 cells was significantly downregulated and that of N-cadherin and vimentin proteins was significantly upregulated in comparison with parental A549 cells (Supplementary Figure S1E). These data suggest that we have established A549/DDP as a stable DDP-resistant cell line, which undergoes EMT.

MiR-224 is upregulated in DDP-resistant LA cells (A549/DDP and SPC-A1/DDP). To determine the correlation of dysregulated miRNAs with DDP resistance of LA cells, miRNA microarrays were used to further identify those differentially expressed miRNAs between A549/DDP and parental A549 cells (Figure 1A). The microarray data showed that a total of 44 miRNAs were differentially expressed between A549/DDP and parental A549 cells $(P < 0.05;$ Supplementary Table S1). By the fold-change analysis, we found that 11 miRNAs in A549/DDP cell line showed at least a 4.0-fold change in expression level compared with parental A549 cell line. When compared with parental A549 cell line, seven miRNAs were significantly upregulated and other four miRNAs were significantly downregulated in A549/DDP cell line (Figure 1B). Then, qRT-PCR assay was performed to confirm the microarray data. The results validated 11 miRNAs whose expression levels were significantly different between A549/DDP and A549 cells, and the expression of the 11 miRNAs showed good accordance with the microarray data (Figure 1C). Among the 11 miRNAs, miR-224 was found to be most abundantly expressed in DDP-resistant A549/DDP cells $(P < 0.01)$. Also, we detected the expression of miR-224 in another DDP-resistant LA cell line (SPC-A1/DDP), and found that miR-224 was significantly upregulated ~6.8-fold in SPC-A1/DDP cell line, compared with parental SPC-A1 cell line $(P < 0.01;$ Figure 1D). These data suggest that upregulation of miR-224 might have important roles in the development of DDP resistance in LA cells.

Effects of miR-224 expression on the *in vitro* chemosensitivity of DDP-resistant LA cells to DDP. To investigate the roles of miR-224 in the DDP resistance of DDP-resistant LA cells, anti-miR-224 or anti-miR-NC was transiently transfected into A549/DDP and SPC-A1/DDP cells. Forty-eight hours after transfection, qRT-PCR assay indicated that expression level of miR-224 in anti-miR-224-transfected A549/DDP and SPC-A1/DDP cells was significantly inhibited by about 57.8 and 44.3% $(P < 0.01;$ Figure 2A and Supplementary Figure S2A). Then, we determined the effect of anti-miR-224 on colony formation ability of A549/DDP cells when exposed to DDP treatment (0.0, 2.0 or $4.0\,\mu g\,ml^{-1})$. As shown in Figure 2B and Supplementary Figure S2B, the capacity of colony formation in anti-miR-224-transfected A549/DDP and SPC-A1/DDP cells was significantly reduced compared with anti-miR-NC-transfected cells. Compared with that to anti-miR-NC-transfected cells, the IC$_{50}$ value of DDP in anti-miR-224-transfected A549/DDP or SPC-A1/DDP cells was significantly decreased by ~39.8 or 51.8% $(P < 0.05;$ Figure 2C and Supplementary Figure S2C). At the same time, it was observed that the IC$_{50}$ value of DDP in anti-miR-224-transfected A549/DDP or SPC-A1/DDP cells still showed higher values than that in parental A549 and SPC-A1, suggesting that reducing miR-224 expression only could partially revert the DDP-resistant LA cells to a DDP-sensitive phenotype. Next, we analysed the effects of anti-miR-224 on cell cycle and apoptosis of A549/DDP cells when exposed to DDP treatment by flow cytometry. Compared with anti-miR-NC-transfected cells, the percent of anti-miR-224-transfected A549/DDP or SPC-A1/DDP cells in G_0/G_1 phase of cell cycle increased and the percentage of cells in S phase decreased with different doses of DDP (Figure 2D and Supplementary Figure S2D). Also, anti-miR-224 could significantly increase DDP-induced apoptosis of A549/DDP or SPC-A1/DDP cells (Figure 2E and Supplementary Figure S2E). Therefore, downregulation of miR-224 could reverse the DDP resistance of DDP-resistant LA cells by inducing cell arrest in G_0/G_1 phase and apoptosis enhancement when exposed to DDP treatment.

To further investigate the effects of miR-224 expression on the chemosensitivity of LA cells to DDP, pGCMV/miR-224 or pGCMV/miR-NC vector was stably transfected into A549 cell line (A549/miR-224 or A549/miR-NC). qRT-PCR assay confirmed the
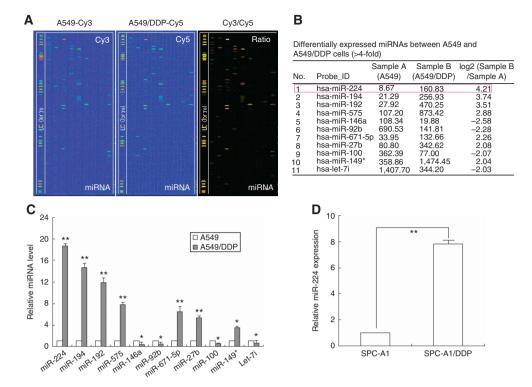
Figure 1. MiR-224 is upregulated in DDP-resistant LA cells. (A) Microarray chip analysis of miRNA expression profiles in A549/DDP and parental A549 cells. miRNAs from A549 or A549/DDP cells were labelled with Cy3 or Cy5, respectively. **(B)** Differential miRNAs' (>4.0-fold) expression between A549 and A549/DDP. Relative expression levels of several representative miRNAs with differential expression levels were presented. **(C)** qRT-PCR detection of differential miRNAs' (>4.0-fold) expression in A549 and A549/DDP cells. **(D)** Relative miR-224 expression level is different between SPC-A1 and SPC-A1/DDP cells after DDP treatment. Quantification by qRT-PCR of miR-224 expression level in A549 and A549/DDP cells before and after DDP treatment. U6 was used for normalisation. Data are expressed as the mean ± s.d. of three individual experiments. NS indicates $P > 0.05$ and * or ** indicates $P < 0.05$ or < 0.01, respectively.

upregulated expression level of miR-224 in A549/miR-224 and SPC-A1/miR-224 cells, compared with that in A549/miR-NC or SPC-A1/miR-NC cells ($P < 0.01$; Figure 3A and Supplementary Figure S3A). When exposed to DDP treatment (0.0, 1.0 or 2.0 μg ml^{-1}), the capacity of colony formation in A549/miR-224 or SPC-A1/miR-224 cells could be enhanced when compared with that in A549/miR-NC or SPC-A1/miR-NC cells ($P > 0.05$; Figure 3B and Supplementary Figure S3B). Compared with that in control cells, the IC$_{50}$ value of DDP in A549/miR-224 or SPC-A1/miR-224 cells was increased by ∼61.6 and 66.8% ($P < 0.05$; Figure 3C and Supplementary Figure S3C). Next, flow cytometry was used to analyse the effects of miR-224 upregulation on cell cycle and apoptosis of parental A549 or SPC-A1 cells when exposed to DDP treatment. Compared with control cells, the percent of A549/miR-224 or SPC-A1/miR-224 cells in G$_0$/G$_1$ phase of cell cycle decreased and the percentage of cells in S phase increased with different doses of DDP ($P < 0.05$; Figure 3D and Supplementary Figure S3D). Compared with that of control cells exposed to DDP treatment, the apoptosis of A549/miR-224 or SPC-A1/miR-224 cells was significantly decreased ($P < 0.05$; Figure 3E and Supplementary Figure S3E). Thus, upregulation of miR-224 could reduce the *in vitro* sensitivity of parental A549 or SPC-A1 cells to DDP.

p21$^{WAF1/CIP1}$ was a functional target of miR-224. It is generally accepted that miRNAs exert their function by downregulating the expression of their downstream target genes. Thus, miR-224 can execute its chemoresistance-promoting function by inhibiting targets that are involved in the regulation of the G1/S transition and apoptosis. Then, the targets of miR-224 were predicted through at least three publicly available algorithms (TargetScan,

PicTar and miRanda), and p21$^{WAF1/CIP1}$, a potent cyclin-dependent kinase (CDK) inhibitor, was selected as a putative target. *In silico* analysis revealed that 3'-UTR of human p21$^{WAF1/CIP1}$ (2131–2151 nt) contains a potential miR-224-binding site (Figure 4A). To determine whether the 3'-UTR region of p21$^{WAF1/CIP1}$ mRNA is a direct functional target of miR-224, we cloned a 305-bp fragment of p21$^{WAF1/CIP1}$ 3'-UTR harbouring the potential binding site into downstream of the pEZX-Luc vector to generate the pEZX-luc-p21/3'-UTR-wt vector (Figure 4B). At 48 h after this vector and pGCMV/miR-224 or pGCMV/miR-NC vector were co-transfected into HEK 293T cells, the luciferase activity was determined. The luciferase activity was significantly suppressed by about 57.3% in HEK293 cells co-transfected with pEZX-luc-p21/3'-UTR-wt and pGCMV/miR-224 ($P < 0.05$; Figure 4C), compared with control. To further confirm that the p21$^{WAF1/CIP1}$ 3'-UTR is a target for miR-224, we mutated the miR-224-binding site on p21$^{WAF1/CIP1}$ 3'-UTR (p21$^{WAF1/CIP1}$ 3'-UTR-mut) and subcloned it into pEZX-luc vector to generate the pEZX-luc-p21/3'-UTR-mut vector (Figure 4B). These vectors were co-transfected into A549 cells with pGCMV/miR-224 (or pGCMV/miR-NC) or anti-miR-224 (or anti-miR-NC), and luciferase activity was measured. Interestingly, the luciferase activity was decreased by almost 46.3% by pGCMV/miR-224 ($P < 0.05$) when the wild-type 3'-UTR of p21$^{WAF1/CIP1}$ was present, and the activity was increased by ∼23.5% ($P < 0.05$) when miR-224 was blocked (Figure 4D). However, the mutations of p21$^{WAF1/CIP1}$ 3'-UTR prevented the expression of miR-224 from affecting luciferase activity. These results indicate that the 3'-UTR of p21$^{WAF1/CIP1}$ is likely a target of miR-224. Then, qRT-PCR and western blot assays were performed to analyse the effects of miR-224 expression on the expression of p21$^{WAF1/CIP1}$ mRNA and protein in LA cells stably transfected
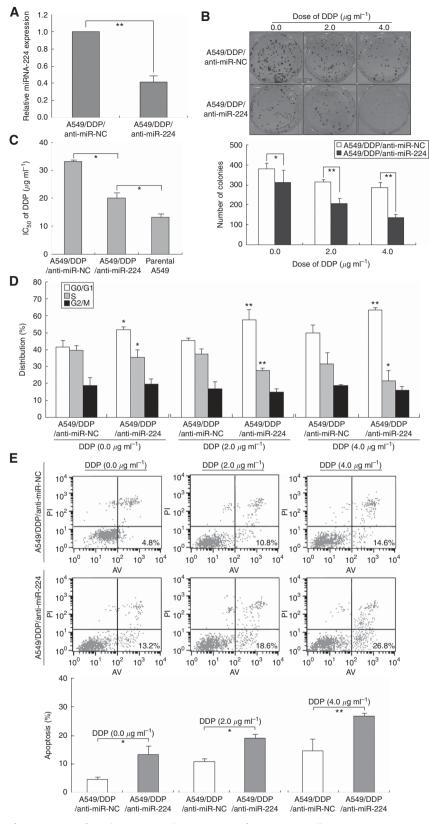
Figure 2. Downregulation of miR-224 significantly increases the sensitivity of A549/DDP cells to DDP. (A) Forty-eight hours after transfection with anti-miR-224 or anti-miR-NC, qRT-PCR detection of miR-224 expression in those cells. U6 was used as an internal control. (B) A549/DDP cells transfected with anti-miR-224 show more DDP sensitivity than cells transfected with anti-miR-NC. Indicated anti-miR-224- or anti-miR-NC-transfected A549/DDP cells were plated in triplicate and exposed to a range of DDP doses (0.0, 2.0 and $4.0\,\mu\mathrm{g\,ml^{-1}}$). The forci formation was indicated. (C) MTT analysis of the IC_{50} values of DDP in anti-miR-224- or anti-miR-NC-transfected A549/DDP and parental A549 cells. (D) Flow cytometric analysis of cell cycle in anti-miR-NC- or anti-miR-224-transfected A549/DDP cells combined with various concentrations of DDP (0.0, 2.0 and $4.0\,\mu\mathrm{g\,ml^{-1}}$). (E) Flow cytometric analysis of apoptosis in anti-miR-NC- or anti-miR-224-transfected A549/DDP cells combined with various concentrations of DDP (0.0, 2.0 and $4.0\,\mu\mathrm{g\,ml^{-1}}$). Data are expressed as the mean ± s.d. of three individual experiments. * or ** indicates $P<0.05$ or <0.01, respectively.
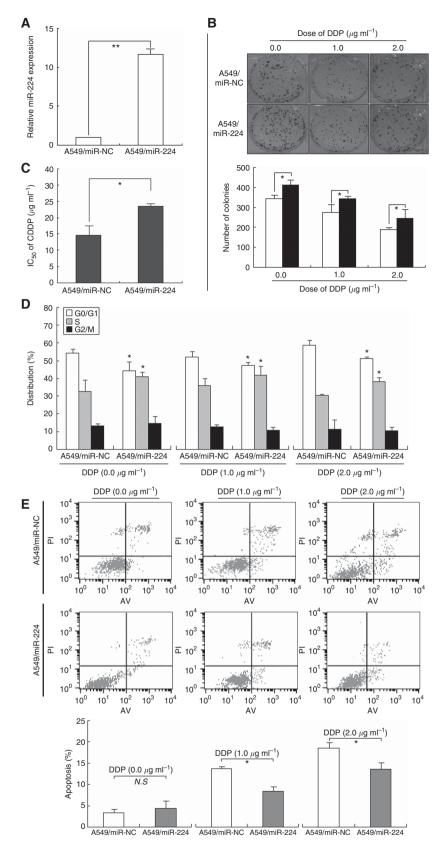
Figure 3. Upregulation of miR-224 significantly reduces the sensitivity of parental A549 cells to DDP. (A) qRT-PCR detection of miR-224 expression in stably transfected A549/miR-224 or A549/miR-NC cells. U6 was used as an internal control. **(B)** A549/miR-224 cells show less DDP sensitivity than A549/miR-NC cells. Indicated A549/miR-224 or A549/miR-NC cells were plated in triplicate and exposed to a range of DDP doses (2.0, 4.0 and 6.0 μg ml^{-1}). The forci formation was indicated. **(C)** MTT analysis of the IC$_{50}$ values of DDP in A549/miR-224 or A549/miR-NC cells. **(D)** Flow cytometric analysis of cell cycle in A549/miR-224 or A549/miR-NC cells combined with various concentrations of DDP (0.0, 1.0 and 2.0 μg ml^{-1}). **(E)** Flow cytometric analysis of apoptosis in A549/miR-224 or A549/miR-NC cells combined with various concentrations of DDP (0.0, 1.0 and 2.0 μg ml^{-1}). Data are expressed as the mean ± s.d. of three individual experiments. NS indicates $P > 0.05$ and * or ** indicates $P < 0.05$ or < 0.01, respectively.
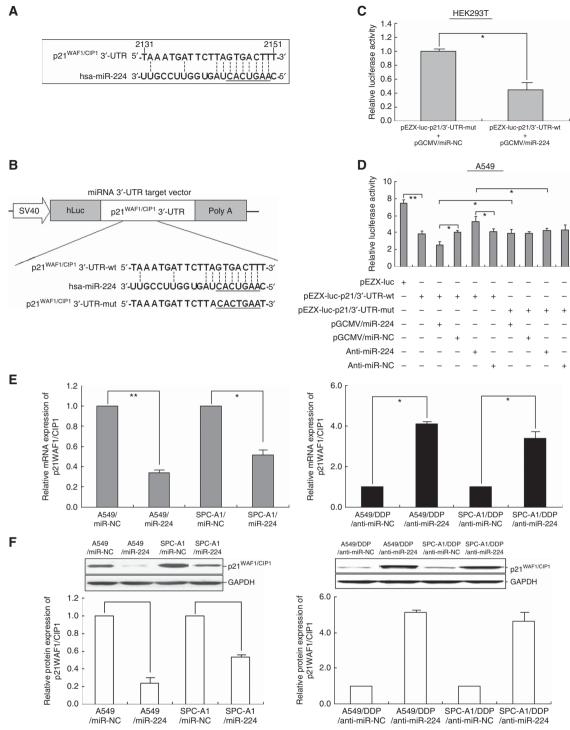
Figure 4. MiR-224 binds to the 3′-UTR of human p21$^{WAF1/CIP1}$ mRNA. (**A**) Sequence of the miR-224-binding site within the human p21$^{WAF1/CIP1}$ 3′-UTR (2131–2151 bp) predicted with TargetScan, miRBase and PicTarget. (**B**) Mutation was generated on the p21$^{WAF1/CIP1}$ 3′-UTR sequence in the complementary site for the seed region of miR-224, as shown. A human p21$^{WAF1/CIP1}$ 3′-UTR fragment containing wild-type or mutant miR-224-binding sequence was cloned downstream of the luciferase reporter gene in pEZX-luc vector. (**C**) pEZX-luc vector containing p21$^{WAF1/CIP1}$ mRNA 3′UTR and pGCMV/miR-224 or control pGCMV/miR-NC were co-transfected into HEK293T cells. Cells lysates were prepared after 48 h for measuring luciferase activity, which was normalised to Renilla luciferase activity. (**D**) Relative luciferase activity was analysed after wild-type or mutant 3′-UTR reporter plasmids were co-transfected with pGCMV/miR-224 or anti-miR-224 in A549 cells. The histogram shows the mean ± s.d. of the normalised luciferase activity from three independent experiments. (**E**) qRT-PCR detection of p21$^{WAF1/CIP1}$ mRNA expression in A549 or A549/DDP cells stably transfected with pGCMV/miR-224 or transiently transfected with anti-miR-224. GAPDH was used as an internal control. (**F**) Western blot detection of p21$^{WAF1/CIP1}$ protein expression in A549 or A549/DDP cells stably transfected with pGCMV/miR-224 or transiently transfected with anti-miR-224. GAPDH was used as an internal control. Data are expressed as the mean ± s.d. of three individual experiments. * or ** indicates $P < 0.05$ or < 0.01, respectively.

with pGCMV/miR-224 (or pGCMV/miR-NC) or transiently transfected with anti-miR-224 (or anti-miR-NC). When compared with that of pGCMV/miR-NC or anti-miR-NC-transfected cells, the expression of p21$^{WAF1/CIP1}$ mRNA in A549/miR-224 or SPC-A1/miR-224 cells was significantly downregulated ($P < 0.01$), but the expression of p21$^{WAF1/CIP1}$ mRNA in A549/DDP/anti-miR-224 or SPC-A1/DDP/anti-miR-224 cells was significantly upregulated ($P < 0.05$; Figure 4E). Further data from western blot assay confirmed the results of qRT-PCR analysis (Figure 4F). Therefore, miR-224 could negatively regulate the expression of p21$^{WAF1/CIP1}$ by directly targeting the 3'-UTR of p21$^{WAF1/CIP1}$ transcript.

To further investigate the roles of p21$^{WAF1/CIP1}$ in the DDP resistance of LA cells, pcDNA/p21 or pcDNA/control was stably transfected into A549/DDP cells. Both qRT-PCR and western blot assays confirmed that the expression of p21$^{WAF1/CIP1}$ mRNA and protein was significantly upregulated in A549/DDP/p21 cells in comparison with A549/DDP/control cells (Figure 5A). Compared with that to A549/DDP/control cells ($34.12 \mu g\,ml^{-1}$), the IC$_{50}$ value of DDP to A549/DDP/p21 cells ($17.68 \mu g\,ml^{-1}$) was decreased by about 48.2% ($P < 0.05$; Figure 5B). Flow cytometric analysis of cell cycle indicated that the percent of A549/DDP/p21 cells in G$_0$/G$_1$ phase of cell cycle increased and the percentage of cells in S phase decreased with different doses of DDP ($P < 0.05$; Figure 5C). The increased apoptosis was observed in A549/DDP/p21 cells when exposed to different doses of DDP treatment ($P < 0.05$; Figure 5D). These data indicated that upregulation of p21$^{WAF1/CIP1}$ could increase the sensitivity of A549/DDP cells to DDP. Further, we determined whether upregulation of p21$^{WAF1/CIP1}$ could rescue the effects of miR-224 overexpression on the chemosensitivity of LA cells. Forty-eight hours after pcDNA/p21 was transfected into A549/miR-224 or A549/miR-NC cells, qRT-PCR and western blot assays confirmed that pcDNA/p21 could recover the expression of p21$^{WAF1/CIP1}$ mRNA and protein (Figure 6A). Also, upregulation of p21$^{WAF1/CIP1}$ could rescue the increased IC$_{50}$ value of DDP to A549 cells induced by miR-224 overexpression (Figure 6B). In addition, upregulation of p21$^{WAF1/CIP1}$ could abrogate the effects of miR-224 overexpression on the G$_1$/S transition and DDP-induced apoptosis of parental A549 cells (Figure 6C and D). Thus, upregulation of p21$^{WAF1/CIP1}$ could mimic the effect of miR-224 downregulation on the chemo-sensitivity of A549/DDP cells and rescue the effect of miR-224 overexpression on the chemosensitivity of A549 cells.

Finally, siRNA targeting p21$^{WAF1/CIP1}$ (siRNA/p21) was transiently transfected into A549 cells. Forty-eight hours after transfection, qRT-PCR and western blot assays showed that the expression of p21 mRNA and protein in siRNA/p21-transfected A549 cells was significantly downregulated in comparison with siRNA/control-transfected cells (Supplementary Figure S4A). Also, the IC$_{50}$ value of DDP to A549/siRNA/p21 cells ($21.58 \mu g\,ml^{-1}$) was increased by 53.4% in comparison with that to A549/siRNA/control cells ($14.06 \mu g\,ml^{-1}$) ($P < 0.05$; Supplementary Figure S4B). When A549 cells were transfected with siRNA/p21 combined with DDP treatment, flow cytometry was performed to detect cell cycle and apoptosis. Cell cycle analysis showed that siRNA-mediated p21 downregulation could lead to the decreased G$_0$/G$_1$ phase rate and the increased S phase rate of A549 cells when exposed to different doses of DDP treatment (0.0, 1.0 and $2.0 \mu g\,ml^{-1}$) (Supplementary Figure S4C). Compared with that in A549/siRNA/control cells, the decreased apoptosis was observed in A549/siRNA/p21 cells when exposed to different doses of DDP treatment (Supplementary Figure S4D). Thus, siRNA/p21 could decrease the sensitivity of parental A549 cells to DDP. Likewise, we determined whether downregulation of p21$^{WAF1/CIP1}$ could rescue the effects of miR-224 downregulation on the chemosensitivity of A549/DDP cells. Forty-eight hours after siRNA/p21 and anti-miR-224 was co-transfected into A549/DDP cells, qRT-PCR and western blot assays showed that siRNA/p21 could recover the

expression of p21$^{WAF1/CIP1}$ mRNA and protein (Supplementary Figure S5A). Also, siRNA/p21 could rescue the increased IC$_{50}$ value of DDP to A549/DDP cells induced by anti-miR-224 (Supplementary Figure S5B), and siRNA/p21 could abrogate the effects of anti-miR-224 on the G$_1$/S transition and DDP-induced apoptosis of A549/DDP cells (Supplementary Figure S5C and D). Thus, downregulation of p21$^{WAF1/CIP1}$ could mimic the effect of pGCMV/miR-224 on the chemosensitivity of A549 cells and rescue the effect of anti-miR-224 on the chemosensitivity of A549/DDP cells.

Previously, other miRNAs including miR-423 and miR-663 could target and reduce p21$^{WAF1/CIP1}$ in other human tumour cells. Thus, we will test whether either of these miRNAs can also lead to DDP resistance in LA cells via downregulation of p21$^{WAF1/CIP1}$. First, we detected the expression of miR-423 and miR-663 in A549 and A549/DDP cells by qRT-PCR assay (Supplementary Figure S6A). The expression of miR-423 in both cells showed no difference ($P > 0.05$), whereas the expression of miR-663 in A549/DDP cells was higher than that in A549 cells ($P < 0.05$). Then, we analysed the effects of miR-663 on p21$^{WAF1/CIP1}$ expression (Supplementary Figure S6B). Forty-eight hours after miR-663 mimics (or miR-NC mimics) and anti-miR-663 (or anti-miR-NC) was transfected into A549 and A549/DDP cells, qRT-PCR and western blot assays were performed. anti-miR-663 increased p21$^{WAF1/CIP1}$ expression in A549/DDP cells, whereas miR-663 mimics decreased its expression in A549 cells ($P < 0.05$). Also, we analysed the effects of miR-663 expression on the sensitivity of LA cells to DDP (Supplementary Figure S6C). Compared with that in anti-miR-NC-transfected cells, the IC$_{50}$ value of DDP in anti-miR-663-transfected A549/DDP cells was increased by 16.5% ($P < 0.05$). Compared with that in miR-NC-transfected cells, the IC$_{50}$ value of DDP in miR-663-transfected A549 cells was decreased by 21.5% ($P < 0.05$). In addition, we analysed the effects of miR-663 expression on cell cycle and apoptosis of A549/DDP cells. Compared with that of miR-NC-transfected cells, the percent of miR-663-transfected A549/DDP cells in G$_0$/G$_1$ phase of cell cycle decreased and the percentage of cells in S phase increased with different doses of DDP ($P < 0.05$; Supplementary Figure S6D). Compared with that of control cells, the apoptosis of miR-663-transfected A549/DDP cells was also increased ($P < 0.05$; Supplementary Figure S6E). Thus, down-regulation of miR-663 could moderately reverse the resistance of A549/DDP cells to DDP by downregulating p21$^{WAF1/CIP1}$. These data provide stronger evidence that the formation of DDP-resistant phenotype of LA cells is mediated via a p21WAF1/CIP1 pathway, which might be post-transcriptionally regulated by miRNAs.

Effects of miR-224 expression on the *in vivo* chemosensitivity of A549 cells to DDP. We further investigated the role of miR-224 on the *in vivo* sensitivity of LA cells to DDP in a mice xenograft model. Then, s.c. tumours were formed in nude mice followed by treatment with DDP. The tumours developed from A549/miR-224 were apparently bigger than those formed from A549/miR-NC cells after the DDP treatment at day 28 (Figure 7A and B). To confirm this, we measured tumour volume at 28 days after inoculation. Following the treatment with DDP, the average volume of tumours formed from A549/miR-NC and A549/miR-224 cells was 312.4 and 487.6 mm^3, respectively, and thus, the upregulation of miR-224 led to a 56.1% promotion of tumour growth ($P < 0.05$; Figure 7C). Next, tumour homogenates were subjected to qRT-PCR for miR-224 and western blot for p21$^{WAF1/CIP1}$, and we found that the expression of miR-224 in xenografts formed from A549/miR-224 cells was significantly upregulated and the expression of p21$^{WAF1/CIP1}$ protein was significantly decreased ($P < 0.05$; Figure 7D and E). Immunostaining showed that the positivity of p21$^{WAF1/CIP1}$ protein in xenografts from A549/miR-224 cells was significantly weeker than that in xenografts from
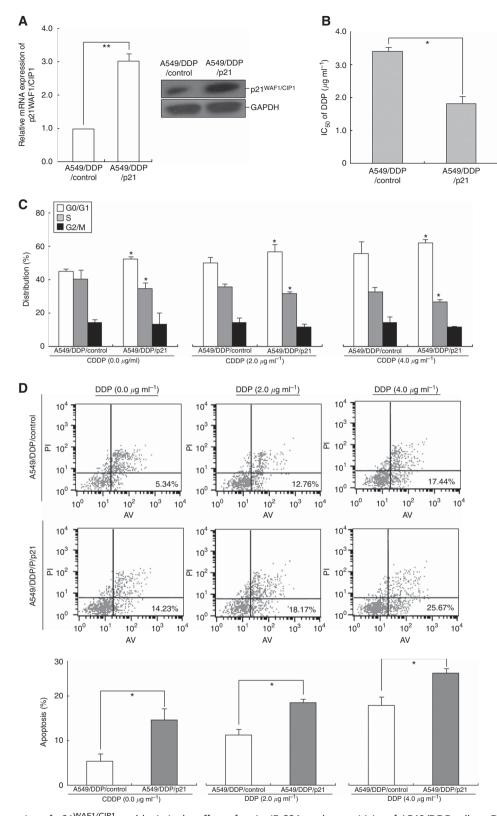
Figure 5. Overexpression of p21$^{WAF1/CIP1}$ could mimic the effect of anti-miR-224 on the sensitivity of A549/DDP cells to DDP. (A) qRT-PCR and western blot detection of p21$^{WAF1/CIP1}$ mRNA and protein expression in stably transfected A549/DDP/control or A549/DDP/p21 cells. (B) MTT analysis of the IC$_{50}$ values of DDP in A549/DDP/control or A549/DDP/p21 cells. (C) Flow cytometric analysis of cell cycle in A549/DDP/control or A549/DDP/miR-224 cells combined with various concentrations of DDP (0.0, 2.0 and 4.0 μg ml^{-1}). (D) Flow cytometric analysis of apoptosis in A549/DDP/control or A549/DDP/miR-224 cells combined with various concentrations of DDP (0.0, 2.0 and 4.0 μg ml^{-1}). Results represent the average of three independent experiments (mean ± s.d.). * or ** indicates $P < 0.05$ or < 0.01, respectively.
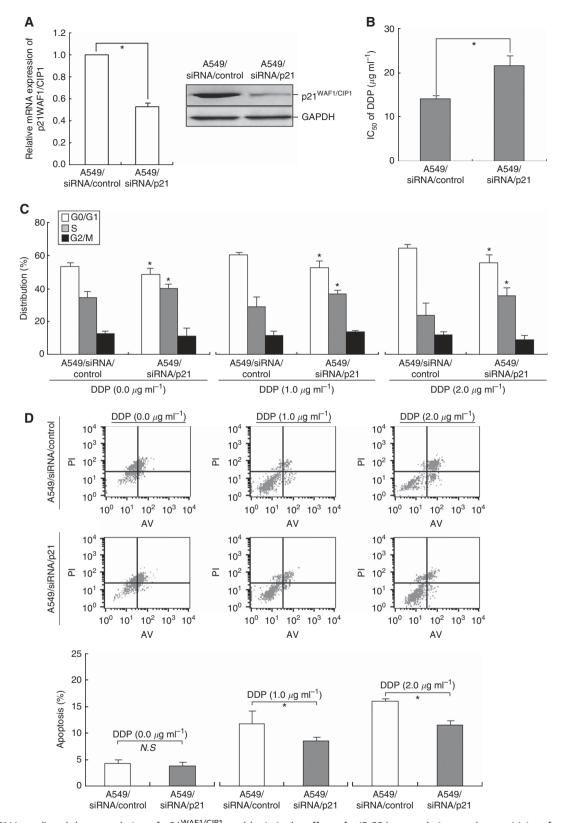
Figure 6. siRNA-mediated downregulation of p21$^{WAF1/CIP1}$ could mimic the effect of miR-224 upregulation on the sensitivity of parental A549 cells to DDP. (**A**) Forty-eight hours after transfection with siRNA/p21 or siRNA/control, qRT-PCR and western blot detection of p21$^{WAF1/CIP1}$ mRNA and protein expression. (**B**) MTT analysis of the IC$_{50}$ values of DDP in siRNA/control or siRNA/p21-transfected A549 cells. (**C**) Flow cytometric analysis of cell cycle in siRNA/control or siRNA/p21-transfected A549 cells combined with various concentrations of DDP (0.0, 1.0 and 2.0 μg ml^{-1}). (**D**) Flow cytometric analysis of apoptosis in siRNA/control or siRNA/p21-transfected A549 cells combined with various concentrations of DDP (0.0, 1.0 and 2.0 μg ml^{-1}). Data are expressed as the mean ± s.d. of three individual experiments. NS indicates $P > 0.05$ and * or ** indicates $P < 0.05$ or < 0.01, respectively.
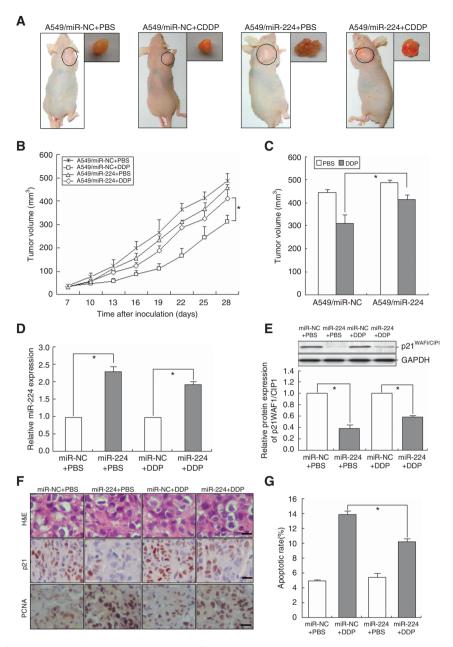
Figure 7. Upregulation of miR-224 reduces the *in vivo* sensitivity of A549 cells to DDP. Mice were treated with DDP (3.0 mg kg^{-1} body weight; i.p., thrice) or with 0.1 ml PBS (pH 7.4; i.p., thrice). (**A**) Representative features of tumours 28 days after inoculation using A549/miR-224 or A549/miR-NC cells treated with PBS or DDP. (**B**) Growth of tumours in the mice injected with A549/miR-224 or A549/miR-NC cells treated with DDP or PBS. The inoculation was done in 10 mice. (**C**) Tumour volume at day 28 after the inoculation. Left (white column), average tumour volume at day 28 after the inoculation of A549/miR-224 or A549/miR-NC cells in mice treated with PBS; right (grey column), average tumour volume at day 28 after the inoculation of A549/miR-224 or A549/miR-NC cells in mice treated with DDP. (**D**) qRT-PCR detection of relative miR-224 expression in tumours developed from A549/miR-224 or A549/miR-NC cells treated with DDP or PBS. Data were presented as the mean ± s.e.m. (*n* = 3). (**E**) Western blotting detection of p21$^{WAF1/CIP1}$ protein expression in tumours developed from A549/miR-224 or A549/miR-NC cells treated with DDP or PBS. Data were presented as mean ± s.e.m. (*n* = 3). (**F**) Immunostaining of p21$^{WAF1/CIP1}$ and PCNA protein expression in tumours developed from A549/miR-224 or A549/miR-NC cells treated with DDP or PBS. Upper: H&E staining; intermediate and lower: immunostaining; bars, 100 μm. (**G**) Detection of apoptosis in tumours developed from A549/miR-224 or A549/miR-NC cells treated with DDP or PBS. Results represent the average of three independent experiments. * or ** indicates *P* < 0.05 or < 0.01, respectively.

A549/miR-NC cells (Figure 7F). Also, following the treatment with DDP, the number of PCNA-positive cells in xenografts formed from A549/miR-224 cells were higher than that in xenografts from A549/miR-NC cells (Figure 7F), and TUNEL staining assay indicated that the rate of apoptotic tumour cells in xenografts formed from A549/miR-224 cells was lower than that in xenografts formed from A549/miR-NC cells (Figure 7G). Taken together,

upregulation of miR-224 could reduce the *in vivo* chemosensitivity of A549 cells to DDP.

MiR-224 and p21$^{WAF1/CIP1}$ regulate the expression of G$_1$/S transition and apoptosis-related proteins in DDP-resistant A549 cells. To further investigate the roles of p21$^{WAF1/CIP1}$ in the miR-224-regulated G1/S transition of cell cycle in DDP-resistant A549

cells, we analysed the expression of p21$^{WAF1/CIP1}$ pathway-related proteins in anti-miR-224- or pcDNA/p21-transfected A549/DDP cells, including p-cdk1, total cdk1, ppRb, pRb, E2F1, cyclinD1, cyclinA and cyclinE. The expression of p-cdk1, ppRb and E2F1 proteins was significantly downregulated in anti-miR-224- or pcDNA/p21-transfected A549/DDP cells compared with control cells ($P < 0.05$; Figure 8A). However, the expression of cyclinD1, cyclinA and cyclinE proteins showed no obvious difference between anti-miR-224- or pcDNA/p21-transfected cells and control cells ($P > 0.05$). Meanwhile, we determined the changes of CDK1 activity and showed that both anti-miR-224 and pcDNA/p21 could lead to the inhibition of CDK1 activity in A549/DDP cells ($P < 0.01$; Figure 8B). It has been reported that p21$^{WAF1/CIP1}$ can regulate the G$_1$/S transition of cell cycle by activating CDKs and cyclin complexes, so the expression of cyclins might not be affected. Our results indicated that miR-224 might affect the G$_1$/S transition of cell cycle through the p21$^{WAF1/CIP1}$-pRb pathway in A549/DDP cells.

Although the role of p21$^{WAF1/CIP1}$ in apoptosis is still controversial, there are studies showing that p21$^{WAF1/CIP1}$ also possesses pro-apoptotic functions against human malignancies (Yang et al, 2008; Wu et al, 2011). Here we showed that overexpression of p21$^{WAF1/CIP1}$ could mimic the apoptosis-promoting functions of anti-miR-224, and siRNA-mediated downregulation of p21$^{WAF1/CIP1}$ could rescue the apoptosis-promoting functions of anti-miR-224 in A549/DDP cells. First, we found that the expression levels of cleaved caspase-3 and PARP proteins in anti-miR-224- or pcDNA/p21-transfected A549/DDP cells were significantly higher than those in anti-miR-NC- or pcDNA/control-transfected cells ($P < 0.05$), but the expression levels of total caspase-3 and PARP proteins showed no significant difference ($P > 0.05$). Also, we found that both anti-miR-224 and pcDNA/p21 could induce the decreased expression of anti-apoptosis Bcl-2 family proteins (Bcl-2 and Bcl-xL) and the increased expression of pro-apoptosis Bcl-2 family proteins (Bax and Bak) (Figure 8C). Thus, anti-miR-224 might promote apoptosis of A549/DDP cells through the intrinsic mitochondrial death pathway by preventing the downregulation of p21WAF1/CIP1.
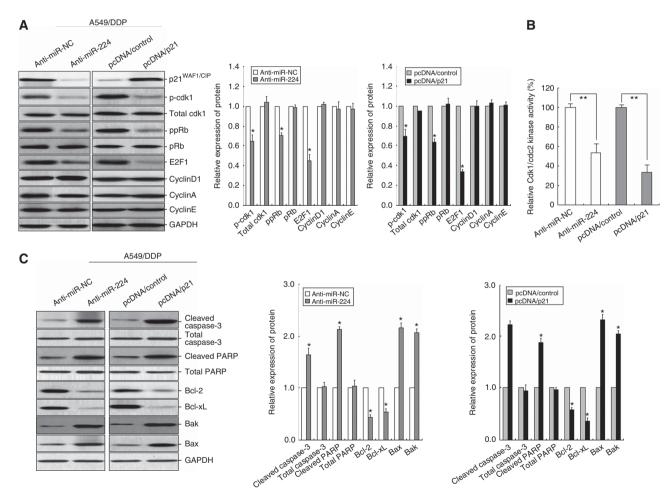

Figure 8. Western blot detection of cell cycle- or apoptosis-related protein expression in A549/DDP cells transiently transfected with anti-miR-224 or stably transfected pcDNA/p21. Forty-eight hours after A549/DDP cells were transfected with anti-miR-224 or anti-miR-NC, cells were collected. Also, the stably transfected A549/DDP cells (A549/DDP/p21 and A549/DDP/control) were collected. (**A**) Western blot detection of cell cycle-related proteins (p-cdk1, total cdk1, ppRb, pRb, E2F1, cyclinD1, cyclinA and cyclinE) in anti-miR-224 (or anti-miR-NC)-transfected A549/DDP and A549/DDP/p21 (or A549/DDP/control) cells. (**B**) Densitometric analysis of HH1 phosphorylation is depicted as the relative level in Cdk1/cdc2 kinase activity. HH1 phosphorylation was determined in anti-miR-NC- or anti-miR-224-transfected A549/DDP cells and A549/DDP/control or A549/DDP/p21 cells. The activity for the control untreated cell extract was set to 1. (**C**) Western blot detection of apoptosis-related proteins (cleaved caspase-3, total caspase-3, cleaved PARP, total PARP, Bcl-2, Bcl-xL, Bax and Bak) in anti-miR-224 (or anti-miR-NC)-transfected A549/DDP and A549/DDP/p21 (or A549/DDP/control) cells. GAPDH protein was used as an internal control. Data are expressed as the mean ± s.d. of three individual experiments. * or ** indicates $P < 0.05$ or < 0.01, respectively.

Expression of miR-224 in LA tissues is negatively correlated with p21$^{WAF1/CIP1}$ expression and responses of LA patients to DDP-based chemotherapy. To investigate the correlation between miR-224/p21$^{WAF1/CIP1}$ dysregulation and response to DDP-based chemotherapy and prognosis of patients, the expression of miR-224 and p21$^{WAF1/CIP1}$ mRNA or protein was detected in a total of 64 LA patients (stage IIIB or IV) who received DDP-based combination chemotherapy combined with docetaxel, gemcitabine or vinorelbine. Tumours were divided into two groups: responding (CR + PR) and non-responding (SD + PD). First, qRT-PCR was used to detect the expression of miR-224 and p21$^{WAF1/CIP1}$ mRNA expression, and we showed that the relative level of miR-224 expression in responding tumour tissues ($n = 26$) was significantly lower than that in non-responding tumour tissues ($n = 38$) ($P = 0.002$; Figure 9A). However, the relative level of p21$^{WAF1/CIP1}$ mRNA expression in responding tumour tissues was significantly higher than that in non-responding tumour tissues ($P = 0.018$; Figure 9B). Immunostaining of p21$^{WAF1/CIP1}$ protein expression indicated that the staining of p21$^{WAF1/CIP1}$ protein was stronger in the responding than in the non-responding LA tissues (Figure 9C).

Then, ROC curve analysis was performed to establish the optimal cutoff value for the mean level of miR-224 expression, which yielded a value of $^{\Delta}C_t$ (7.18). Patients were divided into two groups: low-miR-224 group ($^{\Delta}C_t < 7.82$; $n = 31$) and high-miR-224 group ($^{\Delta}C_t > 7.82$; $n = 33$). The correlation of miR-224 expression with clinicopathological factors of LA patients was shown in Supplementary Table S2. The cisplatin-based chemotherapy response rate of patients with low-miR-224 tumours was 64.5%, as opposed to 36.4% for patients with high-miR-224 tumours, suggesting that miR-224 expression in tumour tissues was

negatively correlated with response to DDP-based chemotherapy ($P = 0.024$). The correlation of miR-224 expression with prognosis of LA patients was investigated by Kaplan–Meier analysis and log-rank test. As shown in Figure 9D, the status of miR-224 expression was found to be closely correlated with both progression-free survival (PFS) and overall survival (OS) of patients ($P = 0.0032$ and 0.008, respectively) (Figure 9E and F). The median PFS time for the high-miR-224 negative group was 5.7 months, as opposed to 11.3 months for the low-miR-224 group. Also, the median OS time for the high-miR-224 group was 10.8 months, as opposed to 18.8 months for the low-miR-224 group. In addition, when all of the clinicopathological factors were evaluated, multivariate analysis using the Cox proportional hazard model indicated that status of miR-224 might be an independent factor for prediction of poor PFS (HR: 1.59, 95% CI: 1.12–2.67; $P = 0.014$) and OS (HR: 2.15, 95% CI: 1.73–3.03; $P = 0.007$) in advanced LA patients (Supplementary Table S3). Furthermore, by linear regression analysis, it was found that there was an inverse correlation between relative miR-224 and p21$^{WAF1/CIP1}$ mRNA expression in LA tissues from patients who received DDP-based combination chemotherapy (Figure 9F). These data suggested that miR-224 expression in tumour tissues was negatively correlated with p21$^{WAF1/CIP1}$ expression and responses of LA patients to DDP-based combination chemotherapy.

DISCUSSION

DDP, a DNA damaging agent, has for many years been used as a systematic chemotherapeutic agent for several human tumour
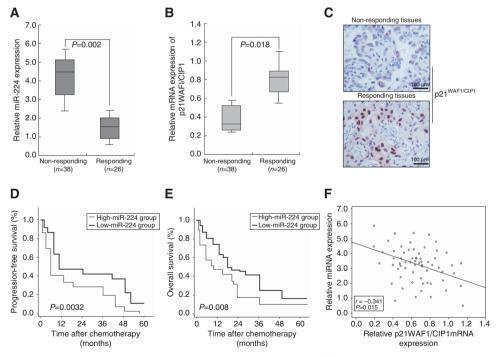

Figure 9. Expression of miR-224 in LA tissues was negatively correlated with p21$^{WAF1/CIP1}$ and responses of patients to DDP-based chemotherapy. (A) qRT-PCR detection of relative miR-224 expression in responding ($n = 26$) and non-responding ($n = 38$) LA tissues ($P = 0.002$). Abundance of miR-224 was normalised to U6 RNA. **(B)** qRT-PCR detection of relative p21$^{WAF1/CIP1}$ mRNA expression in responding ($n = 26$) and non-responding ($n = 38$) LA tissues ($P = 0.018$). Abundance of p21$^{WAF1/CIP1}$ was normalised to GAPDH. **(C)** Immunostaining of p21$^{WAF1/CIP1}$ protein expression in responding and non-responding LA tissues. Bars, 100 μm. **(D)** The PFS curves for the high-miR-224 expression group ($n = 33$) and the low-miR-224 expression group ($n = 31$). The PFS rates between the two groups were significantly different ($P = 0.0032$). **(E)** The OS curves for the high-miR-224 expression group ($n = 33$) and the low-miR-224 expression group ($n = 31$). The OS rates between the two groups were significantly different ($P = 0.008$). **(F)** A statistically significant inverse correlation between miR-224 and p21$^{WAF1/CIP1}$ mRNA levels in 64 cases of LA tissues (Spearman's correlation analysis, $r = -0.341$; $P = 0.015$). Data are expressed as the mean ± s.d. of three individual experiments. Corresponding P-values analysed by Spearman correlation test are indicated.

types, including LA. Unfortunately, intrinsic or acquired tumour cell resistance to DDP severely limits its therapeutic efficacy (Shen et al, 2012). Multiple mechanisms have been proposed for the development of DDP resistance in LA, including the reduced intracellular accumulation of the drug, increased levels of glutathione and anti-apoptotic proteins, and decreased pro-apoptotic proteins (Wang et al, 2004; Stewart et al, 2007). Growing evidence has shown that miRNAs have regulatory roles in the pathogenesis of malignant tumours, through the suppression of genes involved in cell growth, differentiation, development, apoptosis, metastasis and chemo- or radioresistance (Farazi et al, 2011; Farazi et al, 2013). It is likely, therefore, that they can also modulate sensitivity and resistance to anticancer drugs in substantial ways (Ma et al, 2010; Zheng et al, 2010). However, the mechanisms responsible for chemotherapy resistance by miRNAs have not been clearly identified. In the current study, we established DDP-resistant cell line from human LA A549 cells to seek an understanding of the molecular mechanisms of DDP resistance with respect to miRNAs. At first, we approached this issue by obtaining the miRNA differential expression profile between the parental and its DDP-resistant cell line (A549/DDP) by using the array-based miRNA assay. Microarray data showed that a total of 11 miRNAs in DDP-resistant LA cells showed at least a 4.0-fold change in expression level compared with that in parental LA cells. Among these miRNAs, seven miRNAs (miR-224, 194, 192, 575, 671-5p, 27b and 149*) was significantly upregulated and four miRNAs (miR-146a, 92b, 100 and Let-7i) was significantly downregulated in DDP-resistant LA cell line. Specially, miR-224 was found to be the most upregulated miRNA in DDP-resistant LA cell line compared with that in parental LA cells. Furthermore, DDP treatment could lead to the upregulated expression of miR-224 in DDP-resistant LA cells but have no effects on its expression in parental LA cells. These are attracting us to explore in-depth the roles of miR-224 upregulation in the formation of DDP-resistant phenotypes in LA cells.

miR-224 has been known to be upregulated in many human cancers, including HCC, breast cancer, glioma, cervical cancer and colorectal cancer. Wang et al (2008) showed that miR-224, the most significantly upregulated miRNA in HCC patients, increases apoptotic cell death as well as proliferation and targets apoptosis inhibitor-5 (API-5) to inhibit API-5 transcript expression. Li et al (2010) showed that miR-224 could promote migration and invasion of HCC cells by regulating PAK4 and MMP9 expression. Likewise, Zhang et al (2013) testified the involvement of miR-224 in cell proliferation, migration, invasion and anti-apoptosis in HCC by affecting the expression levels of CDC42, CDH1, PAK2 and BCL-2. This miR-224-residing locus in Xq28 is reciprocally regulated by HDAC1, HDAC3, and histone acetylase protein, E1A binding protein p300 (EP300), and EP300 displays increased binding to the Xq28 locus. By functional analyses, Wang et al (2012) further elucidated that overexpression of EP300 may account, in part, for the upregulation of miR-224 expression in HCC. Also, the prognostic values of miR-224 in human cancers are investigated. Shen et al (2013) showed that miR-224 upregulation was associated with aggressive progression and poor prognosis in cervical cancer and could be identified for the first time as an independent marker for predicting the clinical outcome of cervical cancer patients. miR-224 expression was found to be significantly associated with poor disease-free survival and OS in glioma patients with high pathological grades (Lu et al, 2013). More importantly, upregulation of miR-224 is also reported to promote growth in colorectal cancer cells and control cell invasion in breast cancer cells by targeting PHLPP1 and PHLPP2 or RKIP, respectively (Huang et al, 2012; Liao et al, 2013). These experimental data, taken together, support an important role of altered miR-224 during tumour progression and metastasis.

To date, there is only one report about the correlation of miR-224 expression with lung cancer. By studying tumour–microenvironment interactions using zonal transcriptional profiling of squamous cell lung carcinoma and in situ hybridisation, Wu et al (2013) found that miR-224 was moderately expressed in the inner tumour and tumour front compared with that in the adjacent lung tissue. However, the roles of miR-224 in lung carcinogenesis and the effects of miR-224 expression on malignant phenotypes of lung cancer cells are still unclear and remain to be further elucidated. Here we will investigate the emerging roles of miR-224 in DDP resistance of human LA cells. By loss-of-function studies, downregulation of miR-224 could reverse the in vitro DDP resistance of DDP-resistant LA cells by affecting G_1/S cell cycle transition and inducing apoptosis enhancement. Meanwhile, a stably expressing exogenous miR-224 parental LA cell line was established for gain-of-function studies. We showed that upregulation of miR-224 could reduce the sensitivity of parental LA cells to DDP both in vitro and in vivo. Likewise, we also found that upregulation of miR-224 could affect G_1/S transition and reduce the DDP-induced apoptosis in parental LA cells. Importantly, by analysis of miR-224 expression in LA tissues collected from patients who received DDP-based chemotherapy, the relative level of miR-224 expression in responding tumour tissues was significantly lower than that in non-responding tumour tissues, suggesting that miR-224 expression in tumour tissues was negatively correlated with responses of LA patients to DDP-based chemotherapy. Sequentially, to further explore the molecular mechanisms by which miR-224 exerts its function, the determination of its functional target gene is essential. Analyses using the TargetScan, PicTar and miRanda algorithms' databases revealed that more than 100 genes were predicted to be the potential targets of miR-224. According to the functions of these genes and the effect of miR-224 on LA cells, p21$^{\text{WAF1/CIP1}}$ was chosen as the interesting gene in further study. Our data clearly suggest that miR-224 promotes the DDP resistance of LA cells by directly targeting p21$^{\text{WAF1/CIP1}}$. This conclusion is based on several pieces of evidence. First, anti-miR-224 significantly upregulates the expression of p21$^{\text{WAF1/CIP1}}$ mRNA and protein in A549/DDP cells, whereas pGCMV/miR-224 significantly downregulates the expression of p21$^{\text{WAF1/CIP1}}$ mRNA and protein in parental A549 cells. Second, the luciferase activity assay indicated that miR-224 could bind the 3′-UTR of the p21$^{\text{WAF1/CIP1}}$ transcript. Third, overexpression of p21$^{\text{WAF1/CIP1}}$ could mimic the effect of miR-224 inhibitor in A549/DDP cells, whereas silencing of p21$^{\text{WAF1/CIP1}}$ could partially reverse the effect of miR-224 inhibitor in A549 cells. Fourth, downregulation of p21$^{\text{WAF1/CIP1}}$ could mimic the effect of pGCMV/miR-224 in A549 cells, whereas upregulation of p21$^{\text{WAF1/CIP1}}$ could partially reverse the effect of pGCMV/miR-224 in A549/DDP cells. Finally, p21$^{\text{WAF1/CIP1}}$ was negatively correlated with miR-224 in LA tissues. These data suggest that miR-224 targets p21$^{\text{WAF1/CIP1}}$ and downregulates its expression in LA.

p21$^{\text{WAF1/CIP1}}$, a potent CDK inhibitor, inhibits the activity of cyclin-CDK2 or cyclin-CDK4 complexes and blocks DNA replication by binding to proliferating cell nuclear antigen (Abbas and Dutta, 2009). The members of the Cip/Kip family, specifically p21$^{\text{Waf1/CIP1}}$, are responsible for cell cycle control, blocking the transition from phase G1 to phase S. Besides being an inhibitor of cell cycle progression, p21$^{\text{WAF1/CIP1}}$ was also considered as a mediator of the apoptotic pathway (Fujiwara et al, 2008). Although the role of p21$^{\text{WAF1/CIP1}}$ in apoptosis is still controversial, with contradictory findings of both stimulation and inhibition of apoptosis, there are many researches indicating that p21$^{\text{WAF1/CIP1}}$ also possesses pro-apoptotic functions against cancer. Chen et al (2008) show that induction of p21 by RNAa has antitumour activity in vitro in bladder cancer cells by promoting cell cycle arrest and enhancing apoptotic cell death. In HCC, it was reported that p21$^{\text{WAF1/CIP1}}$ transcriptional activation induced by

dsRNAs (dsP21-322) markedly inhibits the viability of HCC cells and causes a significant increase in HCC cell apoptosis, which was correlated with decreased expression levels of the anti-apoptotic proteins' Bcl-xL and survivin, and increased expression of cleaved caspase-3, caspase-9 or PARP (Wu *et al*, 2011). In addition, the enforced expression of p21$^{\text{WAF1/CIP1}}$ was found to enhance the apoptotic response to chemotherapeutic agents. Li *et al* (1997) reported that overexpression of p21$^{\text{WAF1/CIP1}}$ in cells lacking functional retinoblastoma protein might mediate sensitivity to anticancer drugs by inhibiting E2F2 phosphorylation, which contributed to increased S-G2 cell cycle delay and increased cell susceptibility to apoptosis. Wei *et al* (2010) reported that p21$^{\text{WAF1/CIP1}}$ gene transcriptional activation exerted cell growth inhibition and enhanced chemosensitivity to DDP in lung carcinoma cell. Genetic and epigenetic abnormalities can induce lower expression of p21$^{\text{WAF1/CIP1}}$, which may be linked to chemoresistance. Recently, the correlation between miRNAs and p21$^{\text{WAF1/CIP1}}$ in human cancers is reported. Lin *et al* (2011) reported that miRNA-423 promotes cell growth and regulates G1/S transition by targeting p21$^{\text{WAF1/CIP1}}$ in HCC. Yi *et al* (2012) also reported that miR-663 promotes the proliferation and tumorigenesis of nasopharyngeal carcinoma by targeting p21$^{\text{WAF1/CIP1}}$. By identifying multiple miRNAs that can suppress p21$^{\text{WAF1/CIP1}}$ expression by directly targeting its 3′-UTR, Wu *et al* (2010) found that 8 of these 28 p21-regulating miRNAs are located in the chromosome 19 miRNA cluster, the largest miRNA gene cluster in humans, and they can clearly promote cell proliferation and cell-cycle progression in choriocarcinoma cells. However, the roles of miRNAs/p21$^{\text{WAF1/CIP1}}$ in the DDP resistance of human LAs are unclear. In this study, functional characterisation of miR-224 indicated that the miRNA could promote *in vitro* and *in vivo* DDP resistance of LA cells by targeting p21$^{\text{WAF1/CIP1}}$. By analysis of p21$^{\text{WAF1/CIP1}}$ expression in LA tissues, the expression of p21$^{\text{WAF1/CIP1}}$ mRNA and protein in responding tumour tissues was found to be significantly higher than that in non-responding tumour tissues, and negatively correlated with miR-224 expression. Further researches showed that miR-224 might affect the G_1/S transition of cell cycle and apoptosis in DDP-resistant LA cells through the p21$^{\text{WAF1/CIP1}}$-pRb pathway and the intrinsic mitochondrial death pathway. However, we also noticed that up- or downregulation of p21$^{\text{WAF1/CIP1}}$ only could partially reverse the effect of pGCMV/miR-224 or miR-224 inhibitor on DDP-resistant or parental LA cells. Our data indicated that p21$^{\text{WAF1/CIP1}}$ was an important mediator for miR-224-promoting DDP resistance of LA cells. This might be due to the fact that one miRNA can target a diverse set of mRNAs, and one gene can be potentially regulated by multiple miRNAs. In this study, we also found that miR-663 could moderately promote the DDP resistance of LA cells via downregulation of p21$^{\text{WAF1/CIP1}}$. These provided stronger evidence that the formation of DDP-resistant phenotype of LA cells was mediated via a p21WAF1/CIP1 pathway.

In summary, our data establish a functional link that miR-224 and p21$^{\text{WAF1/CIP1}}$ in LA, and show that miR-224 could promote DDP resistance in LA cells via regulating G1/S transition and apoptosis by targeting p21$^{\text{WAF1/CIP1}}$. Thus, miR-224/p21$^{\text{WAF1/CIP1}}$ signature might predict the responses of LA patients to DDP-based chemotherapy and represent potential targets for therapeutic intervention. Of course, this study has several limits. First, further studies are needed to identify other undefined miR-224 targets, which may also affect cellular phenotypes at other levels. Second, as the size of tissue sample is small, further investigation of a larger patient population will be necessary to confirm our findings. Third, as the presence of EMT phenotypic cells in tumour cells may be one of the reasons that tumour patients are typically drug-resistant. Thus, whether miR-224 affects the formation of EMT phenotypes in DDP-resistant LA cells by targeting p21$^{\text{WAF1/CIP1}}$ will be further investigated.

AUTHOR CONTRIBUTIONS

HW conceived and carried out the experiments. L-JZ carried out partial experiments and collected clinical samples. Y-CY conceived experiments and collected clinical samples. HW and RW analysed data. Z-XW and RW designed the study and supervised the experiments. All authors were involved in writing the paper and finally approved.

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