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Original Article

Knockdown of Myeloid Cell Leukemia-1 by MicroRNA-101 Increases Sensitivity of A549 Lung Cancer Cells to Etoposide

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Abstract

Background: Studies have shown that myeloid cell leukemia-1 (Mcl-1) is the target gene for microRNA -101 (miRNA-101), and decreased levels of miRNA-101 are associated with elevated levels of Mcl-1 and lung cancer survival. The objective of the present study was to investigate the effect of miRNA-101 on the sensitivity of A549 lung cancer cells to etoposide.

Methods: The study was conducted during 2018 and 2019 at Arak University of Medical Sciences, Arak, Iran. The effect of miRNA-101 on Mcl-1 expression was assessed using reverse transcription-quantitative polymerase chain reaction 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and trypan blue exclusion assays were performed to determine the effect of treatments on cell survival and proliferation, respectively. The interaction between miRNA-101 and etoposide was evaluated using the combination index analysis of Chou-Talalay. Apoptosis was quantified using ELISA cell death assay. ANOVA and Bonferroni's tests were used to determine statistical differences between the groups ($P<0.05$). GraphPad Prism software (version 6.01) was used for data analysis.

Results: The results showed that miRNA-101 clearly inhibited the expression of Mcl-1 and reduced the growth of A549 cells, relative to blank control and negative control miRNA ($P<0.05$). Transfection of miRNA-101 synergistically enhanced the sensitivity of the A549 cells to etoposide. Apoptosis assay data also showed that miRNA-101 triggered apoptosis and augmented the etoposide-mediated apoptosis.

Conclusion: Up-regulation of miRNA-101 inhibited cell survival and proliferation, and sensitized A549 cells to etoposide by suppressing Mcl-1 expression. miRNA-101 replacement therapy can be considered as an effective therapeutic strategy in non-small cell lung cancer.

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Keywords ● Apoptosis ● Etoposide ● Lung neoplasms ● Myeloid cell leukemia sequence 1 protein ● MicroRNA-101

What's Known

- Over-expression of myeloid cell leukemia-1 is associated with tumor progression, poor prognosis, and unfavorable clinical outcomes in non-small cell lung cancers.
- Down-regulation of microRNA-101 is associated with the up-regulation of myeloid cell leukemia-1, tumor progression, and poorer prognosis.

What's New

- Up-regulation of microRNA-101 inhibits cell proliferation and survival and induces apoptosis in non-small cell lung cancer cells through the regulation of myeloid cell leukemia-1 expression.

Introduction

Lung cancer or lung carcinoma is known as one of the most common deadly cancers in the world. Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) are the two main subtypes of lung cancer.[1-3] Despite many efforts to treat

lung cancer, the efficacy of therapies is limited due to the development of drug resistance.[4] To improve the efficacy of cancer therapy, it is essential to gain a detailed understanding of the cellular and molecular mechanisms underlying drug resistance in NSCLC.

Apoptosis (programmed cell death) is an important cellular process essential for normal cell development and tissue homeostasis. Deregulation of the apoptosis machinery is associated with the development and progression of various cancers, and subsequent poor response to chemotherapy.[5, 6] B-cell lymphoma-2 (Bcl-2) family proteins are the main regulators of apoptosis and include anti-apoptotic (*Mcl-1*, Bcl-xL, Bcl-w, Bfl-1/A1) and pro-apoptotic (Bik, Bim, Bad, Bid, NOXA, PUMA) members.[7] The relative expression ratio of these antagonistic proteins determines cell fate towards survival or death.[8] Increased expression of anti-apoptotic proteins such as myeloid cell leukemia-1 or myeloid cell factor-1 (*Mcl-1*) and Bcl-2 commonly occurs in various malignancies including NSCLC and is associated with tumor development and progression, poor prognosis, and unfavorable clinical outcome.[9, 10] The above findings led to the development of new therapeutic approaches targeting these proteins, such as small interfering RNAs (siRNAs), antisense oligonucleotides (ASOs), and small molecule mimics.[5, 6, 10]

MicroRNAs (miRNAs) are the most abundant class of small and non-coding RNAs. They bind to the three prime untranslated regions (3'-UTR) of target transcripts and regulate gene expression either via inducing translational inhibition or mRNA degradation.[1, 11] MiRNAs participate in a variety of cellular processes such as cell proliferation, differentiation, and apoptosis. Evidence suggests that aberrations in particular miRNAs expression are involved in the initiation and progression of cancer.[12, 13] MiRNAs may act as oncomirs (oncogenic miRNAs) or tumor-suppressive miRNAs.[14] For example, the miRNA-145 expression is reduced in lung cancer cells causing elevated cancer myelocytomatosis (c-Myc), nucleoside X-type motif-1 (NUDT1), octamer-binding transcription factor 4 (OCT4), and epidermal growth factor receptor (EGFR) expression as well as increased tumor cell proliferation, migration, and metastasis. In contrast, miRNA-221 and miRNA-222 are over-expressed in different types of cancers, including NSCLC leading to elevated phosphatase and tensin homolog (PTEN) and Bcl-2 expression, increased cell proliferation, and resistance to TNF-related apoptosis-inducing ligand (TRAIL).[1, 2, 15] Today, miRNA expression profiles have been considered as useful biomarkers for prognostic and evaluation of chemoresistance in patients with lung cancer. MiRNA-101 is a tumor-suppressive miRNA that is significantly down-regulated in various types of cancer cells (bladder, breast, liver, prostate, and lung cancer) and displays an inhibitory effect on cell growth, invasion, and migration.[16, 17] Moreover, it was demonstrated that down-regulation of miRNA-101 is associated with over-expression of *Mcl-1*, tumor progression, and poorer prognosis.[16-19] However, little is known about the cellular role of miRNA-101 on chemoresistance of NSCLC.

Etoposide is one of the first-line chemotherapy drugs for NSCLC. Etoposide inhibits topoisomerase II (an enzyme involved in DNA replication, transcription, and recombination) resulting in the creation of double-strand DNA breaks, the formation of chromosomal aberrations, and subsequent cell death.[20, 21] In the present study, we assessed the effect of combined miRNA-101 and etoposide treatment on A549 NSCLC cells. We hypothesized that miRNA-101 would inhibit cell proliferation and sensitize lung cancer cells to etoposide via suppression of *Mcl-1*.

Materials and Methods

The present study was conducted during 2018 and 2019 at Arak University of Medical Sciences, Arak, Iran. All experimental procedures were evaluated and approved by the Ethics Committee of Arak University of Medical Sciences (code: IR.ARAKMU.REC.1394.169).

Cell Culture

The A549 cell line was purchased from the Pasteur Institute (Tehran, Iran) and propagated in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO, USA), streptomycin (100 mg/ml; Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 U/ml; Sigma-Aldrich, St. Louis, MO, USA) and two mM glutamine (Sigma-Aldrich, St. Louis, MO, USA). The cell cultures were maintained at 37 °C in an atmosphere containing 5% CO_2.

MiRNA Transfection

The negative control (NC) miRNA (scrambled) and miRNA-101 mimics were purchased from Dharmacon (Lafayette, CO, USA) and transfected into the cells at a final concentration of 50 nM. The strand sequences of NC miRNA and miRNA-101 mimics used in the transfection experiments were 5'-UUCUUCGAACGUGUCACGUTT-3'

and 5'- UACAGUACUGUGAUAACUGAA -3', respectively. Transfection was performed using Lipofectamine™2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, miRNA and lipofectamine (4 µl/ml of transfection medium; Invitrogen, Carlsbad, CA, USA) were diluted separately in Opti-MEM I Medium (Invitrogen, Carlsbad, CA, USA) and incubated for five min. Then the diluted miRNA was mixed with the diluted transfection reagent and incubated for 20 min. Next, the mixtures were added to each well-containing medium and cells. After six hours incubation of the cells in a CO_2 incubator, the complete growth medium was added. After 24 and 48 hours transfection, suppression of *Mcl-1* was monitored using a quantitative real-time polymerase chain reaction (RT-qPCR).

RT-qPCR

Total RNA was extracted from the lung cancer cell line using YTzol reagent (Yekta Tajhiz, Tehran, Iran) as described by the manufacturer. Reverse transcription of 1 µg of purified RNA was performed using MMLV reverse transcriptase and oligo-dT primer (Promega, Madison, WI, USA). The primers for Beta-actin were 5'-TCCCTGGAGAAGAGCTACG-3' and 5'-GTAGTTTCGTGGATGCCACA-3', and for *Mcl-1* they were 5'-TAAGGACAAAACGGGACTGG-3' and 5'-ACCAGCTCCTACTCCAGCAA-3'. The reaction system of polymerase chain reaction (PCR) included the following components: 500 nM each of the forward and reverse primers, one µLof reverse transcription product, six µLof nuclease-free distilled water, and 12 µL of SYBR green reagent (Takara Bio, Otsu, Shiga, Japan). The PCR reactions were performed in the LightCycler® 96 System (Roche Diagnostics GmbH, Mannheim, Germany) under the following conditions: at 95 °C for five min, 40 cycles at 95 °C for five seconds, and at 59 °C for 30 seconds. The relative transcript abundance (the amount of *Mcl-1* normalized to the Beta-actin as an endogenous control gene) was calculated using the comparative CT method ($2^{-\Delta\Delta Ct}$).[22]

Cell Proliferation Assay

Cells were plated at a density of 1×10^5 cells/well in a 6-well culture plate with two mL of the medium. The cells were treated with miRNA-101, as described above, after which the cell viability was measured using trypan blue staining at various time points for five days. Next, an equal volume of 0.4% trypan blue stain (Sigma- Aldrich, St. Louis, MO, USA) was added into each cell suspension, and viable cells were measured using a hemocytometer under an inverted microscope (Nikon Instrument Inc., Melville, NY, USA). The results were expressed as the percentage of viable cells in each group relative to the corresponding blank control group.

Cytotoxicity Assay

The effect of miRNA-101 on the sensitivity of A549 cell line to etoposide (Sigma- Aldrich, USA) was assessed using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay. The experiment was subdivided into eight groups, namely miRNA-101, NC miRNA, etoposide, miRNA-101 and etoposide, NC miRNA and etoposide, miRNA blank control, etoposide blank control, and combination blank control. Treatment with only lipofectamine, solvent of miRNA, and 1% dimethyl sulfoxide (DMSO), solvent of etoposide, were considered as miRNA and etoposide blank controls, respectively. In brief, cells were seeded at a density of 5×10^3 cells/well in a 96-well plate and transfected with 50 nM of miRNAs. After six hours of incubation, the cells were treated with etoposide (Sigma- Aldrich, St. Louis, MO, USA) at a concentration range of 0 to 16 µM. After 24 and 48 hours, 10 µl MTT solution (Sigma Aldrich, St. Louis, MO, USA) (5 mg/mL) was added to each well. After four hours of incubation at 37 °C, the supernatants were discarded and 150 µl of DMSO was added to the cells. The absorbance (A) values were read at a wavelength of 490 nm with a microplate spectrophotometer (Awareness Technology, Palm City, FL, USA). The survival rate (SR) was calculated according to the formula:[23]

$SR\ (\%)=(A_{Test}/A_{Control})\times100\%$.

The half-maximal inhibitory concentration (IC_{50} value), defined as the drug concentration that produced 50% cytotoxicity, was calculated by plotting the relative cell survival versus the etoposide concentration and curve-fit to a sigmoidal dose-response (variable slope) model using GraphPad Prism software (version 6.01, GraphPad Software Inc., San Diego, CA, USA).

The Effects of Combined Drug

The Chou-Talalay drug combination index (CI) method was used to determine the synergy, additivity, or antagonism of the miRNA-101/etoposide combinations.[23] The mean values of the survival rates obtained from the MTT assay were used to generate a set of Fraction affected (Fa) values (range 0-1, where Fa=0 is 100% cell survival and Fa=1 is 0% cell survival) and then analyzed with the CompuSyn software (Version: 1.0; ComboSyn Inc., Paramus, NJ, USA). The synergy, additivity, or antagonism were indicated with CI of <1, =1, or >1.

ELISA Cell Death Assay

Apoptosis was detected by using an enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics GmbH; Mannheim, Germany). This assay measures the amount of mono- and oligo-nucleosomes in cytoplasmic fractions of apoptotic cells. The A549 cells were cultivated at a density of 1×10^5 cells/well in a 12-well culture plate and treated with miRNA-101, NC miRNA, and the IC_{50} dose of etoposide; as described in MTT assay. After 24 and 48 hours, cells were collected and ELISA assay was carried out in accordance with the manufacturer's guideline. In brief, the cells were lysed and centrifuged (200 g) for 10 min. Then, 20 µL of the supernatants and 80 µL of anti-DNA-peroxidase and anti-histone-biotin were added to each well of a streptavidin-coated plate and the plate was incubated at ambient temperature for two hours. Following washing with incubation buffer, 100 µL of 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; (Roche Diagnostics GmbH, Mannheim, Germany) solution was transferred to each well. The reaction was stopped with ABTS stop solution, and absorbance was measured at 405 nm (reference wavelength of 490 nm) using a plate reader (Awareness Technology, Palm City, FL, USA).

Statistical Analysis

The data were analyzed using the GraphPad Prism software (version 6.01), and expressed as mean±SD. Repeated measure analysis of variance (ANOVA) was used to ascertain statistical differences between groups. The pair *t* test was used to analyze statistical differences between the two groups. The ANOVA and Bonferroni's tests were used to analyze statistical differences between the multiple groups. P values <0.05 were considered statistically significant.

Results

Down-regulating Effect of MiRNA-101 on Mcl-1 mRNA Expression in A549 Cells

To determine whether miRNA-101 could influence the expression of *Mcl-1* in A549 cells, the lung cancer cells were transfected for 24 and 48 hours with 50 nM miRNA-101 and NC miRNA. Subsequently, the relative expression of *Mcl-1* mRNA was quantified using RT-qPCR analysis. As shown in figure 1, compared with the blank control, miRNA-101 treatment resulted in down-regulation of *Mcl-1* expression in a time-dependent manner (P=0.011). *Mcl-1* mRNA expressions were down-regulated to 77.17% (P=0.031) and 64.32% (P=0.006) after 24 and 48 hours, respectively. Notably, treatment with NC miRNA did not significantly alter *Mcl-1* mRNA.

MiRNA-101 Inhibits the Proliferation of A549 Lung Cancer Cells

As the up-regulation of *Mcl-1* is related to the survival of lung cancer cells, we sought to assess whether miRNA-101 could suppress the proliferation of the A549 cells. Therefore, the cells were transfected with miRNA-101 and negative control miRNA, and then cell proliferation was determined every 24 hours using trypan blue staining. Based on the proliferation curve of A549 cells, we observed a time-dependent anti-proliferative effect of miRNA-101 on A549 cells (P<0.01 compared with the blank control, figure 2). MiRNA-101 decreased the cell proliferation rate of the cells to 86.23% (P=0.086) at 24 hours, 75.23% (P=0.007) at 48 hours, 68.56% (P=0.006) at 72 hours, 65.11% (P=0.005) at 96 hours, and 64.42% (P=0.004) at 120 hours. Compared with the blank control group, the negative control miRNA treatments did not have a significant effect on cell proliferation (P=0.072).

MiRNA-101 Increased the Cytotoxicity of Etoposide in A549 Lung Cancer Cells

To assess whether miRNA-101 could enhance the sensitivity of the lung cancer cells to etoposide, a combination treatment

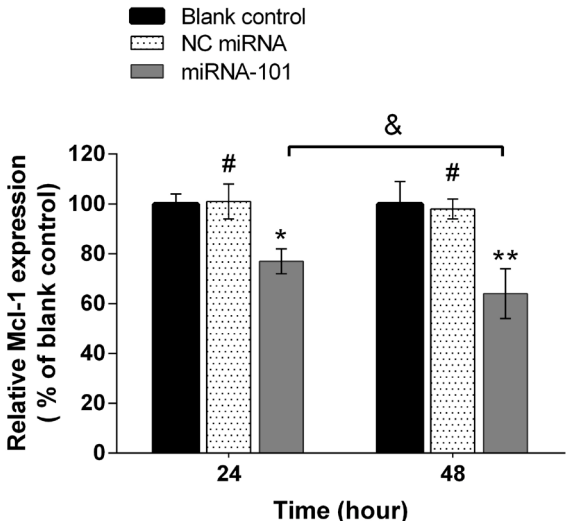

Figure 1: MicroRNA-101 suppressed the expression of myeloid cell leukemia-1 (Mcl-1) mRNA in lung cancer cells. The Mcl-1 expression was measured by reverse transcription quantitative PCR at 24 and 48 hours after treatment of the A549 cells with microRNA-101 and negative control (NC) microRNA. Relative Mcl-1 mRNA levels were measured using the $2^{-\Delta\Delta Ct}$ method. The Mcl-1 mRNAs markedly decreased compared with the blank control and NC microRNA treated cells. Data are expressed as mean±SD (n=3). *P=0.031, **P=0.006, $^{\#}$P=0.066 relative to the blank control, $^{\&}$P<0.045.

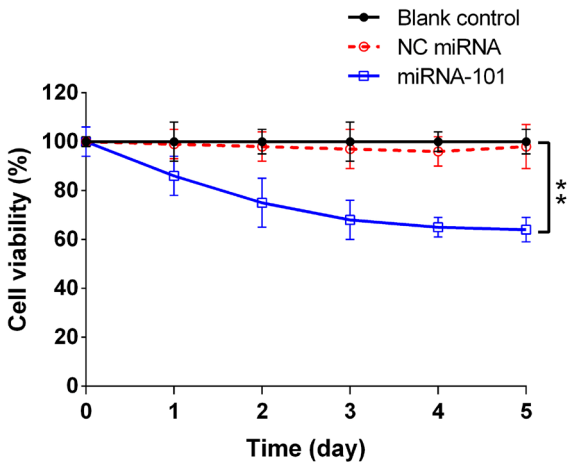

Figure 2: Down-regulation of myeloid cell leukemia-1 by microRNA-101 inhibited the proliferation of A549 cells. The cells were transfected with microRNA-101 and negative control (NC) microRNA for 5 days and then cell viability was assessed using trypan blue staining. Data are expressed as mean±SD (n=3). **P<0.01 relative to the blank control or NC microRNA.

of miRNA-101 and etoposide on A549 cells was performed. As shown in figures 3A and 3C, treatment with etoposide alone induced cytotoxicity in a dose-dependent manner. The results of the MTT assay revealed that 24 and 48 hours after transfection with miRNA-101, cell survival rate significantly decreased to 81.34% (P=0.037) and 75.09% (P=0.023), respectively, relative to the blank control. Moreover, a combination of miRNA-101 and etoposide further reduced the cell survival rate compared with miRNA-101 or etoposide alone (P=0.033 and P=0.029 at 24 and 48 hours, respectively). Surprisingly, transfection with miRNA-101 markedly reduced the IC_{50} values of etoposide from 1.17 µM to 0.65 µM and 0.84 µM to 0.49 µM after 24 and 48 hours, respectively (table 1). Meanwhile, NC miRNA transfection had an insignificant effect on the sensitivity of the A549 cells compared to the miRNA-101 treated cells (P=0.064).

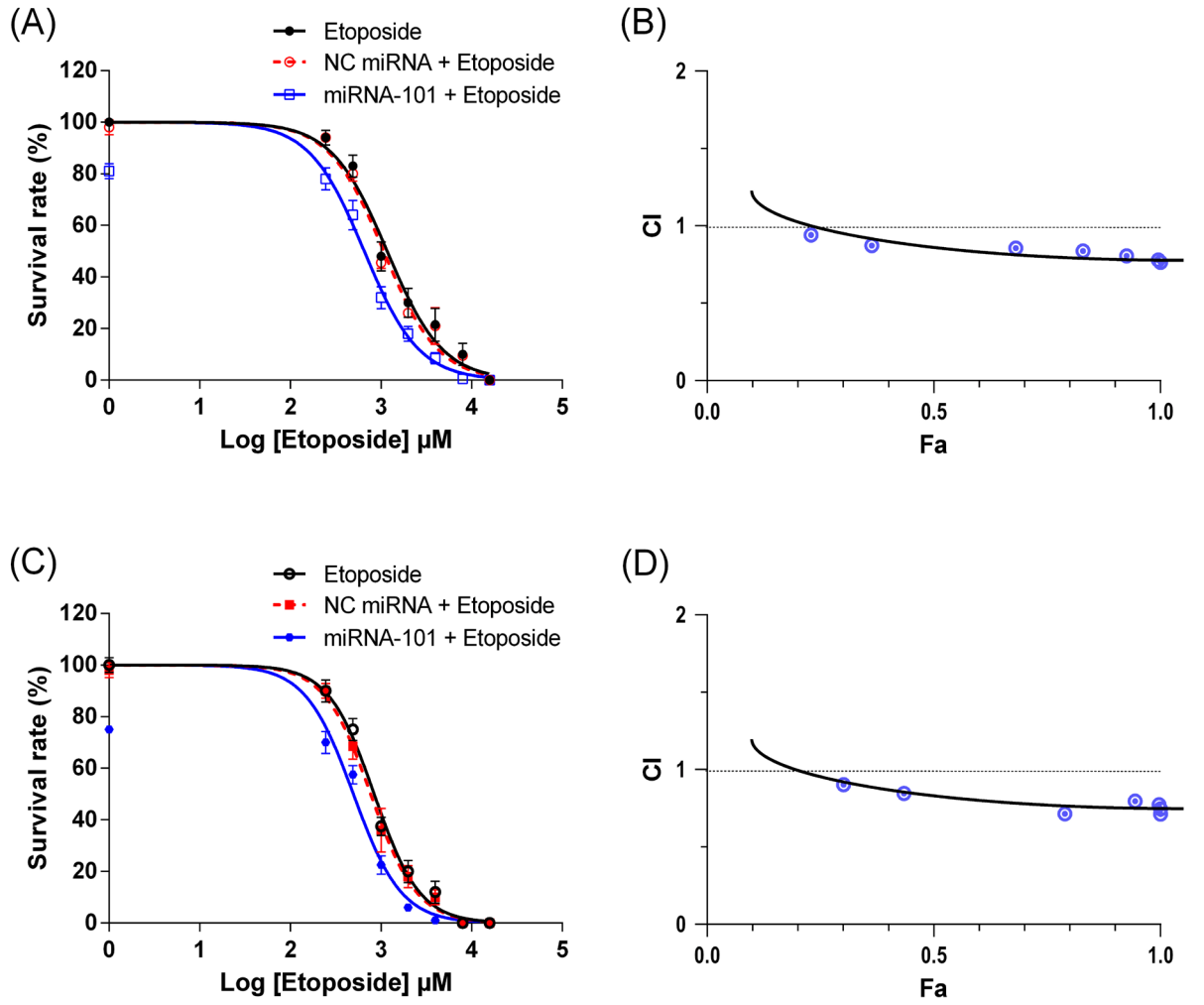

Figure 3: Cytotoxic effects of microRNA-101 and etoposide on cell survival were synergistic. Human A549 cells were exposed to microRNA-101 (50 nM) and different concentrations of etoposide for 24 hours (A and B) and 48 hours (C and D). Cell survival was then measured using the MTT assay. Data are expressed as mean±SD (n=3). Results from the three experiments were used to perform the combination index (CI) analysis using the CI theorem of Chou-Talalay and CompuSyn software. The horizontal dashed line shows CI=1. Fa: Fraction affected.

Table 1: Half maximal inhibitory concentration values of the etoposide alone and in combination with microRNA after 24 and 48 h of treatment

		Treatment			P value
		Etoposide	Etoposide and NC miRNA	Etoposide and miRNA-101	
IC_{50} (μM)	24 hours	1.17±2.23	1.07±1.63	0.65±0.99	0.079# 0.006**
	48 hours	0.84±2.40	0.76±1.40	0.49±1.63	0.001*** 0.065##

IC_{50} values were determined by the sigmoidal dose-response (variable slope) model using Prism software. Data are expressed as mean±SD (n=3). ** and *** Significant difference between etoposide and combination of etoposide and microRNA-101 groups after 24 and 48 hours, respectively. # and ## Significant difference between etoposide and combination of etoposide and negative control (NC) microRNA groups after 24 and 48 hours, respectively. IC_{50}: Half maximal inhibitory concentration

Table 2: Combination index analysis of microRNA-101 and etoposide in A549 cells

Etoposide concentration (μM)	24 hours			48 hours		
	Fa	CI	Combined effect	Fa	CI	Combined effect
0.25	0.23	0.93	S	0.30	0.90	S
0.5	0.36	0.87	S	0.43	0.84	S
1	0.68	0.85	S	0.78	0.71	S
2	0.82	0.83	S	0.94	0.79	S
4	0.92	0.80	S	0.99	0.77	S
8	0.99	0.78	S	1	0.74	S
16	1	0.76	S	1	0.72	S

The Chou-Talalay drug combination index (CI) method was used to determine the synergy (S), additivity, or antagonism of the microRNA-101/etoposide combinations. The synergy, additivity, and antagonism were indicated with CI <1, =, or >1. Fa: Fraction affected

MiRNA-101 Acts Synergistically with Etoposide to Decrease Cell Survival of Lung Cancer Cells

To examine whether inhibition of cell survival was due to the synergistic effect of the miRNA-101 and etoposide, CI analysis was performed on MTT assay results using the non-constant method of Chou-Talalay. The CI-Fa plots showed a synergism (CI<1) in tumor cells, when miRNA-101 (50 nM) was combined with etoposide (0.25-16 μM) (figures 3B and 3D). After 24 hours incubation, the best mean CI value of treatment (CI=0.76) occurred at 16 μM etoposide with a Fa level of 1 (table 2). Moreover, at 1 μM etoposide with a Fa level of 0.78, the most obvious synergistic effect was observed at 48 hours (CI=0.71).

Combining MiRNA-101 with Etoposide Resulted in an Enhanced Apoptotic Effect in Lung Cancer Cells

To analyze whether the observed synergistic cytotoxic effects between miRNA-101 and etoposide were linked to the induction of apoptosis, the effects of either miRNA-101 or etoposide alone, and in combination on apoptosis were assessed using an ELISA apoptosis assay. As shown in figure 4, transfection with miRNA-101 alone enhanced apoptosis by 2.70-fold (P=0.008) after 24 hours, whereas treatment with etoposide alone caused a 5.92-fold (P=0.004) increase in apoptosis (compared with the blank control). In contrast, compared with single-agent therapy, the combination therapy further enhanced apoptosis by 8.85-fold (P=0.003). Moreover, treatment of the cells with miRNA-101 or etoposide alone for 48 hours led to a profound increase in apoptosis (by 3.41- and 6.62-fold, respectively). Additionally, miRNA-101 in combination with etoposide significantly increased apoptosis by 10.27-fold (P=0.001, compared with mono treatment). On the other hand, the cells showed no significant changes in

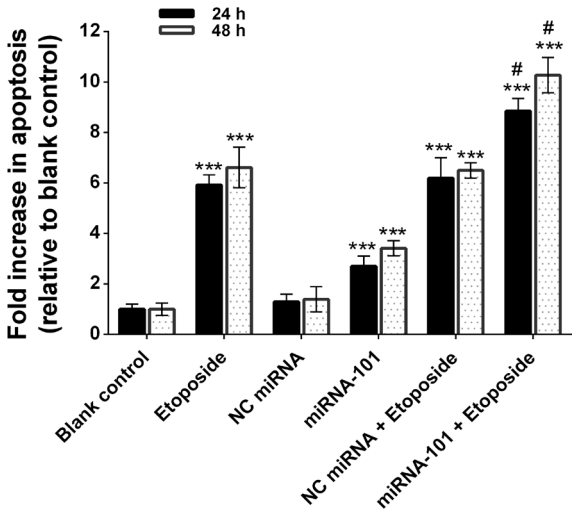

Figure 4: MicroRNA-101 enhanced the apoptotic effect of etoposide in lung cancer cells. The A549 cells were treated with microRNA-101 (50 nM), negative control (NC) microRNA (50 nM), and etoposide (IC_{50} doses of 24 and 48 hours), alone and in combination. Apoptosis was assessed with a cell death ELISA assay kit. The data are expressed as mean±SD (n=3). ***P<0.001 versus blank control, #P<0.001 versus microRNA-101 or etoposide mono treatment.

the extent of apoptosis after adding NC miRNA alone or in combination with etoposide (P=0.087, compared to miRNA-101 or etoposide alone, respectively). These results demonstrate that the sensitization effect of miRNA-101 is linked to the induction of apoptosis.

Discussion

The results showed that overexpression of miRNA-101 suppressed cell growth and survival and triggered apoptosis in NSCLC cells. We provided evidence to prove the role of miRNA-101 in chemoresistance of lung cancer cells, possibly in part through the suppression of *Mcl-1* expression. We also showed that transfection with miRNA-101 alone significantly decreased cell proliferation and survival of A549 cells and triggered apoptosis. These results support the tumor-suppressive effects of miRNA-101 in lung cancer cells.

MiRNAs are a class of single-stranded, non-coding RNAs involved in many cellular processes such as cell development, differentiation, apoptosis, and metabolism.[24] Recent studies have indicated that abnormalities in miRNA expression are associated with the development and progression of cancer. Moreover, dysregulation of miRNAs is associated with the pathogenesis of NSCLC.[25-27] MiRNA-101, as a cancer suppressor, inhibits cell proliferation and invasion, enhances apoptosis, and reduces chemoresistance in different malignancies through different mechanisms.[24] Previously, it has been demonstrated that miRNA-101 down-regulates the expression of enhancer of zeste homologue 2 (*EZH2*), cyclooxygenase-2 (*Cox-2*), zinc finger E-box binding homeobox 1 (*ZEB1*), and *Mcl-1* in several types of cancer including gastric cancer and NSCLC, leading to inhibition of cell proliferation and enhancement of apoptosis.[16-18, 28] Li and others showed that down-regulation of miRNA-101 in breast cancer tissues was positively correlated with the clinical stages, metastasis, and prognosis of patients.[3] Additionally, restoration of miRNA-101 expression significantly inhibited cell proliferation, metastasis, and enhanced apoptosis. Moreover, Zheng and others reported that the expression of miRNA-101 was significantly negatively correlated with the growth and invasion of ovarian cancer cells.[29] These findings were in agreement with our data and further confirmed the critical role of miRNA-101 in the survival and progression of NSCLC cells.

In the present study, we inhibited *Mcl-1* expression by miRNA-101 to explore the role of this miRNA on the sensitivity of NSCLC cells to etoposide. We observed that monotherapy of A549 cells with etoposide inhibited cell survival and induced apoptosis compared to the blank control. The introduction of miRNA-101 to lung cancer cells markedly reduced *Mcl-1* mRNA levels and synergistically enhanced the cytotoxicity of etoposide. Moreover, miRNA-10 enhanced the apoptotic effect of etoposide in lung cancer cells. *Mcl-1* is a Bcl-2 family apoptosis regulator, which was first identified as an induction gene during differentiation of leukemia cells.[30] *Mcl-1* has been shown to block the release of cytochrome-c from mitochondria by interacting with the pro-apoptotic Bcl-2 family members (such as Bim, Bid, and PUMA). This leads to inhibition of Bak and Bax activation and ultimately inhibition of apoptosis.[31, 32] In apoptotic conditions, NOXA displaces *Mcl-1* from these pro-apoptotic proteins, which lead to Bak oligomerization, Bax insertion to the outer mitochondrial membrane, and finally cytochrome-c release.[33, 34] Some studies have reported that *Mcl-1* overexpression is correlated with high levels of cell survival and resistance to diverse chemotherapeutic agents in various cancer cells.[35-37] Furthermore, *Mcl-1* knockdown has been shown to decrease cell viability and sensitize cells to anti-tumor agents.[38-40] In agreement with our data, Yin and others showed that miRNA-101 overexpression promotes cisplatin-induced cell death via the activation of caspase 3 in A549 cells.[24] Su and others showed that ectopic expression of miRNA-101 increased response to chemotherapeutic drugs in hepatoma cells by silencing *Mcl-1*.[18] Moreover, Chen and others reported that enforced expression of miRNA-101 increased the radiosensitization level in NSCLC cell lines.[19] These results were in line with our findings that the up-regulation of miRNA-101 could render NSCLC cells sensitive to chemotherapy via blockage of *Mcl-1*.

Previous studies have shown that different genes and miRNAs are involved in the tumor characteristics of lung cancer.[1, 2] In the present study, we only assessed the effect of miRNA-101 on the expression of the *Mcl-1* gene and cellular parameters in the A549 cell line. In addition to *Mcl-1*, other genes involved in the growth, apoptosis, and drug resistance of lung cancer cells may be targeted by miRNA-101. Therefore, further studies on other genes and several cell lines could provide more comprehensive results and increase the validity of our findings.

Conclusion

Up-regulation of miRNA-101 inhibited cell

proliferation and survival, and induced apoptosis in NSCLC cells. The results demonstrated the role of miRNA-101 in NSCLC drug resistance, possibly in part through the regulation of *Mcl-1* expression. Overall, the data suggest that miRNA-101 can act as a key therapeutic target for NSCLC. However, further studies on animal and human models are required to evaluate the effectiveness of miRNA-101 in the treatment of NSCLC.

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Conflict of Interest: None declared.

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