Int J Clin Exp Med 2018;11(7):6618-6625
www.ijcem.com /ISSN:1940-5901/IJCEM0071680

Original Article

MiRNA520 induces NCI-H1299 cell apoptosis by down-regulating MMP-9 expression

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Received August 22, 2017; Accepted May 9, 2018; Epub July 15, 2018; Published July 30, 2018

Abstract: Lung cancer seriously threats patient's life. MiRNA regulates cell growth and apoptosis. This study explored the effects of miRNA520 on lung cancer cell NCI-H1299 growth and apoptosis. The synthetic miRNA520 and control miRNA were transfected into the NCI-H1299 cells, respectively. MTT assay and flow cytometry were applied to evaluate effects of miRNA520 on NCI-H1299 cell growth and apoptosis. Western blot was used to test the matrix metalloproteinase 9 (MMP-9) expression. MMP-9 siRNA and plasmid were transfected into miRNA520 transfected NCI-H1299 cells, respectively. Cell apoptosis was detected. After miRNA520 transfection, NCI-H1299 cell presented growth inhibition, cell apoptosis, and MMP-9 down-regulation. MMP-9 siRNA transfection enhanced miRNA520 induced NCI-H1299 cell apoptosis. MMP-9 over-expression restrained the miRNA520 induced NCI-H1299 cell apoptosis. MiRNA520 transfection inhibited NCI-H1299 cell growth and induced cell apoptosis. In conclusion, miRNA520 induced NCI-H1299 cell apoptosis by down-regulating MMP-9 expression.

Keywords: miRNA520, MMP-9, NCI-H1299, cell apoptosis

Introduction

Lung cancer incidence and five-year mortality rate gradually elevated following modern life rhythm speeding up and life style diversification. Lung cancer seriously threats patient's health and life [1].

In clinical practice, the treatment of lung cancer is often based on specific situation, mainly including chemotherapy, radiotherapy, and surgery [2]. All of abovementioned methods have played critical roles in lung cancer treatment. However, each method has its own shortcomings and deficiencies. More than 80% of lung cancer patients receive chemotherapy and got good effect at different stages. Chemotherapy presents particularly good effect for non-small cell lung cancer, with the response rate is as high as 60%. The main weakness of chemotherapy is a strong toxic and side effect, as it has killing effect on normal cells and tissue. Moreover, it also has a large impact on bone marrow hematopoietic system. Radiotherapy has the best treatment effect and can be limited to the lesion area. Therefore, it is often necessary to be combined with chemotherapy drugs. Surgery also has critical role in lung cancer, which can achieve radical cure. However, surgical treatment has limitations, which is not suitable for older patients with cardiovascular and cerebrovascular disease. Following molecular target therapy application in various kinds of tumors, lung cancer treatment obtains a new choice. However, lung cancer gene target therapy develops slowly because of its complicated mechanism and lack of effective therapeutic target [3]. Therefore, exploring lung cancer therapeutic targets has important significance in both theory research and clinical practice.

MicroRNA is an important type of non-coding small RNA with various biological functions. For example, microRNA can regulate cell proliferation, death, cell cycle, signal transduction, and selective autophagy [4]. Recent study showed that miRNA520 can regulate cell growth and proliferation in esophagus cancer [5-7]. At present, whether miRNA520 has regulatory effect on lung cancer cells still needs further investigation [8]. Thus, this study aims to explore miRNA520 effect on lung cancer NCI-H1299 cells growth and apoptosis and related mechanism.

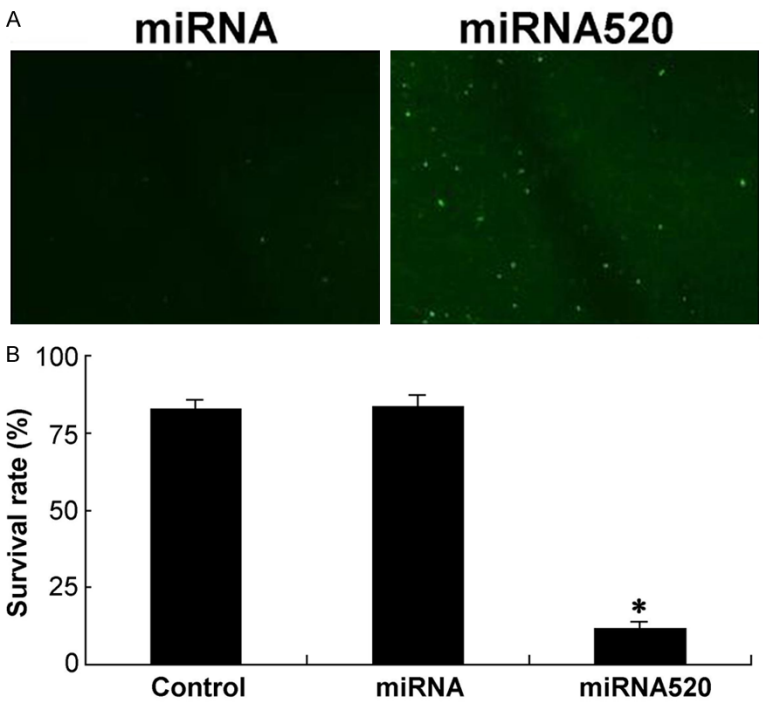

Figure 1. RNA silencing efficacy confirmation (A) and the effect of MiRNA520 transfection on NCI-H1299 cell growth (B). *Statistical significance compared with 1 μg control miRNA transfection.

Matrix metalloproteinase (MMP) is type of protease widely expressed in extracellular matrix [9, 10]. As it often needs Mg^{2+}, Zn^{2+} and Ca^{2+} auxiliary, MMP is called matrix metalloproteinase [11, 12]. At present, a total of 26 subfamilies have been identified in MMP family, which is named MMP1-26 [13-15]. Further research shows that MMP can play a key role in tumor metastasis and invasion through degrading extracellular matrix proteins and destructing tumor tissue barrier [16-18]. It has been reported that MMP-9 plays an important role in lung cancer progress [19-21], however, whether MMP-9 is regulated by microRNA in lung cancer remains to be further discussed.

Therefore, this study intends to investigate the regulatory effect of miRNA520 on NCI-H1299 cells and related mechanism, aiming to provide theoretical basis for lung cancer molecular target therapy.

Materials and methods

Experimental reagents and cell line

MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) was purchased from Santa Cruz. Caspase-3 detection kit and phosphatidylserine flow cytometry kit were got from Beyotime. Lung cancer cell line NCI-H1299 was bought from ATCC. High-glucose DMEM medium and FBS were from Beijing Huamei biological technology co., LTD. Mouse antihuman MMP-9 polyclonal antibody and actin monoclonal antibody were from Sigma. Total RNA extraction kit and reverse transcription kit were from Beijing Dingguo biotechnology co., LTD. MiRNA520 and control miRNA were designed and synthetized by GenePharma. MiRNA520, 5'-GTGCACCGTCGTCGTATCAGTCAGTATGCA-3' and 5'-GGGTCCGAGGTATTCGCACTGGATACGACA-3'. Control miRNA, 5'-ATGGCTGCTGAACCAGTAGA-3' and 5'-CTAGTCTTCGTTTTGAACAG-3'.

Cell culture

NCI-H1299 cells were seeded at 40% and cultured in DMEM medium according to the method reported in reference [9]. The cells were incubated in 5% CO_2 and 37°C for 24 h. Transfection was performed when the cell density reached 80%.

MTT assay

NCI-H1299 cells were tested according to the conventional method [10, 11]. Specially, 1×10^5 NCI-H1299 cells were seeded in 24-well plate at 5% CO_2 and 37°C for 24 h. MTT solution was added at 5 mg/ml for 24 h. Next, 100 μl DMSO was added to stop the reaction for 30 min. The plate was read at 492 nm to obtain absorbance to draw the growth curve [11].

MMP-9 siRNA transfection

MMP-9 siRNA was prepared as follows: MMP-9 siRNA, 5'-CAGATGTAAGTAATAACAGAATGTCAACA-3', and 5'-GATCAGATACAGAATTAAGTAAGTCAACA-3'. Control siRNA 5'-ACTACACCACACCTCA TTTACAGTGGCCA-3', and 5'-TTCACCTGCCACTG ACATCTACAACAGCA-3'. SiRNA was transfected

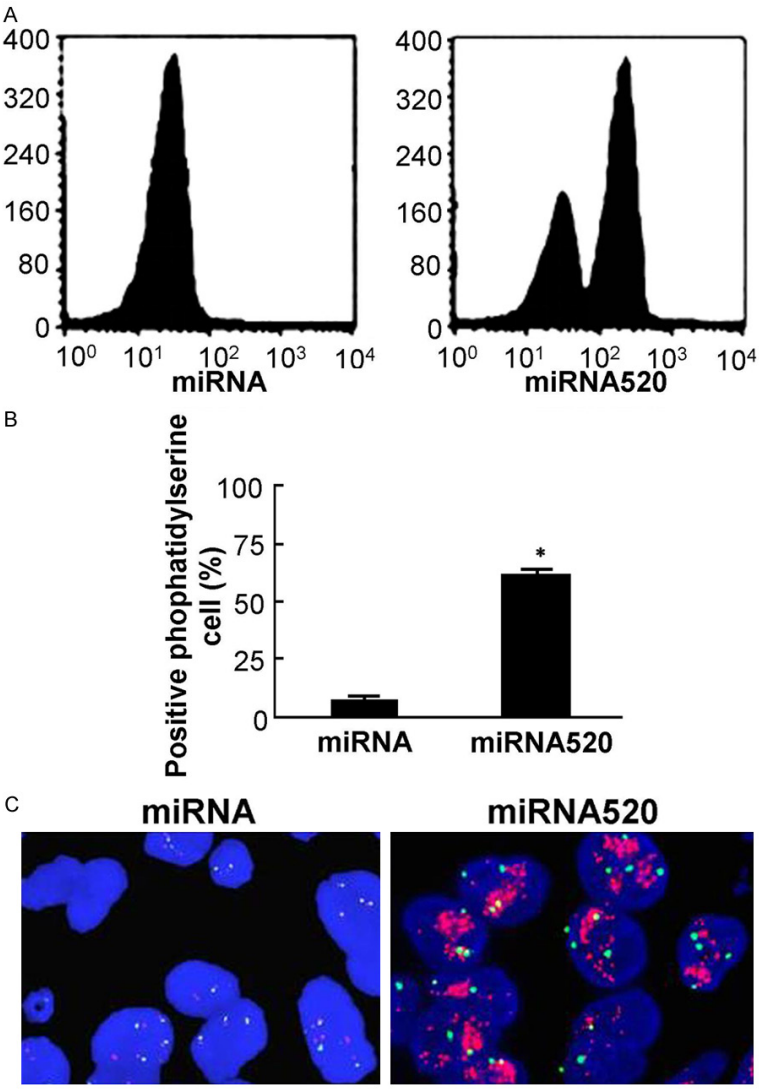

Figure 2. MiRNA520 transfection induced NCI-H1299 cell apoptosis. A and B: Flow cytometry analysis; C: TUNEL assay. *Statistical significance compared with 1 μg control miRNA transfection.

to NCI-H1299 cells according to routine method [12]. Specially, NCI-H1299 cells were cultured in 5% CO_2 and 37°C, and transfection was performed when they reached 80%. MMP-9 siRNA and control siRNA were mixed with lipo2000 for 5 min under room temperature, respectively. Then they were added to the cells, and the following experiments were performed after 24 h incubation.

Flow cytometry

NCI-H1299 cell apoptosis was tested by phosphatidylserine eversion [13]. Specially, NCI-H1299 cells were collected and centrifuged at 1000 r/min for 8 min. Next, the cells were resuspended in PBS solution. Then 250 μl cell suspension was treated with 50 μl FITC-Annexin V buffer and 1 μl reaction liquid at room temperature for 15 min, and tested by flow cytometry.

RT-PCR

Total RNA was extracted and RT-PCR was performed according to the manual [15]. The primers sequences of MMP-9 and actin were as follows: MMP-9, 5'-GGCAAATGGCATTCTGACAT-3' and 5'-AGA TGG TGC ACG ATG CAC AG-3'. Actin, 5'-CTCGACTCTGGTGATGGTGTG-3' and 5'-TCGTACTCCGCCTTGGAGAT-3'. NCI-H1299 cells were collected to extract RNA using Trizol. Then the RNA was reversely transcripted to cDNA for PCR reaction. At last, the product was tested by agarose gel electrophoresis.

Western blot

Western blot was adopted to detect MMP-9 expression in cells [16]. Specially, the cells were digested by enzyme and treated with lysis containing protease inhibitor on ice for 30 min. Total protein was extracted and separated by SDS-PAGE. Next, the protein was transferred to PVDF membrane and blocked by 5% milk/PBS at room temperature for 1 h. Then the membrane was incubated in MMP-9 and actin antibodies at room temperature for 2 h and washed by PBST, respectively. After incubated in secondary at room temperature for 2 h, the membrane was developed for analysis.

TUNEL assay

NCI-H1299 cells were fixed with 4% paraformaldehyde in 0.1 M NaH_2PO_4, pH7.4, and the endogenous peroxidase was inactivated through addition of 3% H_2O_2 followed by incuba-

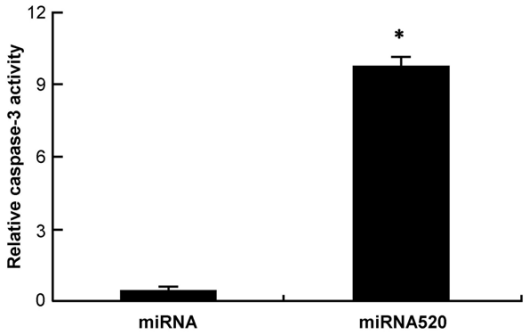

Figure 3. MiRNA520 transfection activated Caspase-3 activity in NCI-H1299 cells. *Statistical significance compared with 1 μg control miRNA transfection.

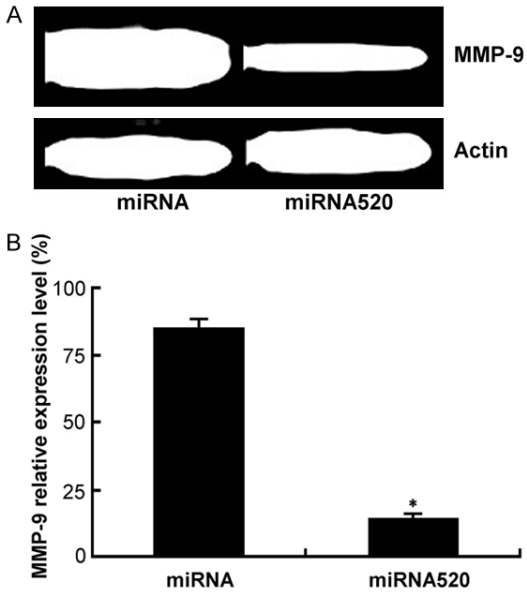

Figure 4. MiRNA520 transfection downregulated MMP-9 mRNA expression. *Statistical significance compared with 1 μg control miRNA transfection.

tion of cells with the solution containing the biotin-dUTP and the terminal deoxynucleotidyl transferase (TdT) for 60 min. After the end-horseradish peroxidase, stained with diaminobenzidine, and counterstained with ethyl green to detect biotin-labeled nuclei. The apoptosis bodies were stained as the brown. The cell nuclei were counted under the fluorescence microscope. The apoptosis index was calculated as the percentage of the apoptotic cells.

Caspase-3 activity detection

Caspase-3 activity was detected according to the instruction provided by Beyotime [17]. Specially, NCI-H1299 cells were collected and resuspended in lysis at room temperature for 30 min. Ac-DEVD-pNA was added to the cells at 5 mM and 37°C for 30 min. At last, the cell suspension was added to 96-well plate and read at 492 nm.

Statistical analysis

All the data analysis was performed on SPSS 14.0 software. The data was presented as mean ± standard deviation. One-way ANOVA was adopted for comparison. $P<0.05$ was considered as statistical significance.

Results

MiRNA520 transfection inhibited NCI-H1299 cell growth

To evaluate the efficacy of RNA silencing, immunofluorescence assay has been performed and showed the silencing efficacy rat was 60% (**Figure 1A**). Compared with control miRNA transfection, NCI-H1299 cells transfected with 1 μg miRNA520 presented significantly lower growth rate ($P=0.0082$, n=3) (**Figure 1B**). As control miRNA transfection showed no statistical difference compared with blank control, we applied control miRNA transfection as control in the following experiments.

MiRNA520 transfection induced NCI-H1299 cell apoptosis

Flow cytometry revealed that the phophatidylserine expression level in NCI-H1299 transfected with 1 μg miRNA520 obviously elevated compared with control ($P=0.012$) (**Figure 2A**, **2B**). Consistently, TUNEL assay also showed increased cell apoptosis after miRNA529 transfection compared with control group (**Figure 2C**).

As shown in **Figure 3**, caspase-3 activity detection demonstrated that caspase-3 activity markedly enhanced in NCI-H1299 transfected with 1 μg miRNA520 obviously elevated compared with control ($P=0.0051$).

MiRNA520 transfection downregulated MMP-9 mRNA and protein expression

As shown in **Figures 4** and **5**, RT-PCR and western blot presented that MMP-9 mRNA and protein levels obviously declined in NCI-H1299

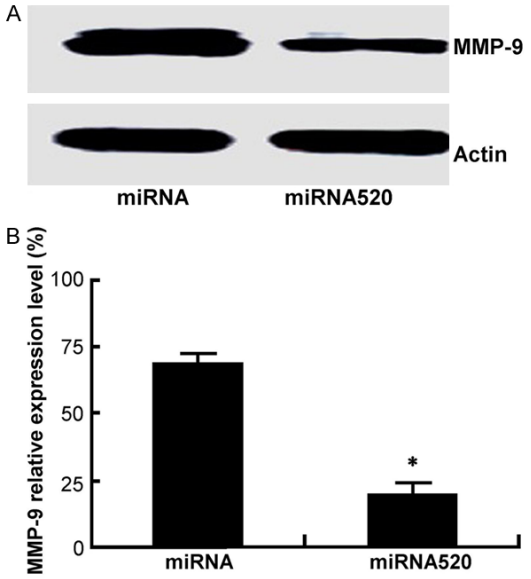

Figure 5. MiRNA520 transfection downregulated MMP-9 protein expression. *Statistical significance compared with 1 μg control miRNA transfection.

transfected with 1 μg miRNA520 obviously elevated compared with control.

MMP-9 knockdown enhanced miRNA520 induced NCI-H1299 cell apoptosis

MMP-9 level was knockdown by siRNA, and the cells were transfected with miRNA520 or control miRNA after 12 h. As shown in **Figure 6**, caspase-3 activity markedly increased in NCI-H1299 cells transfected with 0.1 μg miRNA520 compared with that transfected with control miRNA (*P*=0.0042).

MMP-9 over-expression inhibited miRNA520 induced cell apoptosis

MMP-9 plasmid was applied to up-regulate MMP-9 level. As shown in **Figure 7**, Caspase-3 activity markedly declined in NCI-H1299 cells transfected with 1 μg miRNA520 compared with that transfected with control miRNA (*P*=0.0021).

Discussion

Lung cancer is a serious threat to the patients in clinic [22]. Following industrialization and lifestyle changes, lung cancer show relative high incidence and five-year mortality [23]. Therefore, investigating molecular mechanism of lung cancer cell NCI-H1299 is of great significance for both theory research and clinical practice.

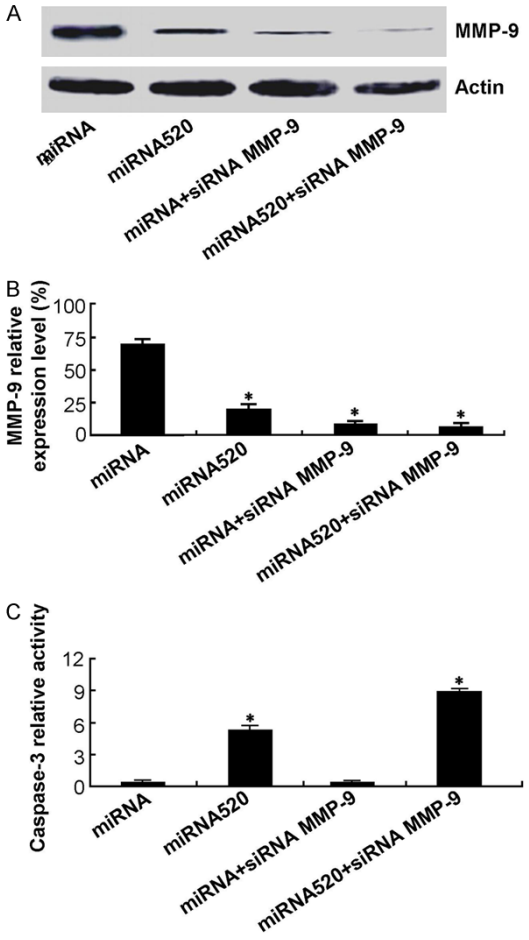

Figure 6. MMP-9 knockdown enhanced miRNA520 induced NCI-H1299 cell apoptosis. *Statistical significance compared with control miRNA transfection.

At present, numerous treatment methods have played a key role in lung cancer, chemotherapy, radiotherapy, and surgery [24]. Unfortunately, the abovementioned therapies have various drawbacks [18], especially toxic and side effect that limits application and curative effect [19]. In recent years, the emergence of molecular target brought dawn for lung cancer patients. Because of complicated process of lung cancer occurrence and development, it is difficult to find common targets for various typies of lung cancer [25]. This study intends to study the potential lung cancer molecular target from molecular and cellular levels.

MiRNA520 can regulate cell growth and survive in other cell types, such as breast cancer cells

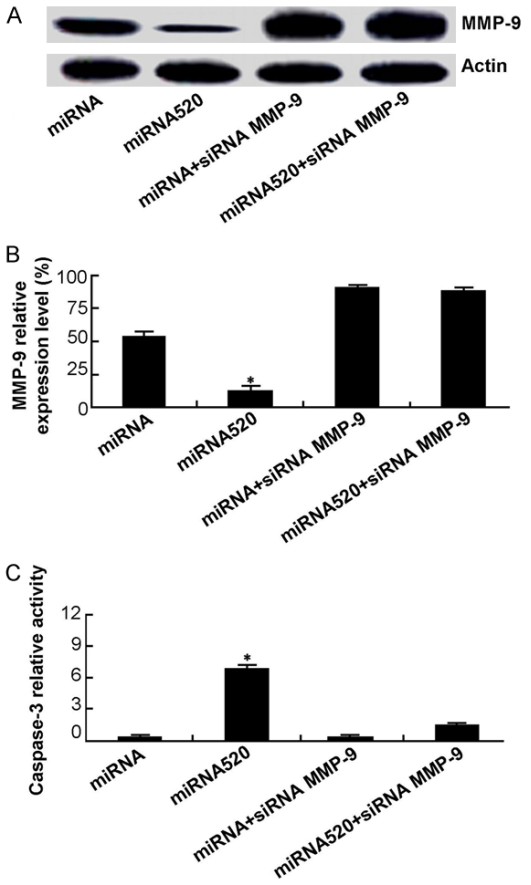

Figure 7. MMP-9 overexpression inhibited miRNA520 induced cell apoptosis. *Statistical significance compared with control miRNA transfection.

and intestinal cancer cells [26]. However, it is still controversy that whether miRNA520 can regulate lung cancer cell growth and apoptosis. This study explored the possible regulatory role of miRNA520 on NCI-H1299 cells. Our results showed that miRNA520 transfection inhibited NCI-H1299 cell growth and promoted apoptosis, indicating that miRNA520 played an important regulatory role on lung cancer cell growth and apoptosis, which was the same as previous reports [27, 28].

This paper has three main results: (1) NCI-H1299 cells presented growth restrain, apoptosis activation, and MMP-9 downregulation after miRNA520 transfection. (2) MMP-9 interference enhanced miRNA520 induced NCI-H1299 cells apoptosis. (3) MMP-9 overexpression reduced miRNA520 induced NCI-H1299 cells apoptosis. These results suggest that miRNA520 transfection restrains NCI-H1299 cells growth and induces apoptosis by downregulating MMP-9.

The latest research showed that MMP-9, a new member of MMP family, played a key role in tumor invasion and metastasis. To our surprise, miRNA520 transfection in NCI-H1299 cells declined MMP-9 level, suggesting that miRNA520 induced NCI-H1299 cells apoptosis may be related to MMP-9. However, how MiRNA520 regulates MMP-9 is still unclear. More importantly, future research should further clarify whether miRNA520 can inhibit tumor metastasis through regulating MMP-9.

This study has four aspects of shortcomings and insufficiencies: (1) This study did not collect different types of cancer and adjacent tissues from patients, neither test MMP-9 protein and mRNA levels in cancer and adjacent tissues by Western blot and RT-PCR. (2) This study did not collect cancer and adjacent tissues from patients receiving chemotherapy, neither test MMP-9 protein and mRNA levels in cancer and adjacent tissues by Western blot and RT-PCR. (3) This study did not establish lung cancer animal model to investigate the possibility of miRNA520 and MMP-9 treatment. (4) This study did not clarify whether miRNA520 can suppress tumor metastasis through regulating MMP-9.

In brief, our results demonstrated that miRNA520 transfection inhibited NCI-H1299 cell growth and induced cell apoptosis. MiRNA520 induced NCI-H1299 cell apoptosis by downregulating MMP-9.

Acknowledgements

These work were supported by The National Natural Science Foundation of China (No. 81400024).

Disclosure of conflict of interest

None.

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