Int J Clin Exp Pathol 2016;9(3):3331-3339
www.ijcep.com /ISSN:1936-2625/IJCEP0021488

Original Article

Overexpression of chromosome kinesin protein KIF4A enhance cisplatin resistance in A549/DDP cells

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Received December 9, 2015; Accepted February 18, 2016; Epub March 1, 2016; Published March 15, 2016

Abstract: Cisplatin-based chemotherapy is the most effective method for treating lung cancer currently; however, cisplatin resistance in lung cancer cells has become a major obstacle to the successful treatment of lung cancer. The specific molecular mechanism of drug-resistant in lung cancer cells remains unclear. So we applied the A549 lung cancer cell line and cisplatin-resistant A549/DDP lung cancer cell line to study the possible molecular mechanism of drug-resistance in lung cancer cells in this study. By RT-PCR and Western blot assays, we found the expression of chromosome kinesin coding gene *KIF4A* was significantly higher in A549/DDP cells than in A549 cells, both at mRNA level and protein level. Then, KIFA4 overexpressed A549 cells and endogenous KIF4A expression interfered A549/DDP cells were correspondingly constructed by cell transfection or RNAi technology, then treated by cisplatin, and changes of cell proliferation and DNA damage in these cells were detected by MTT assay and γH2AX detection. Results showed that after cisplatin treatment, cell proliferation capacity was not significantly decreased and DNA damage was decreased in the KIF4A overexpressed A549 cells, while cell proliferation capacity reduced significantly and DNA damage occurred apparently in KIF4A expression interfered A549/DDP cells. Our research indicates that KIF4A may be involved in the regulatory process of cisplatin resistance in A549 lung cancer cells, and could be used as a new biological target for treatment of cisplatin resistant lung cancer.

Keywords: KIF4A, cisplatin resistance, enhance, A549/DDP cells

Introduction

Lung cancer is one of the most common cancers and has the highest mortality rate in the world, causing about 13 million deaths every year [1-3]. Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers [1-3]. Cisplatin-based chemotherapy is considered as the most effective treatment for lung cancer [4]. In cancer cells, cisplatin can induce DNA damage through intra- and inter-chain cross-linking with DNA, leading to tumor cell death, to achieve the therapeutic purposes [5-7]. However, the cisplatin-resistant lung cancers act as the main obstacle to the successful treatment of lung cancers [8, 9]. Therefore, it is extremely urgent to study the mechanism of drug resistance in lung cancer.

Kinesin superfamily proteins are molecular motor proteins, which can do directional movement along microtubules [10]. KIFs family proteins play important roles in intracellular transport processes, and are very necessary in mitosis and maintaining cell morphology [10]. Chromosome kinesin KIF4A is a multifunctional protein, which is involved in intracellular material transport, cell mitosis regulation, DNA repair, immune cell activation and development of the nervous system [11-16]. KIF4A has been reported to closely relate to the development of a variety of tumors [17, 18], especially in lung cancer tissues. KIF4A mRNA and protein are highly expressed in lung cancer cells, meanwhile inhibiting the expression of KIF4A result in reduced growth and invasive ability in lung cancer cells, indicating that KIF4A plays a key role in the processes of cell growth and proliferation in cancer cells [19, 20]. However, it remains unclear whether KIF4A is associated with drug resistance in lung cancer cells.

Our pilot experiment found that after cisplatin treatment, there was apparent DNA damage in KIF4A low-expressed A549 lung cancer cells, but not in KIF4A high-expressed A549 lung cancer cells. We further explored the role of KIF4A in the processes of drug resistant in lung cancer cells. We found the chemosensitivity to cisplatin was reduced in KIF4A over-expressed A549 cells, while increased in KIF4A expression interfered A549/DDP cells. These results suggest that KIF4A may be use as a new biological target for treatment of cisplatin resistance in lung cancer cells.

Materials and methods

Cell lines, plasmids and reagents

A549 and A549/DDP cell lines kept in our laboratory. They were cultured in RPMI-1640 culture medium supplemented with 10% FBS, and incubated at 37°C in a humidified atmosphere of 5% CO_2. The pEGFP-C1 and pEGFP-KIF4A plasmids were laboratory self-prepared. Lipofectamine 2000 purchased from Invitrogen Inc. (Invitrogen Co., Carlsbad, CA, USA) and cisplatin purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Rabbit anti-KIF4A antibody was laboratory self-prepared. Rabbit anti-GFP antibody, mouse anti-tubulin antibody, mouse anti-γH2AX, and rabbit anti-H2AX antibody were purchased from Sigma. All the secondary antibodies purchased from Jackson ImmunoResearch Inc (West Grove, PA, USA).

Western blot

Cell lysates were prepared with 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP-40, 0.5% dexycholate-Na, and 0.1% SDS plus protease inhibitor cocktail from Sigma Inc. Equal amounts of cell lysis samples were subjected to 12% SDS-PAGE for Western Blot analysis. The primary antibodies, including purified anti-KIF4A antibody (1:1000), anti-GFP antibody, anti-γH2AX antibody, and anti-H2AX antibody, were used for detecting the expression of endogenous KIF4A, over-expressed GFP-KIF4A, endogenous γH2AX, and endogenous H2AX respectively. Tubulin antibody for detecting the expression of endogenous tubulin was used as an internal reference. Secondary antibodies were HRP-conjugated goat anti-rabbit or goat anti-mouse antibodies (1:10000). Chemiluminescence was used for color development.

Immunofluorescence staining

Immunofluorescence staining experiment was performed as follows. Cells were fixed by formalin fixative solution (PBS buffer supplemented with 3% formaldehyde and 2% sucrose) for 15 minutes. After blocking by goat serum (PBS buffer supplemented with 10% goat serum and 0.4% Triton X-100) for 30 minutes, cells were incubated with the primary antibodies, including anti-γH2AX antibody, anti-KIF4A antibody, anti-GFP antibody and anti-tubulin antibody, for 2 hours at room temperature. Then cells were incubated with Texas Red-labeled goat secondary antibody for 1 hour at room temperature in the dark. Finally, cells were incubated with DAPI for DNA staining for 3 minutes at room temperature in the dark.

MTT

A549 and A549-DDP cells were seed in a 96-well tissue culture plates at 5000 cells/well, and cultured in RPMI-1640 medium. MTT analysis was performed after incubation for 0 day, 2 days and 4 days. For MTT assay, 20 μL of MTT (5 mg/mL) was added to each well and incubated for 4 h. After carefully removing the medium, 200 μL DMSO was added to each well. After 30 minutes, the absorbance value of each well was read at a wavelength of 570 nm on a microplate reader (BIO-RAD680, Bio-Rad, Hercules, CA, USA), and cell proliferation curve was drawn according to the absorbance value. All these assays were performed independently in triplicate.

RT-PCR

Total RNA was extracted by Trizol according to the manufacturer's protocol. Then 2 μg RNA was reverse transcribed into cDNA. The cDNA was used as a template for PCR to detect the expression of KIF4A in A549 cells and A549/DDP cells. The expression of β-Actin was set as an internal control. The primer sequences were as follows:

β-Actin: sense primer 5'-TCGTGCGTGACATTAAGGAG-3'; Antisense primer 5'-ATGCCAGGGTACATGGTGGT-3'; KIF4A: sense primer 5'-TGAACTCCCAGTCGTCC-3'; Antisense primer 5'-GCACTGATTACATTTCCC-3'.

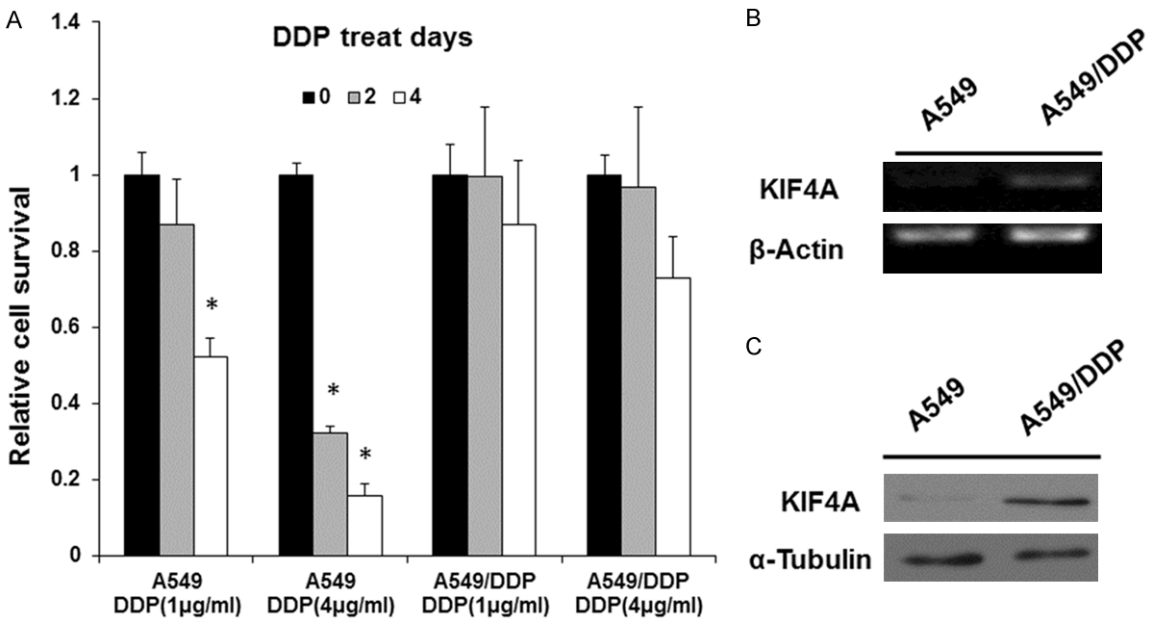

Figure 1. KIF4A is overexpressed in A549/DDP cells. A. MTT analysis was performed to detect cell survival of A549 cells and A549/DDP cells after treatment with DDP. Compared with 0 h, *P < 0.05. B. Western blot was conducted to analyze expression of KIF4A in A549 and A549/DDP cells by using specific anti-KIF4A antibody. α-Tubulin was used as a loading control. C. RT-PCR was performed to detect the mRNA expression level of KIF4A in A549 and A549/DDP cells. Actin was used as a control.

Statistical analysis

All data were analyzed using SPSS 17.0 software package (SPSS, Chicago, IL, USA). All values were expressed as means ± standard deviation (SD) from at least three independent experiments, and statistical significance was defined as $P < 0.05$.

Results

Chromosome kinesin molecule KIF4A is highly expressed in A549/DDP cells

Kinesin molecule KIF4A is closely related to the development of lung cancer [20], but the relationship between KIF4A and drug resistance in lung cancer remains unclear. In order to study the correlation between KIF4A and drug resistance in lung cancer cells, A549 lung cancer cells and cisplatin-resistant A549/DDP lung cancer cells were applied. The chemosensitivity to cisplatin drugs was detected by MTT assay. After treatment by different concentrations of cisplatin, the relative survival rates of A549 cells and A549/DDP cells at 48 h and 96 h were detected. Results showed that the A549 cells were more sensitive to cisplatin in time gradient and concentration gradient with respect to A549/DDP cells (**Figure 1A**).

On this basis, the mRNA and protein levels of *KIF4A* gene in A549 and A549/DDP cells were further examined. The mRNA and protein of KIF4A were analyzed by RT-PCR and Western blot assay, respectively. As shown in **Figure 1B** and **1C**, both the mRNA level and protein level of *KIF4A* were higher in cisplatin-resistant A549/DDP lung cancer cells than in A549 lung cancer cells, and the corresponding ratio was 7.4 and 2.3 times. These results indicate that the chromosomal kinesin molecule KIF4A may be involved in the regulation the chemosensitivity of A549 lung cancer cells to cisplatin.

Chromosome kinesin KIF4A decreases the chemosensitivity of A549/DDP lung cancer cells to cisplatin

The chromosome kinesin molecule KIF4A expressed at a very high level in cisplatin-resistant A549/DDP lung cancer cells suggests that KIF4A may be involved in the regulation of cell resistant to cisplatin in lung cancer cells. In order to study the role of KIF4A in the process of resistant to cisplatin, cell transfection was applied for over expressing exogenous KIF4A in A549 cells, and RNAi technology was also applied for interfering the expression of endogenous KIF4A in A549/DDP cells. Then changes in cell growth and proliferation were studied.

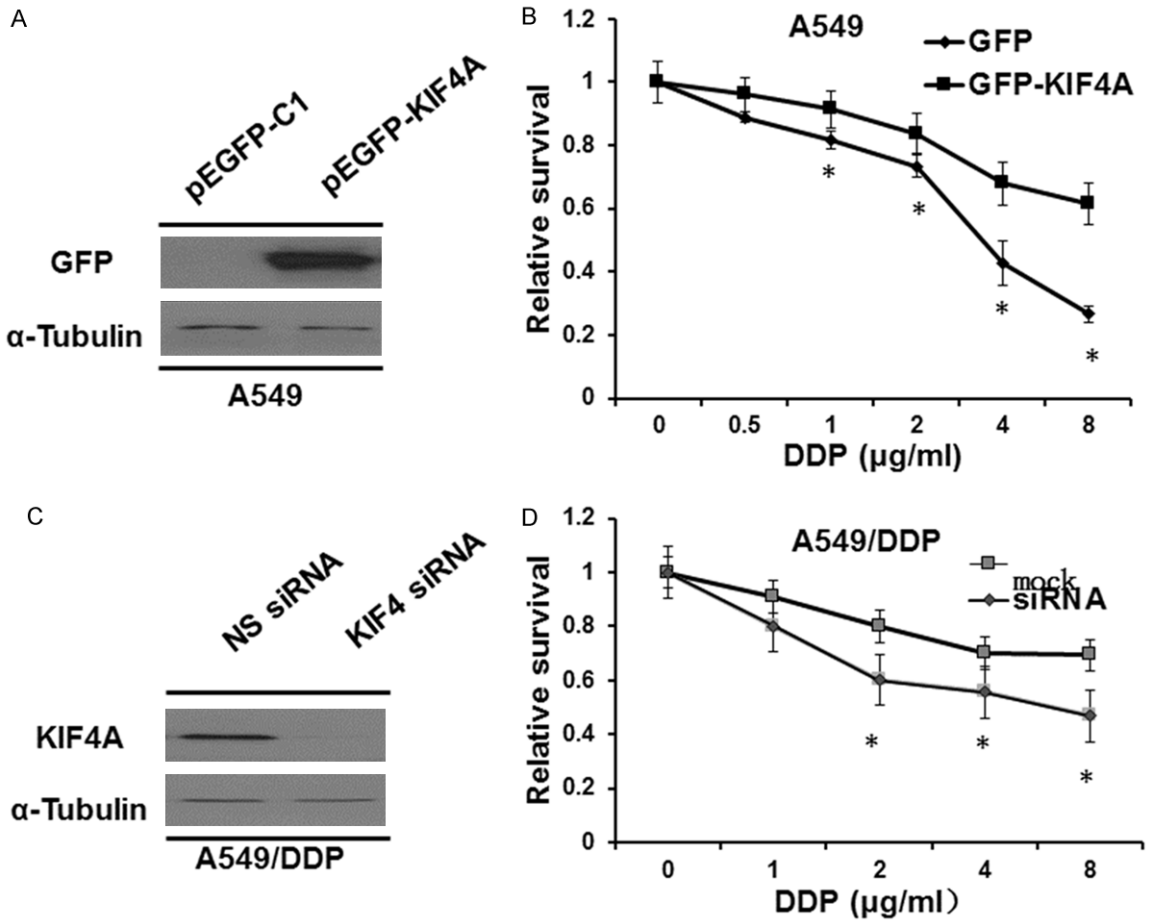

Figure 2. KIF4A overexpression in A549 cells and KIF4A expression interfered in A549/DDP cells reverses the chemosensitivity to cisplatin respectively. A. A549 cells grown in a 6-well plate were transfected with 1000 ng of pEGFP-C1, or pEGFP-KIF4A. Twenty-four hours later, cells were lysed, and the lysates were subjected to SDS-PAGE and immunoblotted with anti-KIF4A and anti-tubulin antibodies. Tubulin was used as a loading control. B. MTT analysis of the chemosensitivity to cisplatin in pEGFP-C1 or pEGFP-KIF4A overexpressed A549 cells. C. A549/DDP cells grown in a 6-well plate were transfected with 1000 ng of NS Si RNA, or KIF4A Si RNA. After 36 hours, cells were lysed, and the lysates were subjected to SDS-PAGE and immunoblotted with anti-KIF4A and anti-tubulin antibodies. Tubulin was used as a loading control. D. MTT analysis of the chemosensitivity to cisplatin in NS Si RNA or KIF4A Si RNA transfected A549/DDP cells.

By MTT assay, we found that after treated with different concentrations of cisplatin for 48 hours, the cell proliferation capacity was significantly decreased with increasing of cisplatin concentration in GFP over-expressed A549 cells. However, at the same treatment concentration of cisplatin, the cell proliferation capacity was far higher in GFP-KIF4A over-expressed A549 cells than in control A549 cells (**Figure 2A** and **2B**). These results suggested that GFP-KIF4A over expression reduced the chemosensitivity of A549 lung cancer cells to cisplatin. Then, we examined the chemosensitivity of *KIF4A* expression interfered A549/DDP cells to cisplatin. Results showed that under the high concentrations of cisplatin treatment, compared with that in the nonsense siRNA transfected A549/DDP cells, the expression of KIF4A was significantly reduced and the cell proliferation capacity was also significantly decreased in KIF4A siRNA transfected A549/DDP cells (**Figure 2C** and **2D**). These results indicate that decreasing the expression of KIF4A in A549/DDP cells would enhance the chemosensitivity of the cells to cisplatin drugs.

Chromosome kinesin KIF4A inhibits the DNA damage of A549/DDP cells induced by cisplatin

The chemotherapy cisplatin achieves the purpose of killing tumor cells by inducing DNA dam-

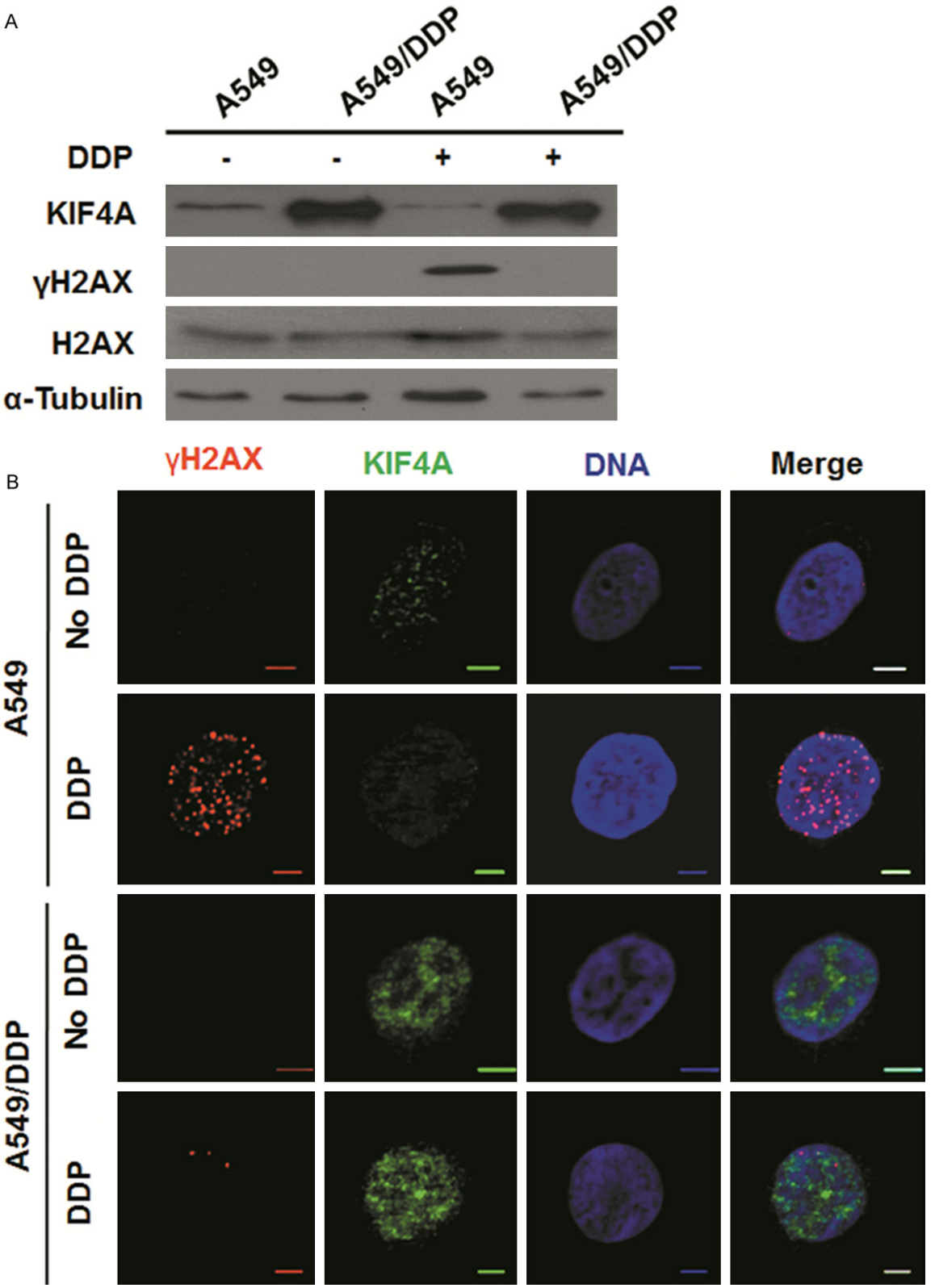

Figure 3. DDP induced DNA Damage in A549 cells not A549/DDP cells. A. Western blotting analysis of γH2AX expression level after DDP treatment in A549 and A549/DDP cells. A549 and A549/DDP cells were treated with DDP for 16 h, and then cell lysates were analyzed by Western blotting with anti-KIF4A, anti-γH2AX or anti-H2AX antibodies. α-Tubulin was used as a loading control. B. Immunostaining analysis of γH2AX expression level after DDP

treatment in A549 and A549/DDP cells. A549 and A549/DDP cells were treated by DDP for 16 h, and then the cells were fixed for immunostaining analysis with mouse anti-γH2AX antibody. DNA was labeled with DAPI. Representative images were shown. The scale bar represents 5 μm.

age through DNA cross-linking [6]. When DNA damage occurs, the histone H2AX in the injury site will be phosphorylated to γH2AX, which clusters at the injury site (foci). Therefore, the expression level and intracellular localization of γH2AX is a sign of DNA damage. As results of Western blot and immunofluorescence staining assays shown (**Figure 3A** and **3B**), after treated with 1 μg/ul cisplatin for 16 hours, the expression level of γH2AX was significantly increased in A549 cells, and γH2AX clustered in the nucleus. These results indicate that there is apparent DNA damage occurred in the cells. Instead, there was no apparent γH2AX expression and nucleus cluster in A549/DDP cells under the condition of 1 μg/ul cisplatin drug treatment (**Figure 3A** and **3B**).

In order to study whether KIF4A was involved in the drug resistant of lung cancer cells, GFP or GFP-KIF4A was overexpressed in A549 cells, then the cells were treated with cisplatin for 16 h, and the changes of γH2AX were detected by Western blot and immunofluorescence staining assay. Western blot results showed after cisplatin treatment, unlike that in the GFP over-expressed A549 cells, intracellular γH2AX expression level was not significantly increased in GFP-KIF4A over-expressed A549 cells (**Figure 4A**). Results of immunofluorescence staining assay also showed that after cisplatin treatment, the amounts of γH2AX staining foci were significantly reduced in KIF4A over-expressed A549 cells relative to the control cells (**Figure 4C**). These results indicated that no obvious DNA damage was observed after treated with cisplatin in GFP-KIF4A over-expressed A549 cells.

Subsequently, intracellular expression levels and localization of γH2AX were compared between nonsense siRNA transfected and KIF4A siRNA transfected A549/DDP cells after cisplatin treatment. Different from that in the nonsense siRNA transfected A549/DDP cells, γH2AX was significantly up-regulated and clustered in the nucleus of KIF4A expression interfered A549/DDP cells (**Figure 4B** and **4D**). This suggested that reduced expression of KIF4A in A549/DDP cells resulted in significant DNA damage after cisplatin treatment. All these results indicate that chromosomal kinesin molecule KIF4A can inhibit the DNA damage of A549 or A549/DDP cells induced by cisplatin.

Discussion

Although there are a large number of studies focused on lung cancer, lung cancer remains as one of the most common cancers with the highest mortality rate in the world [3]. Resistance to antitumor drugs becomes one of the important factors for the death of lung cancer patients [21]. In order to investigate the mechanism of drug resistance in lung cancer, we discussed the key role of chromosome kinesin molecule KIF4A in cisplatin resistance in lung cancer.

It has been reported that kinesin molecule KIF4A were highly expressed in most lung cancer cells and clinical samples [20], while KIF4A was barely detectable in A549 cells. In our study, we also found that KIF4A was barely detectable in A549 cells, while obviously expressed in cisplatin-resistant A549/DDP cells. Meanwhile by MTT assays, we found the chemosensitivity to cisplatin was decreased in KIF4A over-expressed A549 cells while increased in KIF4A expression interfered A549/DDP cells. Moreover, after cisplatin treatment, the amounts of DNA damage were fewer in KIF4A over-expressed A549 cells than controls, while much more in KIF4A expression interfered in A549/DDP cells. Thus, chromosomal kinesin molecule KIF4A reduced the chemosensitivity of A549 and A549/DDP cells to chemotherapeutic cisplatin drugs.

We speculate that KIF4A may be involved in cisplatin resistance in lung cancer cells in two ways. Firstly, as has been reported in the literature, the multidrug resistance ABC (ATP-binding cassette transporter) superfamily proteins played important roles in cancer drug resistance, and two superfamily members transporter protein LRP (lung resistance-related protein) and MVP (major vault protein) were closely related to lung cancer drug resistance [22]. After been expressed within the cytoplasm, the superfamily transporter proteins need to be transported to cell membrane to play their corresponding roles. As a member of kinesin su-

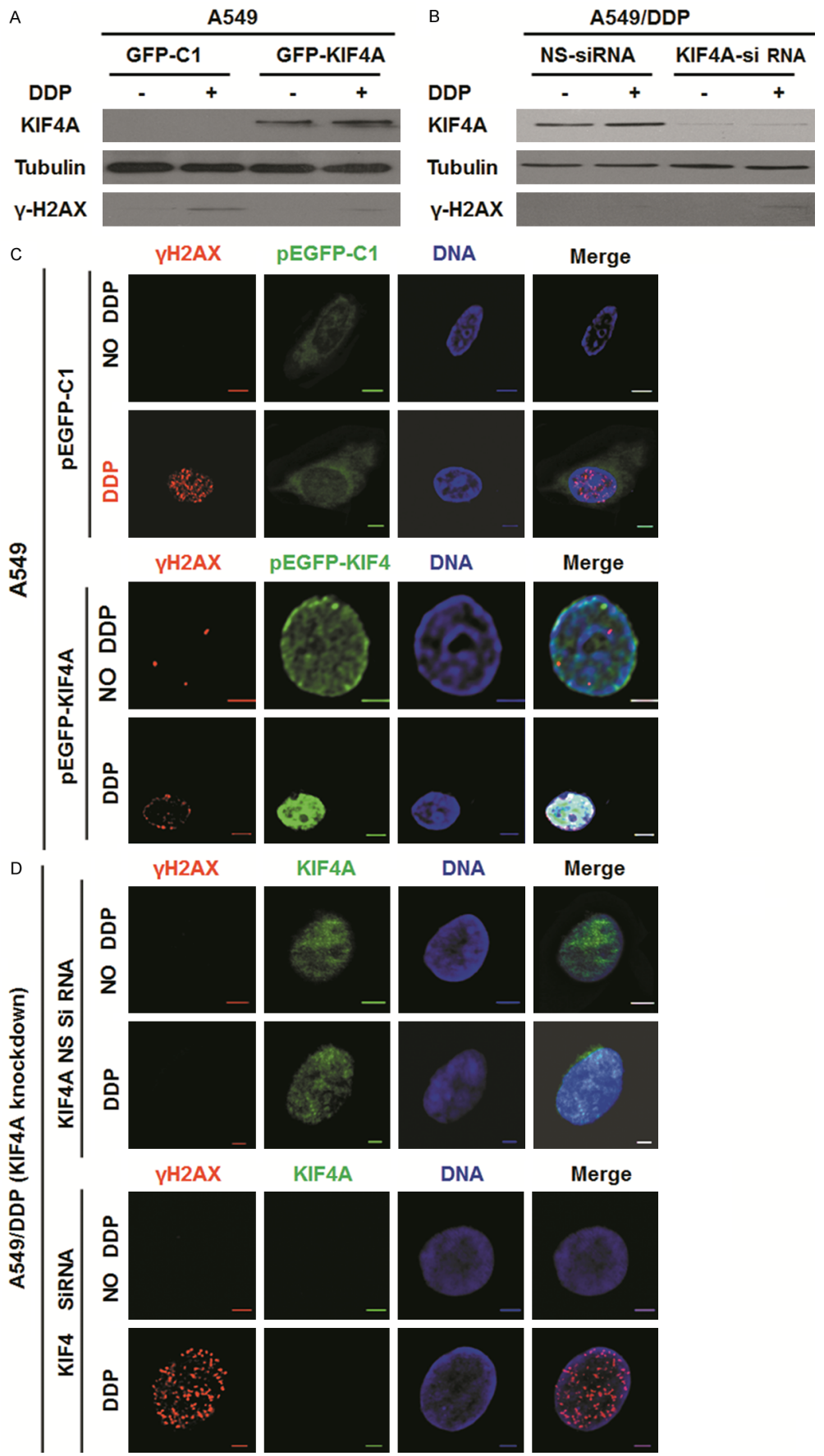
A
A549
GFP-C1
GFP-KIF4A
DDP
KIF4A
Tubulin
γ-H2AX
B
A549/DDP
NS-siRNA
KIF4A-si RNA
C
γH2AX
pEGFP-C1
DNA
Merge
pEGFP-KIF4
A549
NO DDP
DDP
D
KIF4A
A549/DDP (KIF4A knockdown)
KIF4A NS Si RNA
KIF4 SiRNA

Figure 4. The mechanism of KIF4A in inhibiting the induced DNA damage of cisplatin to A549/DDP cells. A. A549 cells were transfected with control pEGFP-C1 or pEGFP-KIF4A for 24 h, and then treated with DDP for 16 h. Cells were lysed, and the lysates were subjected to SDS-PAGE and immunoblotted with anti-KIF4A, anti-γ-H2AX antibody and anti-tubulin antibodies. Tubulin was used as a loading control. B. A549/DDP cells were transfected with control NS Si RNA or KIF4A SiRNA for 36 h, and then treated with DDP for 16 h. cells were lysed, and the lysates were subjected to SDS-PAGE and immunoblotted with anti-KIF4A, anti-γ-H2AX and anti-tubulin antibodies. Tubulin was used as a loading control. C. DDP induced the phosphorylation of H2AX in A549 cells. A549 cells were transfected with control pEGFP-C1 or pEGFP-KIF4A for 24 h, and then treated with DDP for 16 h. The cells were fixed and stained with anti-γH2AX antibody. DNA was labeled with DAPI. D. A549/DDP cells were transfected with control NS Si RNA or KIF4A SiRNA for 36 h, and then treated with DDP for 16 h. The cells were fixed and stained with anti-KIF4A or anti-γH2AX antibodies. DNA was labeled with DAPI. Representative images were shown. The scale represents 5 μm.

perfamily proteins, kinesin molecule KIF4A can transport subcellular structure or biological macromolecule in cells. Therefore, KIF4A may be involved in the process of transporting transporter proteins to cell membrane, and then pumping the chemotherapy cisplatin drugs out of cells. Secondly, previous study shows that KIF4A may also directly participate in the regulation of DNA damage repair pathways [23]. In addition, knockout *KIF4A* gene in mice resulted in increased chemosensitivity of cells to ionizing radiation and DNA damage [23]. Meanwhile KIF4A may affect the processes of DNA damage repair by regulating the expression and function of breast cancer-related genes BRCA2 and homologous repair gene Rad51 [17, 23]. Therefore, high expression KIF4A may antagonize the induced DNA damage caused by cisplatin drugs though promoting the repair of DNA damage in cells.

In conclusion, our study was the first to report the key role of chromosome protein KIF4A in the process of cisplatin resistance in lung cancer. Our findings may provide a new target for the effective prevention and for the treatment of drug-resistant lung cancer.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 81372764, No. 31271485, and No. 31-301138), Program for New Century Excellent Talents in University (NCET-11-1066), Tianjin Research Program of Application Foundation and Advanced Technology (12JC2DJC21400, 14JCYBJC24200), Doctor Foundation of Tianjin Normal University (52XB1104, 52XB1005), and the Natural Science Foundation of Shandong Province (No. ZR2015HM047).

Disclosure of conflict of interest

None.

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