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Original Article

MiR-328 targets histone H2AX and regulates lung cancer cells apoptosis

Ling Zhang[1,2], Chengshan Xu[1], Xinpin Wu[1], Yaqiong Dong[3], Zhe Wang[1], Yuan Luo[1], Lianning Duan[1], Chengrong Lu[1]

[1]*Aviation Medicine Research Laboratory, Air Force General Hospital, PLA, Beijing 100142, China;* [2]*Department of Biochemistry and Molecular Biology, Dalian Medical University, Dalian 116044, China;* [3]*Yongledian Town Community Health Service Center, Beijing 101115, China*

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Abstract: Increasing reports indicate that microRNAs play a key role in cell growth, differentiation, and apoptosis. In this study, we describe how the regulation of miRNA-328 (miR-328), which increases in cancer cells, is involved in the apoptosis of lung cancer cells (A549 and NCI-H1650). Expression analysis has verified that the level of miR-328 is significantly decreased in apoptotic A549 and NCI-H1650 cells. Furthermore, overexpression of miR-328 in lung cancer cells inhibits etoposide-induced cellular apoptosis. Additionally, we identified that histone H2AX is a downstream target of miR-328, which can bind directly to the 3'-untranslated region (3'-UTR) of *H2AX*, subsequently downregulating both the mRNA and protein levels of H2AX. The results from co-expression demonstrated that over-expression of H2AX which lacked 3'-UTR in A549 and NCI-H1650 cells partially reduces the effect of miR-328 on cell apoptosis. Taken together, our results illuminate that miR-328 functions as an apoptosis silencer to regulate lung cancer cell apoptosis through targeting histone H2AX and may become a critical therapeutic target in lung cancer.

Keywords: MiR-328, lung cancer, H2AX, apoptosis

Introduction

Lung cancer is one of the most frequently diagnosed malignant tumors and the leading cause of cancer-related deaths for both men and women, and accounts for over a million deaths worldwide each year [1-3]. The predominant subtypes of lung cancer are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Approximately 80% of lung cancers are classified histopathologically as NSCLS. At early stages of NSCLS, the only treatment is surgery, with a 5-year overall survival rate of 40% [4], whereas chemotherapy is mostly employed for SCLS. Recently, accumulating evidences have shown that microRNAs (miRNAs) are deregulated in lung cancers, and may act as oncogenes or tumor suppressor genes. For example, miR-197 [5], miR-21 [6, 7], and miR-198 [8] are dramatically deregulated in lung cancer apoptosis, whereas, miR-31 [9] is upregulated in apoptosis of lung cancer cells. These studies provide new insights into lung cancer biology and deserve in depth investigation.

MiRNAs are small non-coding RNAs (20-25 nucleotides) that bind partially complementary sites in the 3'-untranslated regions (3'-UTRs) of target genes and regulate gene expression mostly at the post-transcriptional level [10]. In recent years, there has been a considerable interest in understanding the role of miRNAs in disease development, including cancers. Many studies have shown that miRNAs such as miR-372, miR-202, miR-340, miR-509, and miR-1469 [11-15] regulate cellular apoptosis. Recently, dysregulation of miR-328 has been reported in various types of disease, such as cardiac hypertrophy [16], human melanoma [17], and brain metastases [18]. MiR-328 was found to be located on chromosome 16. Some studies have demonstrated that miR-328 acts as tumor suppressor in human melanoma cells [17]. MiR-328 controls cell fate through base pairing with mRNA targets and interferes with the function of regulatory proteins [19]. However, the function of miR-328 in lung cancer is not clear.

In this study, we firstly analyzed the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information (NCBI) and found that miR-328 expression is generally associated with lung cancer. Then we examined the role of miR-328 in apoptosis of lung cancer cells. MiR-328 was reduced when etoposide (VP16)-induced lung cancer cells apoptosis. Further investigation revealed that the histone H2AX is a direct and functional target of H2AX and miRNA-328 regulates apoptosis of lung cancer cells through targeting H2AX. Our results show that miR-328 may act as an oncogene in lung cancer cells.

Materials and methods

The miRNA microarray data

GSE15008, GSE24709, GSE31568, and GSE-61741 datasets were accessible at the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/). The miRNA microarray datasets are based on the platforms of GPL8176 [20] and GPL9040 [21]. Four datasets of microarrays on lung cancer patients and normal controls were retrieved from the GSE database. The analyzed datasets, including GSE15008 (177 pairs of primary lung cancers with their corresponding adjacent normal lung tissues), GSE24709 (n = 19 normal controls, n = 28 lung cancer patients), GSE31568 (n = 76 normal controls, n = 32 lung cancer patients), and GSE61741 (n = 86 normal controls, n = 73 lung cancer patients), have been screened for the complete miRNA repertoire.

Cell culture

Lung cancer cells A549 and NCI-H1650 (H1650) cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, and 1% antibiotics (100 U/ml penicillin and 100 U/ml streptomycin). Cells were incubated at 37°C in a humidified incubator with 5% CO_2. The medium was changed at alternate days and the cells were split before they reached 100% confluence.

Oligonucleotides, plasmids and transfection

miR-328 mimics and miR-328 inhibitor, as well as negative control (NC) and inhibitor NC were chemically synthesized and optimized by Shanghai Gene-Pharma Co. (Shanghai, China). The full-length 3'-UTR of *H2AX* was subcloned into the pIS0 luciferase plasmid [22], a generous gift from Dr. Liu (Cancer Institute and Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China), to generate pIS0-H2AX-3'UTR. A mutant construct of H2AX-3'UTR, named pIS0-H2AX-3'UTR-mut, which carried a substitution of three nucleotides within the core binding sites of H2AX-3'UTR, was created using mutant PCR primers. Primers used in this study are shown in Table S1. Lipofectamine 2000 (Life Technologies Corporation, Grand Island, NY, USA) was used in transfection of oligonucleotides and plasmids into A549 and H1650 cells. All transfection procedures were performed according to the protocols supplied by manufacturers.

RNA extraction and real-time PCR

RNA was extracted from cells with Trizol reagent (Life Technologies Corporation). RNA extraction was performed following the standard Trizol protocol. Reverse transcription was performed using a FastQuant RT Kit (with gDNase) (TianGen, Beijing, China). MiR-328 was reversely transcribed by a looped primer, which binds to six nucleotides at three positions in the miR-328 molecule. Reverse transcription of *H2AX* mRNA was performed according to the manufacturer's protocol. Real-time PCR was performed using SuperReal PreMix Plus (TianGen) according to the manufacturer's recommendations. To detect the expression of *H2AX* mRNA and mature miR-328, expression levels were normalized to β-actin and U6, respectively. Primers for qRT-PCR are shown in Table S1. All of the reactions were run in triplicate.

Luciferase assay

Cells were cultured in 96-well plates and transiently co-transfected with firefly luciferase reporter gene constructs. MiR-328 mimics were co-transfected with pIS0 vectors (pIS0-H2AX-3'UTR and pIS0-H2AX-3'UTR-mut) using Lipofectamine 2000 (Life Technologies Corporation) into A549 cells and H1650 cell, and a scrambled sequence used as NC. Cells were harvested 48 h after transfection using the Dual Luciferase Reporter Assay System according to the manufacturer's protocol (Promega, Madison, WI, USA). The pRL-TK Renilla was used as an internal control. Three independent

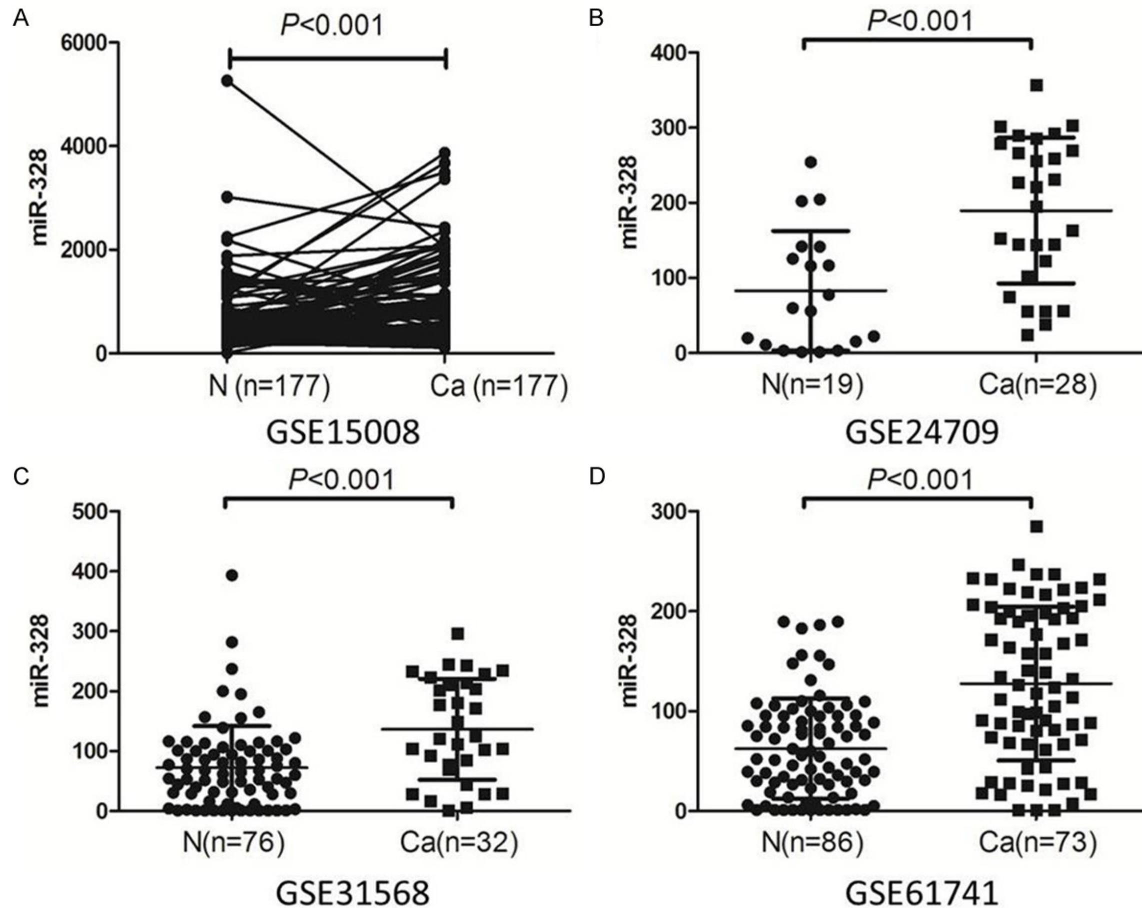

Figure 1. MiR-328 expression levels in lung cancer cells are higher than in normal controls. A. Analysis of the GSE15008 database. MiRNA-328 expression could distinguish lung cancer from normal tissues in 177 pairs of primary lung cancer (Ca) tissue with corresponding adjacent normal lung tissue (N). A minimum of 5 cm was collected from each tumor. B. Analysis of the GSE24709 database. This project analyzed peripheral miRNA blood profiles of patients with lung diseases. The miR-328 expression have been analyzed in N (n = 19 normal controls) and Ca (n = 28 lung cancer patients). C. Analysis of the GSE31568 database. This project analyzed peripheral blood profiles of controls and patients of 14 different diseases, including lung diseases. The miR-328 expression have been analyzed in N (n = 76 normal controls) and Ca (n = 32 lung cancer patients). D. Analysis of the GSE61741 database. This project analyzed peripheral blood profiles of patients of various diseases and controls, including lung diseases. The miR-328 expression have been analyzed in N (n = 86 normal controls) and Ca (n = 73 lung cancer patients).

experiments were performed and all reactions were performed in triplicate.

Flow cytometry

After drug treatment, A549 cells (1×10^6) were washed with 1× binding buffer at room temperature. The cells were incubated with Annexin V for 15 min. The cells were then incubated with propidium iodide (PI) before detection, followed by analysis with flow cytometry method (FCM) (FACS-Calibur; Becton-Dickinson, San Jose, CA, USA). All reagents were obtained from an Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA).

Analysis of cell apoptosis

Cells transfected with negative control (NC) and miR-328 mimics or NC inhibitor and miR-328 inhibitor for 24 h were starved overnight and then treated with etoposide for 48 h. The FCM assay was performed to analyze cellular apoptosis. The Annexin V FITC Apoptosis Detection Kit (BD Biosciences) was used to detect cell apoptosis according to the manufacturer's instructions.

Western blot

Cellular proteins were extracted after treatment of etoposide and western blotting experi-

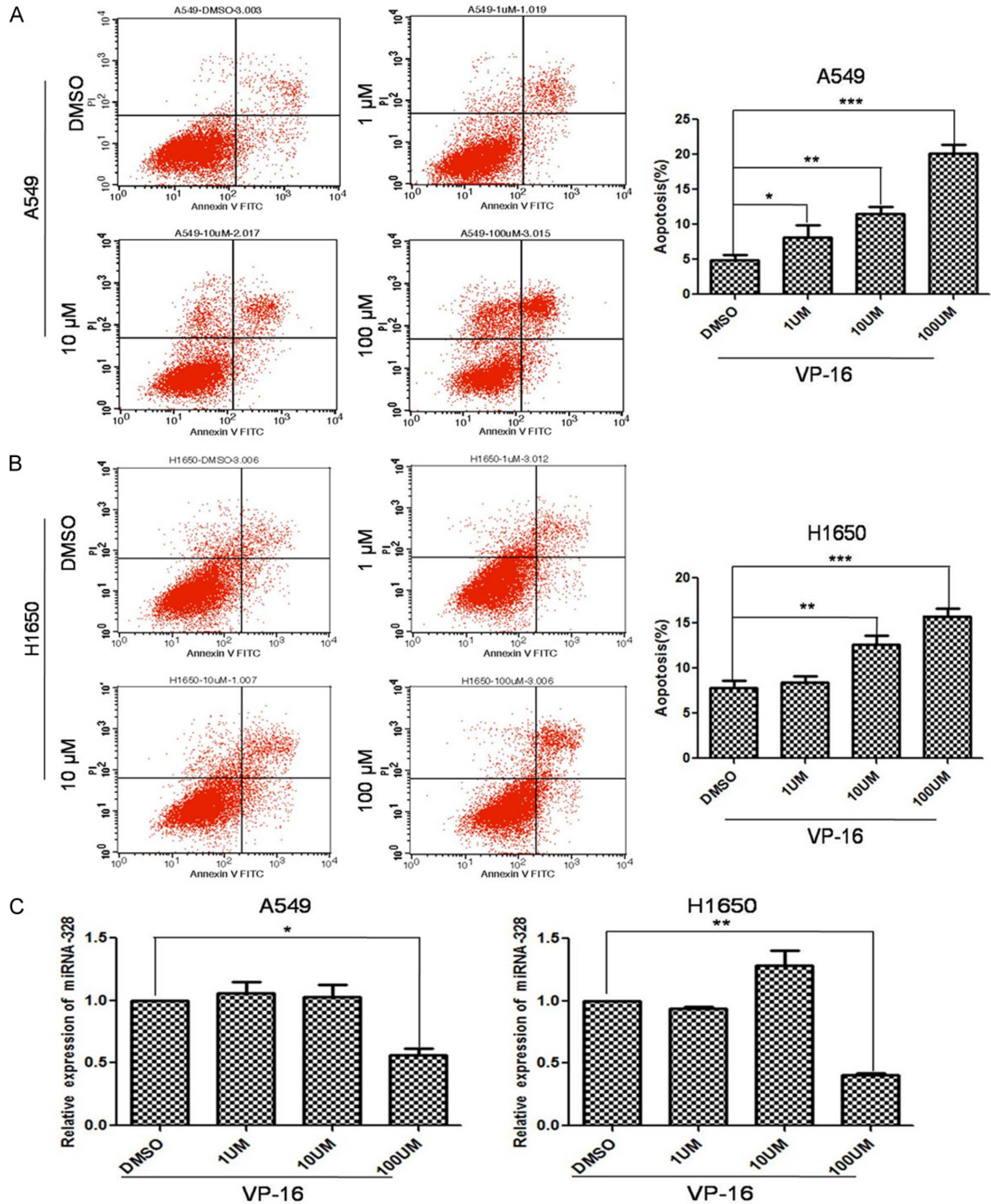

Figure 2. MiR-328 expression is decreased during apoptosis of lung cancer cells. A. The apoptosis of A549 cells was induced by treating with VP16 (1 μM, 10 μM, and 100 μM) for 48 h. The histogram shows the apoptotic cell percentage detected by flow cytometry method (FCM), and error bars denote mean ± SD (right panel). B. Flow cytometry showed apoptosis of H1650 cells after VP16 (1 μM, 10 μM, and 100 μM) treatment. The histogram shows the apoptotic cell percentage detected by FCM, and error bars denote mean ± SD (right panel). DMSO was used as a control. C. The histogram shows the expression of miR-328 in A549 (left panel) and H1650 cells (right panel) 48 h after VP16 treatment (1 μM, 10 μM, and 100 μM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

ments were performed as previously described [23]. Clarified cell lysates were equalized for protein concentration using the BCA protein assay. The protein samples were resolved by

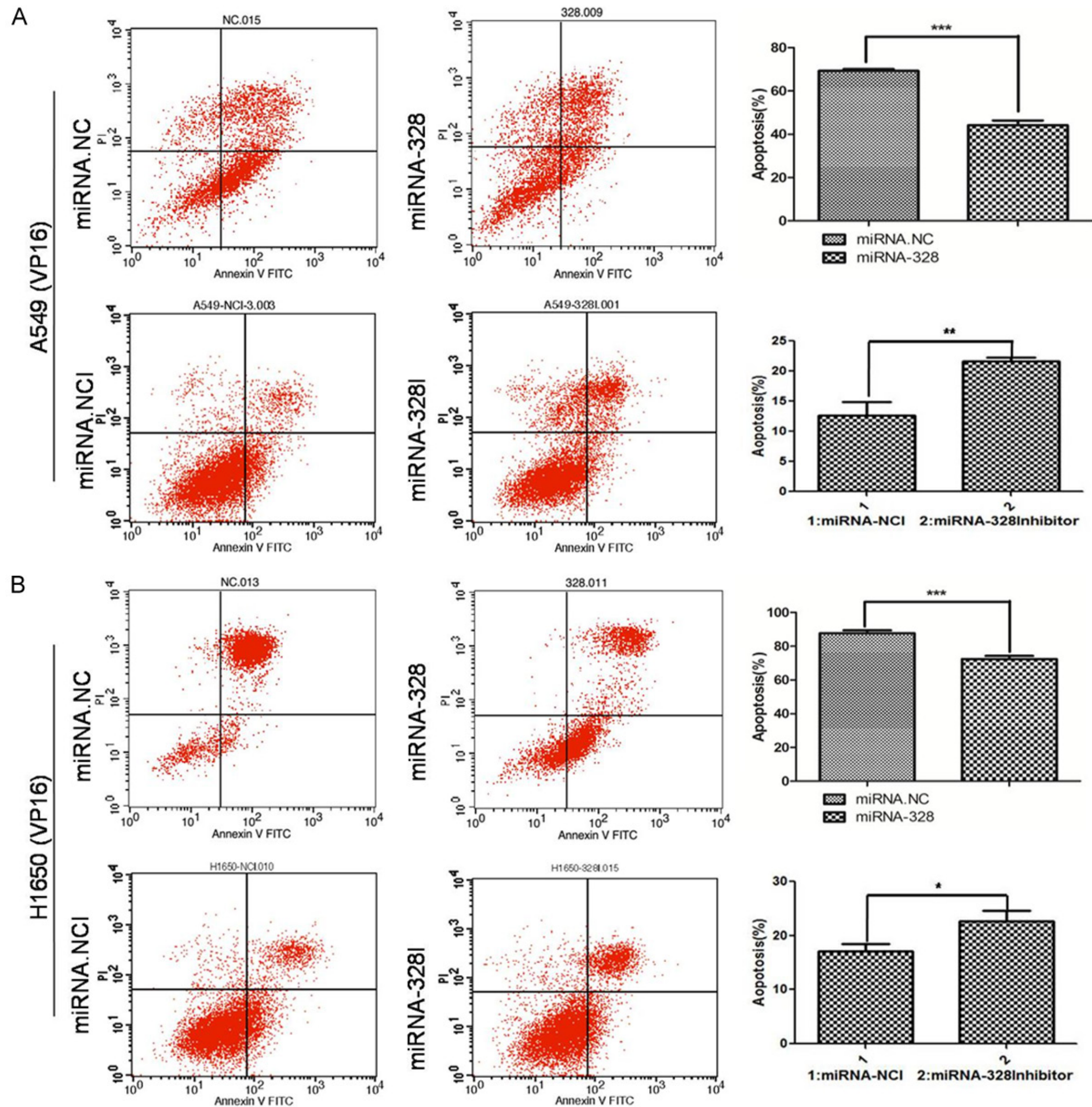

Figure 3. MiR-328 regulates apoptosis of lung cancer cells. A. The apoptosis of A549 cells was induced by treatment with VP16 (100 μM) for 48 h after miRNA-328 mimics (20 nM) and miRNA-328 inhibitors (20 nM) transfection for 24 h. The histogram shows the apoptotic cell percentage detected by flow cytometry method (FCM), and error bars denote mean ± SD (right panel). MiRNA-NC and miRNA-NCI served respectively as the controls for miR-328 and miR-328 inhibitor. B. Flow cytometry was used to detect apoptosis of H1650 cells induced by treating with VP16 (100 μM) after miR-328 mimics (20 nM) and miR-328 inhibitors (20 nM) transfection for 24 h. The histogram shows the apoptotic cell percentage and error bars denote mean ± SD (right panels). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

SDS-PAGE and processed by western blotting. The antibodies against H2AX (Cell Signaling Technology, Beverly, MA, USA), Bcl2 (Abcam, Cambridge, MA, USA), Bim (Abcam), b-actin (Cell Signaling Technology) were respectively used to detect their targeted proteins.

Statistical analysis

Data were presented as mean ± SD from at least three separate experiments, and Student's t-test analysis was performed using SPSS 17.0 software. Statistical significance was set at $P < 0.05$.

Results

MiRNA-328 expression in lung cancers is higher than that in normal controls

To confirm the miR-328 expression *in vivo*, the publically available GSE database from the

Figure 4. Histone H2AX is a target of miR-328. A. MiR-328 targeting site resides at nucleotides 60-66 of the *H2AX* 3'-UTR. Sequences alignment of miR-328 with binding sites on the *H2AX* 3'-UTR. B. MiR-328 is highly conserved in different species. Sequences of the miR-328 binding site within the *H2AX* 3'-UTR of eight species (*Homo sapiens*, *Pan troglodytes*, *Macaca mulatta*, *Rattus norvegicus*, *Canis familiaris*, *Gallus gallus*, *Takifugu rubripes*, and *Danio rerio*). C. Diagram of the luciferase reporter plasmids including plasmid with the full-length *H2AX* 3'-UTR insert (pIS0-H2AX-3'UTR) and plasmid with a mutant *H2AX* 3'-UTR (pIS0-H2AX-3'UTR-mut) which carried a substitution of three nucleotides within the miR-328 binding site. D. Luciferase activity assay demonstrates a direct targeting of the *H2AX* 3'-UTR by miR-328. A549 cells (left panel) and H1650 cells (right panel) were respectively transfected with

miR-328 mimics (20 nM) and pIS0-H2AX-3'UTR/pIS0-H2AX-3'UTR-mut. NC was served as a negative control miRNA. pRL-SV40 Renilla was used for the normalization of transfection efficiency. After 48 h, the luciferase activities were measured. E. Western blotting was used to detect the expression of the H2AX protein after miR-328 (miRNA-328 mimics 20 nM) or miR-328I (miRNA-328 Inhibitor 40 nM) transfection of A549 (left panel) or H1650 (right panel) cells. miRNA-NC was a negative control miRNA and miRNA-NCI was a control for miRNA-328I. F. *H2AX* mRNA in the A549 (left panel) or H1650 (right panel) cell-lines treated as above were measured with real-time RT-PCR. β-actin was used as internal control. G. Overexpression of miR-328 in A549 or H1650 cell lines transfected miRNA-328 mimics (20 nM) or miRNA-328 Inhibitor (40 nM) were measured by real-time RT-PCR. U6 was used as an internal control. **, $P < 0.01$; ***, $P < 0.001$.

NCBI was subjected to analysis for miRNA expression profiles of the lung cancer samples and normal controls. GSE15008, GSE24709, GSE31568 and GSE61741 datasets were downloaded from the GEO database and used to screen the differentially expressed miR-328 in lung cancer. The microarray datasets from the GSE database indicated that miR-328 expression in lung cancer tended to be higher than that in normal controls (**Figure 1**), suggesting that miR-328 could play an important and unknown role in the mechanism of lung cancer development.

MiR-328 expression decreased during apoptosis of lung cancer cells

Etoposide (VP16), a chemotherapy drug derived from a type of plant alkaloid known as a podophyllotoxin, is thought to work by blocking the action of an enzyme in cells called topoisomerase II [24]. Etoposide has been used for the treatment of a wide variety of cancers [25] and can induce apoptosis of many types of cancer cells [26-29]. Here we showed that the number of apoptotic cells was markedly increased after being treated with VP16 both in lung cancer cells A549 and H1650 compared with control DMSO treatment (**Figure 2A** and **2B**). In the meantime, real-time PCR was performed to detect whether the level of miR-328 had changed. The results showed that the miR-328 level was significantly downregulated during apoptosis induced by VP16 at 100 μM (**Figure 2C**). Overall, these data demonstrated that miR-328 may be involved in apoptosis of lung cancer cells.

MiR-328 regulates apoptosis of lung cancer cells

To determine whether miR-328 has a role in apoptotic regulation, we respectively transfected miR-328 mimics and control miRNA, miR-328 inhibitor and control miRNA inhibitor into A549 and H1650 cells, followed by treatment of VP16 for 48 h. FCM was performed to detect the number of apoptotic cells including early and late apoptosis. As is shown, our results indicated that miR-328 could significantly prevent VP16-induced cellular apoptosis, and miR-328 inhibitor dramatically increased apoptosis induced by VP16 (**Figure 3A** and **3B**). Taken together, our results illuminate that miR-328 functions as an apoptosis silencer to regulate lung cancer cells apoptosis, suggesting that miR-328 may act as a tumor promoter.

Histone H2AX is a target of miR-328

To explore the molecular mechanism of miR-328 in the regulation of lung cancer cell apoptosis, we used TargetScan, miRanda and miRbase to identify the potential downstream targets of miR-328. The analysis results showed that *H2AX* is one of the predicted targeting genes and that there is a miR-328 binding site at nucleotides 60-66 of *H2AX* 3'-UTR (**Figure 4A**). Homology search showed that the miR-328 targeting sequence at nucleotides 60-66 of the *H2AX* 3'-UTR is highly conserved in *Homo sapiens*, *Pan troglodytes*, *Macaca mulatta*, *Rattus norvegicus*, *Canis familiaris*, *Gallus gallus*, *Takifugu rubripes*, and *Danio rerio* (**Figure 4B**). To determine whether *H2AX* is regulated by miR-328 through direct binding to its 3'-UTR, we constructed pIS0-H2AX-3'UTR and pIS0-H2AX-3'UTR-mut (3'-UTR was mutated to block binding by miRNA-328) (**Figure 4C**). Co-transfection of the luciferase reporter pIS0-H2AX-3'UTR and miRNA-328 into A549 cells produced nearly 60%-80% decreases in the luciferase activity compared with the negative control (Figure 4D, left panel). This suppressive effect from miRNA-328 was rescued by pIS0-H2AX-3'UTR-mut (**Figure 4D**, left panel). The similar effect was also found in H1650 cells (**Figure 4D**, right panel). Consistent with these results, we found a significance decrease of endogenous H2AX protein and mRNA level in A549 (**Figure 4E** and **4F**, left panels) and H1650 cells (**Figure 4E** and **4F**, right panels) transfect-

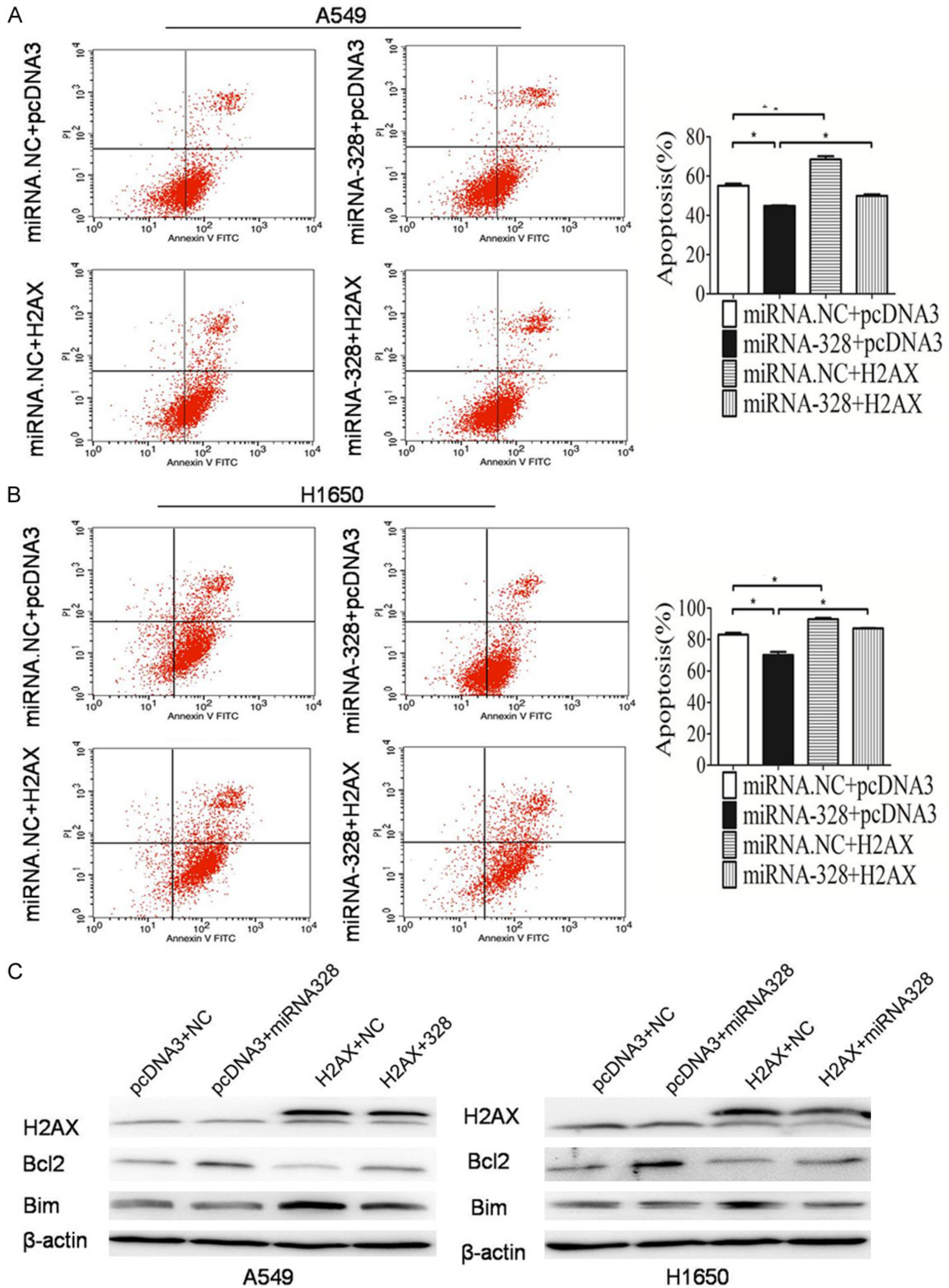

Figure 5. MiR-328 prevents apoptosis through downregulation of H2AX. A. Apoptosis of A549 cell were detected by flow cytometry method (FCM) 48 h after miR-328 mimics or combined with H2AX without 3'-UTR transfection (left panel). Error bars denote mean ± SD (right panel). B. The apoptosis of H1650 cells 48 h after transfection of miR-328 mimics and combined with H2AX without 3'-UTR were detected by FCM (left panel). Error bars denote mean ± SD (right panel). *, $P < 0.05$. C. Western blot analysis was used to detect the expression of BcL-2, Bim, and H2AX in A549 and H1650 cells both treated after miR-328 mimics and combined with lacked-3'-UTR H2AX transfection for 48 h. β-actin was detected as a loading control.

ed with miR-328 mimics (**Figure 4G**, left panel), whereas transfection with miR-328 inhibitor (**Figure 4G**, right panel) induced a significant increase in H2AX expression at mRNA (**Figure 4F**) and protein levels compared with the controls (**Figure 4E**). Taken together, these results suggest that miR-328 down regulates H2AX expression by directly targeting its 3'-UTR.

MiR-328 prevents apoptosis through targeting H2AX

Next, we investigated whether miR-328 functions in cell apoptosis via targeting H2AX. To examine the role of H2AX involvement in apoptosis regulated by miR-328, A549 were co-transfected with the miR-328 mimic and pcDNA3-H2AX, which encoded the full-length coding region of *H2AX* without the 3'-UTR of the *H2AX* mRNA. The results showed that overexpression of miRNA-328 decreased the number of VP16-induced apoptotic cells, whereas co-expression of miR-328 with *H2AX* effectively reversed the change induced by VP-16 (**Figure 5A**). The same phenomenon was also observed in H1650 cells by FCM (**Figure 5B**). There are several proteins including Bcl2 and Bim, which were reported to be related to apoptosis. Here, we found that overexpression of miR-328 increased the level of Bcl2 and decreased the level of Bim. But co-expression of miR-328 with *H2AX* without the 3'-UTR decreased the level of Bcl2 and increased the level of Bim in A549 (**Figure 5C**, left panels) and H1650 (**Figure 5C**, right panels) cells during the treatment. These results suggest that the apoptotic regulation of lung cancer cells by miR-328, at least in part, was dependent on its role in regulating the expression levels of *H2AX*.

Discussion

There have been several previous studies on miRNAs that could have predictive and prognostic relevance in lung cancer. One of the initial studies showed suppression of NSCLC development by the let-7 miRNA family [30]. Expression of several different miRNAs, namely, miR-221, let-7a, miR-137, miR-182, miR-372a, miR-1, miR-30d, miR-486 and miR-499 have previously been associated with survival of lung cancer patients [31, 32]. Despite these reports, there have been no studies looking for miRNA biomarkers to stratify NSCLC patients. Up until now, it has been reported that miR-328 plays a role in the increased migratory potential in NSCLC cells [33].

However, the molecular and epigenetic mechanism of miR-328 involved in apoptosis in lung cancer remains elusive. MiRNA expression profiles reveal important clinical tools for the pathology of lung cancer [20]. Our study demonstrated that miR-328 expression is associated with lung cancer. MiRNA expression profiles indicate that miR-328 expression in lung cancers is more than in normal controls. This is the first time that miR-328 has been found to be involved in the regulation of lung cancer cell apoptosis. Our findings provide important resources to explore further the molecular mechanisms of tumor development and identify lung cancer-related molecules.

It is generally known that H2AX is a variant of the histone H2A family and is believed to participate in DNA repair in the nucleus [34]. We have previously reported that knockout or knock down of H2AX in cells can block apoptosis, suggesting that H2AX plays a crucial role in the regulation of apoptosis [35-37]. An increasing amount of published data has demonstrated that H2AX is also involved in apoptosis of cancer cells [37-39]. H2AX is strongly related to genesis, development and drug sensitivity of tumors, therefore, it is known as a novel tumor suppressor protein [38, 40]. Further exploration has indicated that H2AX is also required for the apoptosis of lung cancer cells [41], as observed in other types of cancer cells [37, 40]. It has been reported that H2AX was involved in apoptosis of lung cancer A549 cells [42].

In this study we used detailed experiments to prove that *H2AX* is a target of miR-328. By interacting directly with the 3'-UTR of *H2AX* mRNA, miR-328 regulates H2AX expression at the post-transcriptional level. Overexpression of miR-328 could prevent VP16-induced apoptosis and significantly decrease endogenous H2AX protein in tumor cells. This finding expands the list of miRNA members involved in regulating cellular apoptosis. In conclusion, we have shown for the first time that the level of miR-328 was significantly downregulated during the apoptosis of lung cancer cells. The screening of GSE datasets from the GEO database showed that the level of miR-328 expression in lung cancer is higher than in normal controls. We identified *H2AX* as a direct and func-

tional target of miR-328. Overall, our data revealed that miR-328 regulates apoptosis through downregulation of H2AX. This finding might lead to unique therapeutic options for treating human cancers.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Chengrong Lu, Aviation Medicine Research Laboratory, Air Force General Hospital, PLA, No. 30 Fucheng Road, Haidian District, Beijing 100142, China. Tel: 86-10-66928553; Fax: 86-10-66928553; E-mail: luchengrong@263.net

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Table S1. Primers used in this study

The primers for plasmid construction	
pIS0-H2AX-3'UTR-F	5'-TTGAGCTCACAAGAAGACGCGAATCAT-3'
pIS0-H2AX-3'UTR-R	5'-GCTCTAGACTGAAAGTCCTGGGCTAAG-3'
pIS0-H2AX-3'UTR-mut-F	5'-CAAAGGCCCTTTTAAGTATCACCACCGCCCTCAT-3'
pIS0-H2AX-3'UTR-mut-R	5'-ATGAGGGCGGTGGTGATACTTAAAAGGGCCTTTG-3'
H2AX-F	5'-TCAGCTCTCCCTCCATCTTC-3'
H2AX-R	5'-TGTGCCTGTTACCAAGTGCT-3'
MiRNA-328 reverse-transcribed primer	5'-CTCAACTGGTGTCGTGGAGTCGG-3'
	5'-CAATTCAGTTGAGACGGAAGG-3'
MiRNA-328-F	5'-ACACTCCAGCTGGG CTGGCCCTCTCTGCCC-3'
MiRNA URP (universal reverse primer)	5'-TGGTGTCGTGGAGTCG-3'
U6-F	5'-CTCGCTTCGGCAGCACA-3'
U6-R	5'-AACGCTTCACGAATTTGCGT-3'