Original Article
MLF1 protein is a potential therapy target for lung adenocarcinoma

Xiaojun Li[1], Shengping Min[2], Hongtao Wang[3], Yuanbing Shen[2], Wei Li[2], Yuqing Chen[2], Xiaojing Wang[2]

[1]Department of Thoracic Surgery, First Affiliated Hospital, Bengbu Medical College, Bengbu, Anhui China; [2]Department of Respiration, Anhui Clinical and Preclinical Key Laboratory of Respiratory Disease, First Affiliated Hospital, Bengbu Medical College, Bengbu, Anhui China; [3]Department of Immunology, Bengbu Medical College, Bengbu, Anhui China

Abstract: Myeloid leukemia factor 1 (MLF1) is a protein involved in myeloid cell differentiation which regulates the cell cycle and the expression of numerous genes. The role of MLF1 in hematologic cancers is well established; however, its role in lung adenocarcinoma is unknown. Here, we investigated the role of MLF1 in lung adenocarcinoma using a variety of cell lines along with patient samples to determine whether MLF1 plays a significant role in this devastating disease. Lung cancer cell lines (A549, H1975, HCC827, and NCI-H460) and primary lung tissue were used to assess the relative levels of MLF1 in lung adenocarcinoma. The lung adenocarcinoma cell line A549 was infected with a lentivirus to knockdown MLF1, and successful knockdown was confirmed by a real-time polymerase chain reaction (qPCR). Cell proliferation was assessed through fluorescence imaging and MTT assays. Cell cycle analysis was performed utilizing flow cytometry and formation of cell colonies evaluated microscopically. Proliferation of A549 cells was significantly inhibited in cells where MLF1 was silenced compared to controls. Cell cycle analysis indicated that cell cycle phases were not significantly changed upon the silencing of MLF1 in lung adenocarcinoma cells. A significant increase in apoptosis was observed in MLF1-knockdown cells, while a significant decrease in the number of cell colonies formed was observed in MLF1-knockdown cells compared to controls. In most, but not all, human lung adenocarcinoma tissue samples, MLF1 was upregulated. The results show that MLF1 promotes the proliferation and colony forming abilities of lung adenocarcinoma cells and significantly decreases apoptosis while having no impact on the cell cycle. Further studies with larger sample sizes are needed 1) to conclude whether human lung adenocarcinoma upregulates MLF1, 2) to reveal the mechanism of action for MLF1 in lung carcinogenesis and 3) to investigate MLF1 gene therapy for the treatment of lung adenocarcinoma.

Keywords: Lung cancer, myeloid leukemia factor 1, gene therapy

Introduction

Lung cancer has two main types, known as small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC), and lung adenocarcinoma is the main subtype of NSCLC. It is a devastating disease that is rarely diagnosed early (15% of cases) and even with early detection, it has a five-year survival rate that is only around 50% [1]. Typically, lung cancer is diagnosed at the later stages after it has spread to other organs, at which point the five-year survival rate drops dramatically down to only 4% [1]. Worldwide, lung cancer is the leading cause of cancer related deaths [2-4].

Although conventional chemotherapy is still used, targeted therapies, which usually have fewer side effects, are becoming more common. Targeted therapies take advantage of a specific requirement or mutation that the tumors exhibit. Some examples of targeted therapies include angiogenesis inhibitors, such as Bevacizumab (Avastin) and Ramucirumab (Cyramza), which inhibit blood vessel formation and block the supply of nutrients to a tumor, drugs that target epidermal growth factor receptor (EGFR) mutations, such as Erlotinib (Tarceva), Afatinib (Gilotrif) and Gefitinib (Iressa), and drugs that target cells with the anaplastic lymphoma receptor tyrosine kinase (ALK) gene

mutations, such as Crizotinib (Xalkori), Ceritinib (Zykadia), and Alectinib (Alecensa) [1]. Although targeted therapies for lung cancer are showing promise they are still associated with dangerous side effects and are not effective in treating all cases of lung cancer [3].

The MLF1 gene codes for a 30 kDa intracellular protein that is most commonly known for its role in regulating the commitment of hematopoietic progenitor cells into the myeloid lineage and restricting erythroid formation [5]. MLF1 interacts with a number of other proteins and is thought to act as transcription factor due to its DNA binding domains and ability to modulate gene expression. Many of the normal functions of MLF1 involve cell cycle regulation [6]. In cancer, translocations of the MLF1 gene and the nucleophosmin gene have been associated with acute myeloid leukemia and myelodysplastic syndrome [7].

Materials and methods

Cell lines and culture conditions

All cell lines (A549, H1975, HCC827, and NCI-H460) were purchased through American Type Culture Collection (ATCC, USA). Culture media contained Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin, Gibco, USA) and fetal calf serum (10% FCS, Thermo Fisher Scientific, USA). Cells were incubated in 5% CO_2 at 37°C. Media changes were performed every two days.

Detection of endogenous MLF1 transcript expression though real-time polymerase chain reaction

Cells were lysed in Trizol and ribonucleic acid (RNA) purification was performed using the general protocol that accompanied the Trizol kit (Invitrogen, USA). Spectrophotometric analysis was performed using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific, USA) to determine RNA purity and concentration. The integrity of the RNA was verified using electrophoresis through agarose gels. CDNA was synthesized by preparing samples with an M-MLV-RTase kit (Promega, USA) and running samples on an ABI 2720 thermal cycler (ABI Biosystems, USA) according to the manufacturer's recommendations. Briefly, 2.0 µg RNA,

Oligo dT (Shanghai Sangon, China) and DEPC-H_2O were added to PCR vials, then centrifuged and the RT reaction was run for one hour at 42°C. Samples were then placed at 70°C to inactivate the reverse transcriptase for 10 minutes. Detection of products, including the endogenous control GAPDH, was performed using a Takara Bio PCR Thermal Cycler (Dice Real Time TP800, Takara, Japan). Each reaction contained 0.5 µl cDNA, 10 µl SYBR green premix (DRR041B, Takara, Japan), 0.5 µM of forward and reverse primers and 8.0 µl water. The run cycle consisted of: 45 denaturation cycles for 15 seconds at 95°C and annealing and elongation each for 30 seconds at 60°C. Samples were placed for one minute at 95°C to allow denaturation and then were cooled to 55°C. Absorbance readings were collected while increasing the temperature in 0.5°C -steps for 4 seconds until reaching 95°C. Relative MLF1 mRNA expression (normalized to GAPDH) was calculated with the $2^{-\Delta\Delta Ct}$ technique.

Lentiviral infection of A549 cells

A549 cells line were used for the remaining knock down experiments. Cells in a logarithmic growth phase were detached with trypsin (Shanghai Chemical Reagent Company, China), re-suspended in DMEM with antibiotics and 10% FCS, seeded at a density of 5×10^4 cells per well in six-well plates, and incubated at 37°C in 5% CO_2. Cells at 30% confluence were divided into two groups: 1) MLF1-knockdown cells were infected with the MLF1-siRNA GFP lentivirus (Jikai, China), whereas, 2) control cells were infected with an empty GFP lentivirus (Jikai, China). No cytotoxic effects were noticed at the MOI used after 12 hours. After 24 hours media was aspirated and replaced with fresh media. 80% transfection efficiency was verified after 72 hours by GFP fluorescence. Protein and RNA were collected from cells for use in subsequent experiments.

qPCR detection of MLF1 knock down efficiency

After five days, confluent wells in a 12-well plate were harvested for RNA. RNA was isolated in Trizol as described above and the transcript expression was detected using the previously described qPCR method. Again, the $2^{-\Delta\Delta Ct}$ technique was employed to determine knockdown efficiency of the MLF1 lentivirus.
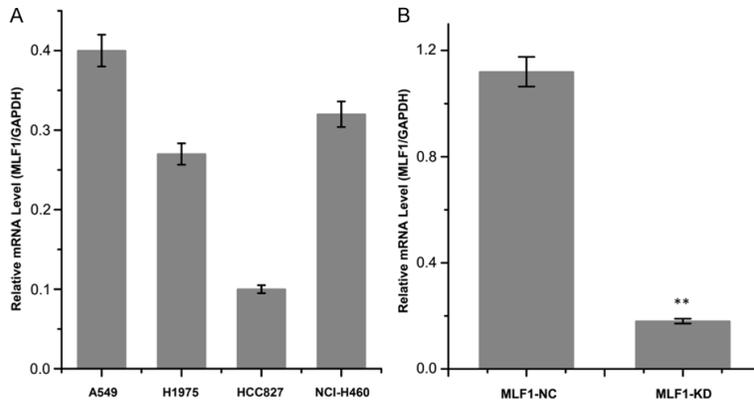
Figure 1. MLF1 mRNA expression assessed by qPCR. Levels of MLF1 mRNA were assessed in four different lung cancer cell lines (A) MLF1IP mRNA expression varied across the four cell lines: A549, H1975, HCC827, and NCI-H460 with the highest levels observed in A549 cells. GAPDH was used as an endogenous control. To knock down MLF1 gene expression A549 cells were infected with MLF1-siRNA lentivirus. (B) Post-lentiviral infection, relative MLF1 mRNA expression was significantly inhibited in the A549 MLF1-siRNA knockdown (MLF1-KD) cells as compared to the A549 negative control (MLF1-NC) cells (**$P<0.01$). Again, GAPDH was used as an endogenous control.

Cell proliferation assays

Infected cells were detached with trypsin and resuspended in a standard medium at confluence. 2,000 infected cells were resuspended in 100 μl and plated in 96-well plates and incubated at 37°C with 5% CO_2. After cells were allowed to attach for 24 hours the expression of GFP was assessed over 5 days using a Cellomics ArrayScan VTI imager (Thermo Fisher Scientific, USA). Cell proliferation curves were compiled from this data to determine growth kinetics. Cell growth was also assessed using MTT assays (Beijing Ding Guo Biotechnology, China) which were performed following the manufacturer's recommendations. Cells were trypsinized, placed in a standard medium, plated at 2,000 cells in 100 μl per well in 96-well plates, and incubated at 37°C and 5% CO_2. To perform MTT assays, after 2 days 10 μl of MTT (5 mg/ml) was added to each well and plates were incubated for 4 hours. Next, media containing MTT was removed and a 100 μl stop solution was added (dimethyl sulfoxide (DMSO), Shanghai Pharmaceutical Group, Shanghai, China). Plates were agitated for 10 minutes and microplate readers were used to determine optical density values at a wavelength of 490 nm using an absorbance microplate reader (Biotek Elx800, USA) to determine proliferation rates.

Flow cytometric analysis of cell cycle using propidium Iodide

Infected cells were grown to 80% confluence, detached with trypsin, counted using a hemocytometer, and then centrifuged for 5 minutes at 1500 rpm. Cells were washed with ice cold PBS (pH 7.2-7.4) and again centrifuged at 1500 rpm for 5 minutes and fixed with ice cold 70% ethanol for 1 hour. Cells were centrifuged again and washed with PBS to remove ethanol. Cells were then resuspended in 1.0 ml to 1.5 ml of a solution which contained 2 mg/ml propidium iodide (PI) liquor (P4170, Sigma, USA), 10 mg/ml RNase mother liquor (EN-0531, Fermentas, USA), and PBS. Cells were then filtered through a 40 μm mesh to remove clumps and flow cytometry was performed using a FACSCalibur (Becton-Dickinson, USA).

Apoptosis detection assay

Control and MLF1-siRNA infected cells were trypsinized, placed in DMEM with 10% FCS and PBS washes performed before assessing apoptosis using Annexin V-APC and PI (eBioscience, USA) according to the manufacturer's recommendations. Briefly, 1×10^5 cells were resuspended in 100 μl of $1 \times$ Binding Buffer and stained with either Annexin V-FITC reagent (5 μl), PI reagent (10 μl), both, or nothing (control). Cells were gently mixed for 15 minutes in the dark at room temperature and then 400 μl of l Binding Buffer was added. Flow cytometry to measure apoptosis was conducted after one hour.

Colony formation assay

Control and MLF1-siRNA infected cells were trypsinized, counted with a hemocytometer, seeded onto six well plates at a density of 800 cells per well, and half of the culture media was changed every 3 days for a total of 14 days. After 14 days media was removed followed by a PBS wash and then 4% paraformaldehyde was

Table 1. MILF1 Expression in 17 human lung tumors and 4 healthy controls by RT-qPCR

Tissue sample serial[#]	GAPDH expression	MLF1IP expression	$-\Delta\Delta Ct$	Relative MLF1IP expression (MLF1IP/GAPDH)	Direction of significant dysregulation[*]
A2354	16.97	27.32	-0.12	1.59	↑
A2355	17.64	25.93	0.81	1.47	↑
A2356	18.81	22.17	2.24	1.18	-
A2357	14.34	28.92	2.07	2.02	↑
A2358	16.98	28.75	0.64	1.70	↑
A2359	17.97	26.34	0.72	1.47	↑
A2360	16.19	27.01	-1.18	1.67	↑
A2361	18.92	23.05	0.31	1.22	↑
A2362	17.80	18.72	-0.57	1.05	-
A2363	16.77	23.02	-0.69	1.37	↑
A2364	18.70	15.33	-0.81	0.82	↓
A2365	14.80	29.21	-0.11	1.97	↑
A2365	12.32	23.87	1.24	1.94	↑
A2365	15.98	24.22	0.93	1.52	↑
A2365	13.03	26.76	-0.22	2.05	↑
A2365	11.62	25.33	1.03	2.18	↑
A2367	15.82	26.51	1.07	2.18	↑
A2369	11.75	29.11	-1.10	1.68	↑
A2370	15.39	26.82	1.18	1.74	↑
A2373	17.41	18.45	1.22	1.06	-
A2376	12.23	25.13	1.09	2.05	↑

[*]Significant dysregulation defined as either >1.20 fold-change or <0.83 (1/1.2) fold-change. [#]Clinical records (Department of Pathology, the First Affiliated Hospital; Bengbu Medical College) for all lung tumor resection cases performed between January 2014 and December 2016 were screened. After screening, a total of 17 lung adenocarcinoma cases (mean age: 55 years, rang: 48-72); sex: 12 male, 5 female) were finally enrolled in this study. MLF1 transcript levels were assessed with qPCR in 17 lung adenocarcinoma tissues and 4 normal lung control tissues. 13 cancerous lung samples demonstrated increased levels of MLF1 gene expression, one lung cancer sample displayed downregulation of MLF1 gene expression, and 3 lung cancer samples showed no change in MLF1 gene expression compared to the control lung samples.

added for 30 minutes to fix the cells (Shanghai Sangon, China). Cells were washed again with PBS to remove paraformaldehyde and stained for 20 minutes with 500 µl Giemsa (ECM550 Chemicon). Giemsa was washed off with distilled water three times, and the cells were left to dry. Digital cameras were used to take pictures of the colonies through microscopes (MicroPublisher 3.3RTV; Olympus, Japan) and pictures were used to determine colony numbers.

MLF1 expression in lung cancer patient samples

MLF1 transcript levels were assessed in diseased lung samples from 17 lung cancer patients and 4 normal control lung tissue samples. All samples were obtained following protocols approved by the Ethics Committee (IRB) of the First Affiliated Hospital of Bengbu Medical

College (Bengbu, China), including the collection of informed consents from all donors used in this study. RNA was isolated from homogenized tissue samples as before using Trizol. Briefly, tissues were flash frozen and sliced into 3 mm3 sections. Tissue slices were placed in 1 ml of Trizol. After homogenization samples were placed at 4°C for 10 seconds then centrifuged at 5000 rpm for 3 minutes. Supernatant was collected and RNA extracted using a Trizol kit (Invitrogen), then reverse transcribed into cDNA (M-MLV, Promega) and qPCR performed to determine quantitative expression of MLF1 gene in samples.

Statistical analysis

SPSS v19 software was used to perform statistical analyses (IBM, USA). Data are represented as means of three independent experiments and the variance is displayed as standard devi-
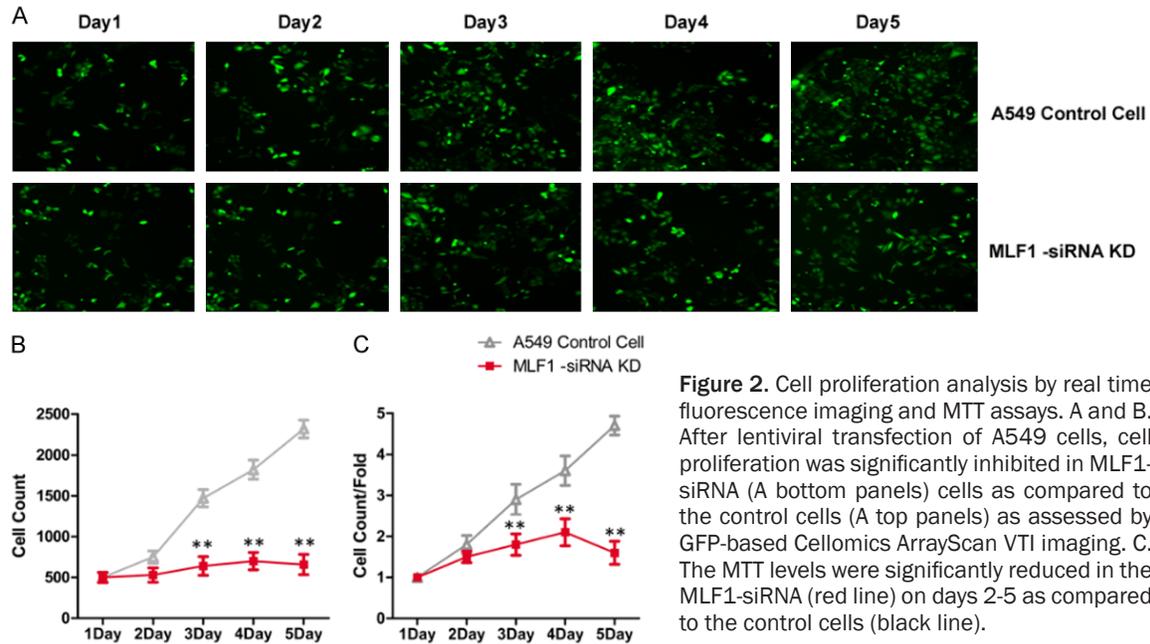
Figure 2. Cell proliferation analysis by real time fluorescence imaging and MTT assays. A and B. After lentiviral transfection of A549 cells, cell proliferation was significantly inhibited in MLF1-siRNA (A bottom panels) cells as compared to the control cells (A top panels) as assessed by GFP-based Cellomics ArrayScan VTI imaging. C. The MTT levels were significantly reduced in the MLF1-siRNA (red line) on days 2-5 as compared to the control cells (black line).

ation (SD). Significance was determined with Student's t-test and a *p*-value of less than 0.05 was deemed significant *a priori.*

Results

Numerous lung cancer cells lines were used in this study including A549, H1975, HCC827 and NCI-H460 to investigate the role of MLF1 in lung cancer. Expression of the MLF1 transcript was detected in all of the cell lines tested. MLF1 expression levels varied among the different lung cancer cell lines, with A549 having the highest expression followed by NCI-H460 and H1975 (**Figure 1A**). HCC827 cells demonstrated the lowest expression levels of MLF1 mRNA in the cell lines tested (**Figure 1A**). Since A549 cells expressed the highest levels of MLF1 mRNA, they were utilized for the remaining knockdown experiments. MLF1 expression in lung cancer patient samples showed that in most, but not all, human lung cancer tissue samples MLF1 was upregulated (**Table 1**).

To investigate the effects of MLF1 knock down on the activity of lung cancer cells, A549 cells were stably infected with lentiviral vectors containing GFP with or without siRNA for MLF1 to tag cells and knockdown gene expression, respectively. Upon infection of A549 cells with a lentivirus that contained siRNA for MLF1 (MLF1-KD) a significant decrease in MLF1 gene expression was observed compared to the control cells infected with only GFP lentivirus (MLF1-NC, **Figure 1B**, **P<0.01).

Next, the effects of MLF1 expression on cell proliferation were assessed using a real time fluorescence microscopy assay along with a cell activity assay. Over a period of 5 days, using the GFP-based Cellomics ArrayScan VTI imaging assay, a significant decrease in cell proliferation was observed in A549 cells infected with MLF1-siRNA compared to control infected cells (**Figure 2A** and **2B**). To validate these proliferation results, an MTT assay was performed on the cells once daily for 5 days. A similar decrease in cell proliferation was observed in MLF1-siRNA infected A549 cells compared to controls using the MTT assay (**Figure 2C**).

Since MLF1 has been shown previously to be involved in cell cycle regulation, we wanted to determine whether the changes in cell behavior observed here were due to changes in the cell cycle. This was done using flow cytometry of PI stained cells to determine the ratio of cells in each phase of the cell cycle. No significant difference was observed in the G1, S and G2/M phase cell counts in MLF1-siRNA infected A549 cells compared to the control infected cells (**Figure 3A**). Additionally, apoptosis of cells was assessed through flow cytometric analysis of
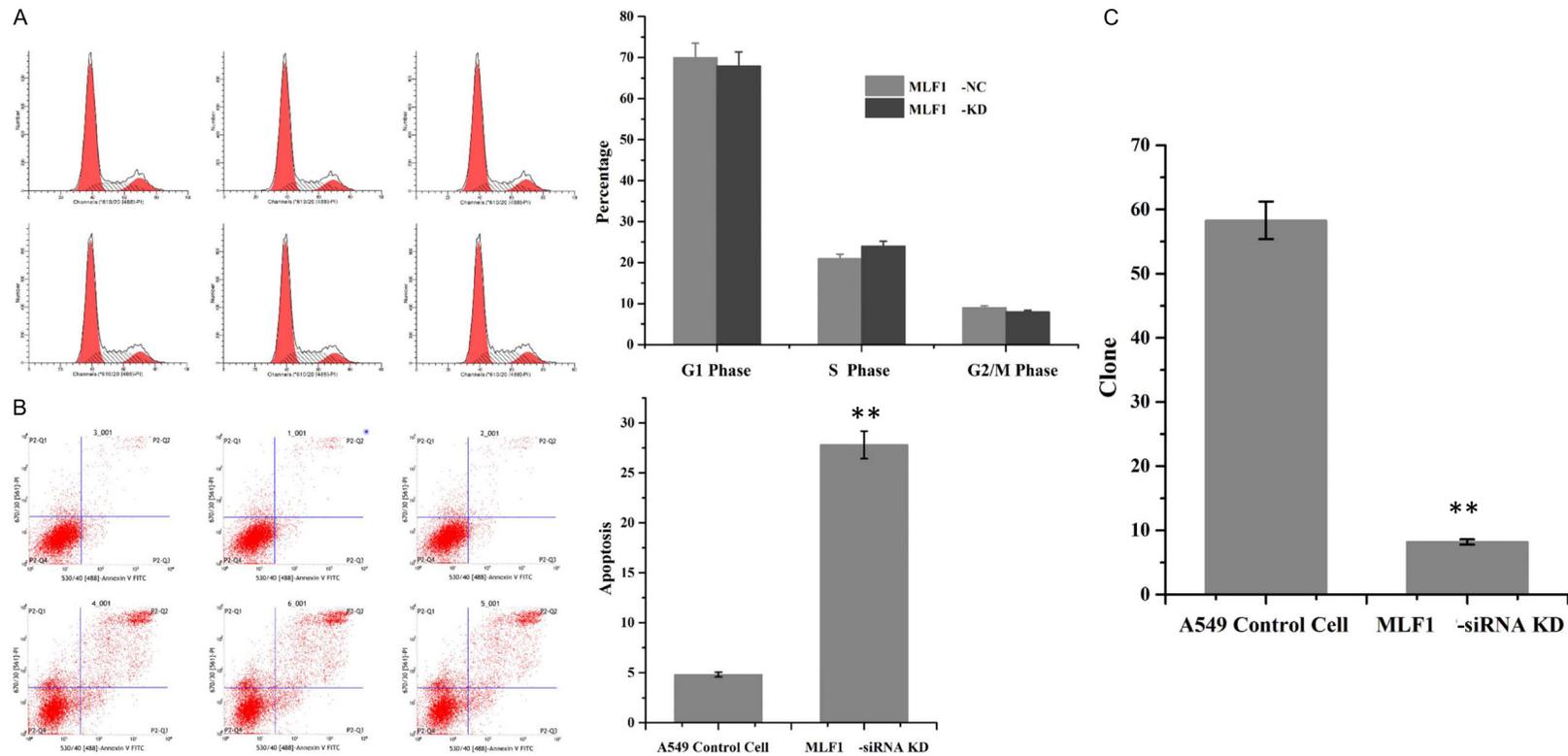
Figure 3. Cell cycle analysis, apoptosis and colony forming ability of MLF1-siRNA infected A549 cells. A. Cell cycle analysis was performed using flow cytometric analysis of PI stained cells. After lentiviral transfection of A549 cells, G1, S and G2/M phase cell counts were not significantly changed in MLF1-siRNA infected A549 cells (A bottom panels) as compared to control cells (A top panels). B. Cell apoptosis was assessed by Annexin V-FITC/PI Assay. A549 cell that were infected with MLF1-siRNA (B bottom panels) demonstrated significantly increased levels of apoptosis as compared to control cells (B bottom panels, **P<0.01). C. The colony forming ability of A549 cells was assessed using light microscopy. After lentiviral transfection of A549 cells with MLF1-siRNA, the cells displayed a significantly reduced number of cell colonies compared to the control cells (**P<0.01).

Annexin V-FITC/PI stained cells. A significant increase in apoptosis was observed in MLF1-siRNA infected A549 cells (**Figure 3B**, top panels) compared to the control infected cells (**Figure 3B**, bottom panels, **P<0.01*).

Colony formation assays are ideal *in vitro* techniques to determine the aggressiveness and stemness of cancer cells. Therefore, the ability of A549 cells, infected with MLF1-siRNA or control vectors, was assessed. Control A549 cells infected with control lentivirus (GFP) readily formed cell colonies (**Figure 3C**), however, A549 cells infected with MLF1-siRNA formed significantly fewer colonies than the controls (**Figure 3C**, **P<0.01*).

Discussion

Lung cancer is the leading cause of cancer-related deaths worldwide, and targeted therapies are showing great promise in effectively treating certain patients [8, 9]. MLF1 is a protein which has a number of functions, including regulation of the cell cycle, and its role in other cancers has been established [6, 7, 10, 11]. Whether MLF1 plays a role in lung cancer is unknown, so we investigated the role of MLP1 in lung cancer using a variety of cell lines along with lung cancer patient samples. All of the lung cancer cell lines (A549, H1975, HCC827, and NCI-H460) expressed the MLF1 transcript, with the highest expression observed in A549 cells. To determine whether the knockdown of MLF1 impacted lung cancer cell behavior, the A549 cells were infected with a lentivirus that contained siRNA for MLF1. Effective knockdown was confirmed by qPCR. Imaging analysis and biochemical assays both revealed significantly less proliferation in the A549 cells with a lower expression of MLF1 (MLF1-KD) compared to the controls. No changes in cell cycle progression were observed in the MLF1-KD cells, but a significant increase in apoptosis was observed in the MLF1-KD cells compared to the controls. Most of the human lung samples revealed an increased expression of MLF1.

This study, for the first time, identified MLF1 as a factor that promotes lung cancer cell growth and aggressiveness. MLF1, through its inhibition of apoptosis, is able to significantly increase the growth of A549 lung cancer cells. Targeting MLF1 in lung cancer cells, using an siRNA or other gene targeting approach, would diminish the anti-apoptotic effects of MLF1 in these cells leading to cell death, which may translate into tumor regression *in vivo*. Furthermore, MLF1 knock down significantly impaired the ability of A549 lung cancer cells to form colonies. These results indicate that MLF1 contributes to the survival of colony forming lung cancer cells which are typical stem-like cancer cells [12, 13]. Cancer stem cells are resistant to many types of therapy and are often responsible to the reoccurrence of tumors [14, 15]. Hence, the inhibition of MLF1 may decrease the number of colony forming stem cells in tumors contributing to less resistance and reoccurrence. Observing the variable expression of MLF1 in lung cancer patient samples may indicate that certain patients (high MLF1 expressers) will benefit more from targeted MLF1 therapies than others which agrees with the current paradigm of targeted therapies [16, 17].

In summary, MLF1 promotes the proliferation and colony forming ability of lung cancer cells while significantly diminishing apoptosis, while having no impact on the cell cycle. Therefore, MLF1 targeting therapies may benefit certain groups of lung cancer patients. Future studies, with larger sample sizes, are warranted to establish MLF1s mechanism of action in lung cancer and whether MLF1 gene therapies will benefit patients.

Conclusions

In summary, MLF1 promotes the proliferation and colony forming abilities of lung cancer cells and significantly decreases apoptosis, while having no impact on the cell cycle. Further studies with larger sample sizes are needed 1) to conclude whether human lung cancers upregulate MLF1, 2) to reveal the mechanism of action for MLF1 in lung carcinogenesis and 3) to investigate MLF1 gene therapy for the treatment of lung cancer.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Xiaojing Wang and Yuqing Chen, Department of Respiration, Anhui Clinical and Preclinical Key Laboratory of Respiratory Disease, First Affiliated Hospital, Bengbu Medical College, Bengbu 233000, Anhui, China. Tel: +86-15105528215; Fax: +86-5523070260; E-mail: wangxiaojing8888@163.com (XJW); Tel: +86-13-695528585; Fax: +86-5523070260; E-mail: bbmc-cyq@126.com (YQC)

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