Original Article
miRNA98 induces apoptosis of HCC827 cell via downregulation of Mcl-1

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Abstract: Lung cancer is the malignant tumor of lung that is seriously threatening the life of patients. microRNAs are small non-coding RNA molecules that function in the post-transcriptional regulation of gene expression. This study aimed to investigate the effect of miRNA98 on the proliferation and apoptosis of lung cancer cell HCC827. miRNA98 and negative control miRNA were synthesized and respectively transfected into HCC827 cells. The proliferation and apoptosis were detected by MTT assay and flow cytometry. The intracellular expression of Mcl-1 was determined by Western blot. Furthermore, HCC827 cells were transfected with miRNA98 or negative control miRNA, followed by transfection of siRNA targeting Mcl-1 (siMcl-1) or plasmid expressing Mcl-1. Mcl-1 expression was detected by Western blot and cell apoptosis was measured by flow cytometry. miRNA98 transfection markedly inhibited the proliferation ($P = 0.0023$) and induced the apoptosis of HCC827 cells ($P = 0.014$). The Mcl-1 expression in miRNA98 group was significantly decreased compared with control miRNA group ($P = 0.022$). The expression of Mcl-1 was obviously reduced after siMcl-1 transfection. The apoptosis in siMcl-1+miRNA98 group was significantly increased compared with miRNA98 group ($P = 0.0042$), however there was no significant difference in the apoptosis between miRNA and siMcl-1+miRNA groups. The expression of Mcl-1 was obviously higher after Mcl-1-expressing plasmid transfection. The apoptosis in Mcl-1+miRNA98 group was significantly reduced compared with miRNA98 group ($P = 0.0018$), but no significant difference in the apoptosis was detected between miRNA and Mcl-1+miRNA groups. In conclusion, miRNA98 induced the apoptosis of HCC827 cells probably through down-regulating intracellular Mcl-1 expression.

Keywords: miRNA98, Mcl-1, HCC827, cell apoptosis

Introduction

With the rapid development of modern industries, heavy air pollution has become a serious threatening to the public health, leading to an increased incidence of lung cancer [1, 2]. This disease has become the number one leading cause of death among all cancers of the respiratory system. Although conventional therapies including chemotherapy, radiotherapy and surgery have played important roles in the treatment of lung cancer, they have several drawbacks such as side effects associated with chemotherapy, high requirement for instruments in radiotherapy, the possible limitations of surgery in elderly patients, etc. [3-5]. It is therefore urgent to develop a novel effective treatment for lung cancer. Recently, molecular targeted therapy has become a research hotspot in cancer treatment [6]. Previous stud-

ies have identified several targets for the lung cancer gene therapy such as tyrosine kinase receptor c-Met, epidermal growth factor receptor and anaplastic lymphoma kinase [2, 4, 5]. Nevertheless, targeted therapy is complicated and selected targets are not always effective. The search for appropriate gene targets has become crucial on the treatment of lung cancer [7-9].

microRNAs are small non-coding RNA molecules found in bacteria, plants, animals and humans, that play an important role in the post-transcriptional regulation of gene expression. These molecules are involved in the regulation of a wide range of biological processes such as cell cycle, apoptosis, organ development, tissue regeneration, aging, and even the pathogenesis of several diseases such as neurodegenerative diseases [10-12]. We have previously

found that expression of miRNA98 in lung cancer tissues was significantly lower compared with adjacent non-cancerous tissues, suggesting abnormal miRNA98 expression might be associated with the occurrence and development of lung cancer [13, 14]. However, the regulatory role and molecular mechanism by how miRNA98 is involved in cancers especially lung cancer has not been fully clarified.

Mcl-1 is a conserved anti-apoptotic protein in the Bcl-2 protein family [15, 16]. An increased intracellular level of Mcl-1 indicates an enhanced anti-apoptotic ability of cells [17, 18]. In recent years, it has been shown that Mcl-1 expression in tumor cells is markedly higher compared with normal cells, revealing a possible association between Mcl-1 and growth or apoptosis of cancer cells [19]. Nevertheless, there are currently very few studies investigating the regulatory mechanism of Mcl-1 expression in cancers [20]. In this study, we aimed to investigate the effect of miRNA98 on the proliferation and apoptosis of lung cancer cell HCC827. Furthermore, Mcl-1 expression was manipulated by transfection of siRNA targeting Mcl-1 (siMcl-1) or Mcl-1-expressing plasmid in order to evaluate the relationship between Mcl-1 and miRNA98-induced changes of cell behaviors. The current study will provide new insights into the targeted gene therapy for lung cancer.

Material and methods

Reagents

DMEM medium, trypsin, fetal bovine serum (FBS), penicillin, streptomycin, EDTA, PBS, poly-L-lysine, Hanks balanced salt solution and dimethyl sulfoxide (DMSO) were purchased from Sangon Biotech. (Shanghai, China). Lipofectamine 2000 transfection reagent was purchased from Shanghai Bioleaf Technology Co (Shanghai, China). MTT cell viability assay kit was purchased from Dingguo Changsheng Biotech (Beijing, China). Annexin V-FITC apoptosis assay kit and caspase-3 activity assay kit were purchased from Sangon Biotech. miRNA98 (5-aaacagatcccgtgtattgt-3 and 5-acgaaagaactgtactttta-3) and negative miRNA (5-agggagactctcatttaaat-3 and 5-tgtgacagttgtccacaaca-3), and siMcl-1 (5-agtgtcctgcggcggggagc-3 and 5-tacggatgtggacgcagcca-3) were

synthesized by Sino-American Biotech. (Beijing, China). Protein extraction kit and BCA kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Mcl-1-expressing plasmid was purchased from Sunbiotech. (Beijing, China). Rabbit anti-human Mcl-1 monoclonal antibody and rabbit anti-human actin antibody were purchased from Sino-American Biotech. HRP-labeled goat anti-rabbit IgG was purchased from Santa Cruz Biotech. (Santa Cruz, CA, USA).

Cell line and culture

HCC827 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in DMEM medium containing 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in an incubator with 5% CO_2. Cells in exponential phase were used for subsequent experiments.

Transfection of miRNA98

The miRNA98 and negative control were transfected into HCC827 cells with Lipofectamine 2000 transfection kit as previously described [21]. Briefly, aliquots of HCC87 cells (2 ml) were inoculated into each well on 6-well plates and cultured at 37°C in a 5% CO_2 incubator. siC-CAT1 (1 μg) or negative control (1 μg) were mixed with 5 μl of liposomal transfection reagent. The mixture was used to transfect the Cells after reaching 70% confluence. After 24 h of incubation, cells were collected and subjected to further analyses.

MTT assay

The proliferation of HCC827 cells in all groups was detected by MTT assay as previously described [22]. Briefly, aliquots of 100 μl of T24 and J82 cells were inoculated into each well of 96-well plates. Cells at 70% confluence were transfected with miRNA98 and negative control miRNA. After 24 h, cells were rinsed 3 times with PBS and 5 μl 0.2 M MTT (Ph 7.4) was added to each well. Medium was discarded after 4 h, and 150 μl DMSO was added to each well. The optical density of dissolved MTT crystals was measured by a plate reader DNM-9602G (Baiyuan Medical Instruments, Jinan, China) at 560 nm. Each sample was measured independently three times.
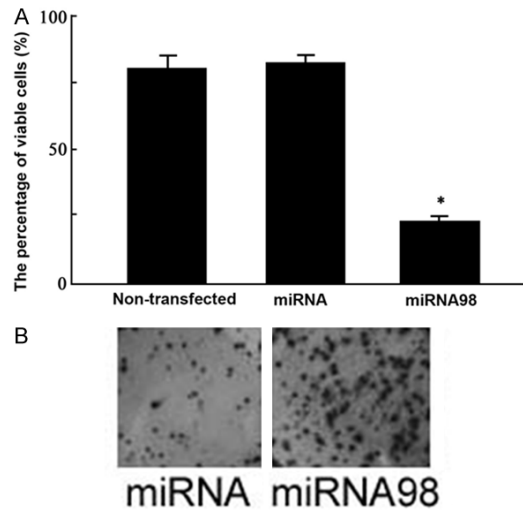
Figure 1. Comparison of cell viability in miRNA, miRNA and non-transfected groups by MTT assay (A) and EdU assay (B). *, P < 0.05 compared with miRNA group.

EdU assay

After transfection, EdU was added into culture medium for cell labelling, and then fixed by formaldehyde, rinsed and stained. After that, cells were washed on coverslips several times. Counterstained cells with Hoechst or DAPI, mount in standard mounting media and image by fluorescence microscopy.

Detection of apoptosis by flow cytometry

The apoptosis of HCC827 cells in all groups was detected by Annexin V-FITC staining as previously described [23]. Briefly, HCC827 cells were transfected with miRNA98 and negative control miRNA. After 24 h, cells were collected and prepared into suspension. Cell suspension (250 µl) was incubated with 50 µl Annexin V-FITC solution and 1 µl binding buffer at room temperature in dark for 20 min. Cell apoptosis was detected by flow cytometry at an emission wavelength of 484 nm and an absorbance wavelength of 625 nm. The experiment was repeated three times and the percentage of cells with phosphatidylserine externalization was calculated.

TUNEL assay

Cells were fixed by 2% formaldehyde in PBS and incubated on ice for 15 min followed by centrifugation and postfix with 70% ice-cold ethanol. Then cells were resuspended in TdT equili-

bration buffer and incubated at 37°C for 10 min with occasional gentle mixing followed by resuspension of the cells in TdT reaction buffer, subsequent wash and addition of TdT staining buffer which was incubated at room temperature for 30 min at dark. After that, cells were centrifuged, washed and resuspended in PBS buffer for fluorescence microscopy analysis.

Detection of apoptosis by caspase-3 activity assay

The apoptosis of HCC827 cells in all groups was also determined by caspase-3 activity assay as described previously [24]. Briefly, HCC827 cells in 96-well plates were transfected with miRNA98 and negative control miRNA. After 24 h, 20 µl lysis buffer was added to each well. Cell lysate was incubated with 5 µl chromogenic substrate at room temperature in dark for 20 min. The optical density of each well was measured by a plate reader at a wavelength of 560 nm.

Western blot

The expression of Mcl-1 in HCC827 cells in all groups was measured by Western blot. Briefly, after transfection, cells at 70% confluence were collected. Total protein was extracted using protein extraction kits and quantified using a BCA kit according to the manufacture's instruction. Equal amounts of total proteins (20 µg) were separated by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes. The membrane was blocked with TBS buffer containing 5% skim milk and 0.1% Tween 20 at room temperature for 2 h, followed by incubation with rabbit anti-human Mcl-1 monoclonal antibody (1:2000 dilution) or rabbit anti-human actin antibody (1:4000 dilution) overnight at pH 7.6 at 4°C with gentle shaking. The peroxidase-labeled goat anti-rabbit secondary antibodies (1:1000) were added and the membranes were incubated at 37°C for 3 h. The membranes were washed twice with TBST for 5 min per each and subjected to ECL detection. The intensity of bands was detected by a Molecular Imager® ChemiDocTM XRS System (Bio-Rad Laboratories). The gray value of bands was analyzed by Image J 6.0 software (Bio-Rad Laboratories). The relative expression of Mcl-1 was calculated as the ratio of the grey value of Mcl-1 to actin.
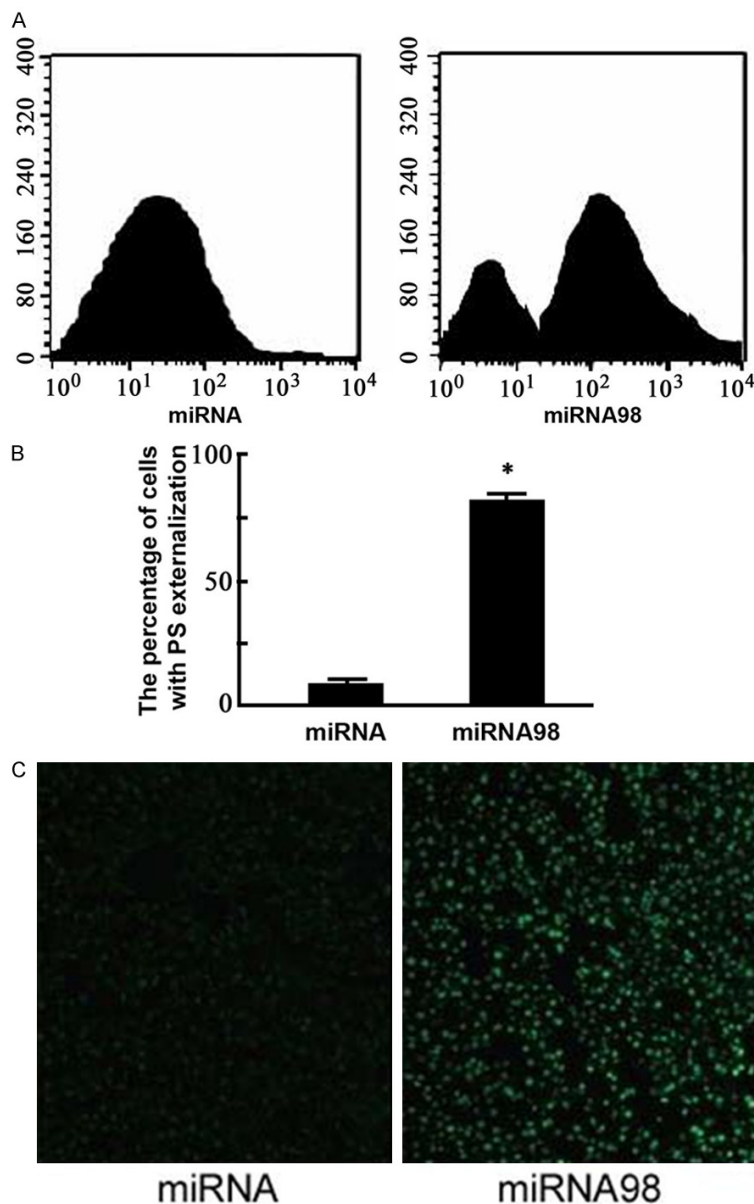
Figure 2. Comparison of HCC827 cell apoptosis in miRNA98 and miRNA by Annexin VFITC staining (A, B) and TUNEL assay (C). *, P < 0.05 compared with miRNA group. PS: Phosphatidylserine.

24 h, cells were collected and subjected to caspase-3 activity assay and Western blot analysis.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Difference between groups was analyzed by two-tailed unpaired student t-test. P < 0.05 is considered statistically significant.

Results

Inhibitory effect of miRNA98 transfection on the proliferation of HCC827 cells

The proliferation of HCC827 cells in all groups were measured by MTT assay and EdU assay. As shown in **Figure 1A**, the percentage of viable cells in miRNA98 was significantly lower compared with miRNA group (P = 0.0023). Consistent with this, EdU assay (**Figure 1B**) also revealed significantly lower cell proliferation (17.1± 2.5%) after transfection of miRNA98 compared with control (100%) (P = 0.001). There was no significant difference in the cell viability between miRNA group and cells without transfection (P > 0.05). Cells in miRNA group were therefore used as a control in subsequent experiments.

miRNA98 transfection induced the apoptosis of HCC827 cells

The apoptosis of HCC827 cells in miRNA98 and miRNA groups were evaluated by Annexin VFITC staining. As shown in **Figure 2A**, **2B**, the percentage of phosphatidylserine externalization in miRNA98 group was significantly higher than that in miRNA group (P = 0.014), suggesting
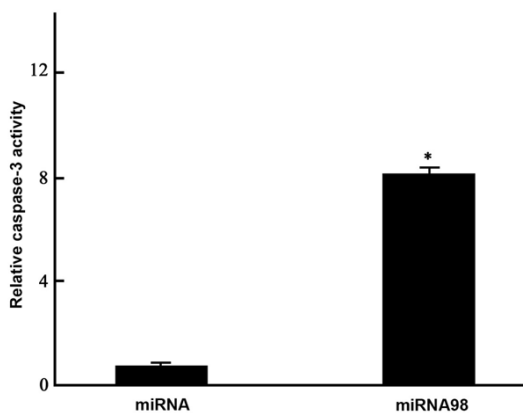
Detection of the effect of Mcl-1 interference or overexpression on HCC827 transfected with miRNA98

In order to investigate the effect of Mcl-1 interference or overexpression on HCC827 cells transfected with miRNA98, cells were transfected with miRNA or control miRNA and incubated at 37°C with 5% CO_2 for 3 h. siMcl-1 or Mcl-1-pressing plasmid (1 µg) were mixed with 5 µl liposomal transfection reagent. The mixture was used to transfect the Cells. After

Figure 3. Comparison of HCC827 cell apoptosis in miRNA98 and miRNA by caspase-3 activity assay. *, P < 0.05 compared with miRNA group.
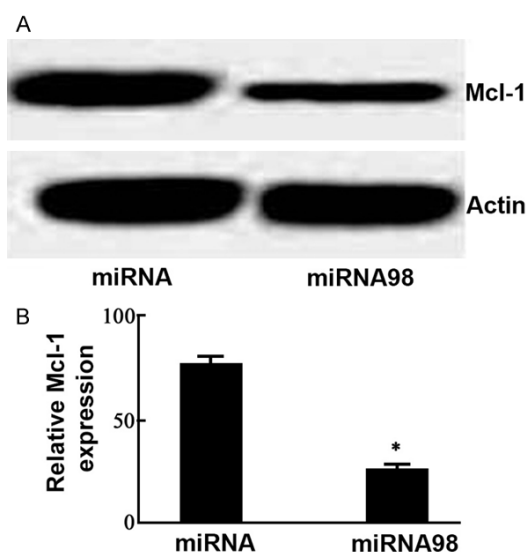

Figure 4. Western blot analyses of Mcl-1 expression in HCC827 cells in miRNA98 and miRNA groups. *, P < 0.05 compared with miRNA group.
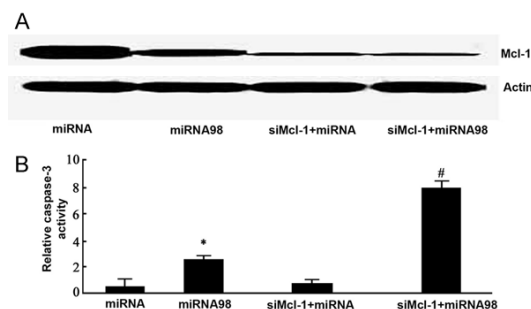

Figure 5. Mcl-1 interference enhanced miRNA98-induced apoptosis of HCC827 cells. A: Western blot analyses of Mcl-1 expression in different groups. B: Caspase-3 activity assay in different groups. *, P < 0.05 compared with miRNA group. #, P < 0.05 compared with miRNA98 group.
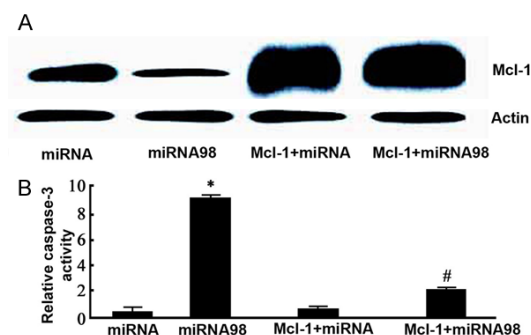

Figure 6. Mcl-1 overexpression inhibited miRNA98-induced apoptosis of HCC827 cells. A: Western blot analyses of Mcl-1 expression in different groups. B: Caspase-3 activity assay in different groups. *, P < 0.05 compared with miRNA group. #, P < 0.05 compared with miRNA98 group.

that miRNA98 markedly induced the apoptosis of HCC827 cells. Consistent with this, TUNEL assay (**Figure 2C**) also showed higher cell apoptosis in cells transfected with miRNA98 (22.5±1.6%) than that in control cells (2.5±0.35%) (P = 0.0035).

miRNA98 transfection induced caspase-3 activation

The apoptosis of HCC827 cells in miRNA98 and miRNA groups was further assessed by caspase-3 activity assay. As shown in **Figure 3**, the relative caspase-3 activity in miRNA98 group was significantly increased compared with mi-

RNA group (P = 0.0031), indicating that miRNA98 transfection substantially induced caspase-3 activation in HCC827 cells.

miRNA98 transfection reduced intracellular Mcl-1 expression

The expression of Mcl-1 protein in HCC827 cells in miRNA98 and miRNA groups was measured by Western blot. As shown in **Figure 4** and Supplementary Figure 1, Mcl-1 expression in miRNA98 group was significantly lower than that in miRNA group (P = 0.022).

Mcl-1 interference enhanced the miRNA98-induced apoptosis of HCC827 cells

In order to detect the effect of Mcl-1 interference on the miRNA98-induced apoptosis of HCC827 cells, miRNA98-transfected cells were

further transfected with siMcl-1. The Mcl-1 expression in siMcl-1+miRNA98 and siMcl-1+miRNA groups was obviously reduced compared with miRNA98 and miRNA groups as demonstrated by Western blot (**Figure 5A** and Supplementary Figure 2), suggesting the effective interference of siMcl-1. The caspase-3 activity in miRNA98+siMcl-1 group was significantly increased compared with miRNA98 group (P = 0.0042), however there was no significant difference in the caspase-3 activity between miRNA and miRNA+siMcl-1 groups (**Figure 5B**).

Mcl-1 overexpression inhibited the miRNA98-induced apoptosis of HCC827 cells

miRNA98-transfected cells were also transfected with Mcl-1-expressing plasmid. As shown in **Figure 6**, the Mcl-1 expression in Mcl-1+miRNA and Mcl-1+miRNA98 groups was obviously increased compared with miRNA and miRNA98 groups (**Figure 6A** and Supplementary Figure 3), confirming the overexpression of Mcl-1 protein. The caspase-3 activity in Mcl-1+miRNA98 group was significantly reduced compared with miRNA98 group (P = 0.0018), and there was no significant difference in the caspase-3 activity between miRNA and Mcl-1+miRNA groups (**Figure 6B**).

Discussion

microRNAs are known to be involved in the regulation of cancer cell activity [3, 6, 7]. In this study, the effect and molecular mechanism of miRNA in lung cancer HCC827 cells were investigated. Results showed that miRNA98 transfection reduced the proliferation and induced the apoptosis of HCC827 cells.

Mcl-1 is an anti-apoptotic protein [3]. In this study, miRNA98 markedly reduced the expression of Mcl-1, which induced the apoptosis of HCC827 cells. Moreover, Mcl-1 interference enhanced the miRNA98-induced apoptosis of HCC827 cells, whereas Mcl-1 overexpression inhibited the miRNA98-induced apoptosis, suggesting that miRNA98 induced apoptosis of these cells through down-regulating the expression of Mcl-1. Nevertheless, the molecular mechanism behind the inhibitory effect of miRNA98 on Mcl-1 expression remains unclear and needs further investigation. miRNA98 might suppress the transcription of Mcl-1 mRNA

by directly binding to the promoter of Mcl-1 gene, which can be verified by a luciferase reporter gene assay. Alternatively, miRNA98 might directly or indirectly induce the degradation of Mcl-1. For instance, miRNA98 might promote the degradation of Mcl-1 by activating the caspase. The speculation can be validated by co-immunoprecipitation technology and in vitro caspase activity assay.

There are several limitations in the current study. First, no clinical data on the Mcl-1 expression in lung cancer tissues and adjacent normal tissues was obtained to support the association between Mcl-1 and lung cancer. Moreover, the molecular mechanism underlying the inhibitory effect of miRNA98 on Mcl-1 expression has not been studied. Lastly, no animal experiments were performed to evaluate the feasibility and outcome of miRNA98-targeted gene therapy for lung cancer in vivo.

In summary, miRNA98 inhibited the proliferation and induced the apoptosis of HCC827 cells probably through down-regulating intracellular Mcl-1 expression, indicating that Mcl-1 might be a potential target for lung cancer treatment. This study will provide a theoretical basis for molecule targeted therapy on lung cancer.

Acknowledgements

Disclosure of conflict of interest

None.

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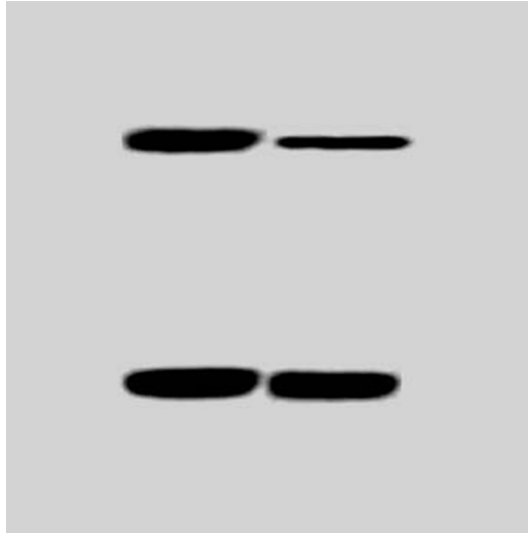
Supplementary Figure 1. Western blot analyses of Mcl-1 expression in HCC827 cells in miRNA98 and miRNA groups.
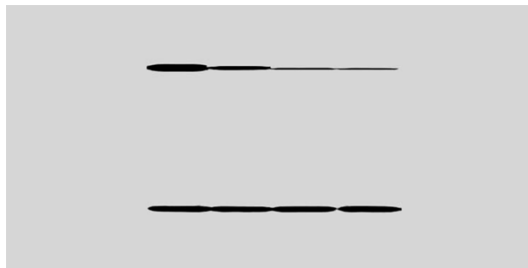
Supplementary Figure 2. Western blot analyses of Mcl-1 expression in different groups.

Supplementary Figure 3. Western blot analyses of Mcl-1 expression in different groups.