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Original Article

Effects of miR-144 on the biological behavior of A549 lung cancer cells

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Abstract: Objective: The aim of this study was to explore the expression of microRNA-144 (miR-144) in human lung cancer, evaluating its effects on the biological behavior of A549 lung cancer cells. Methods: Expression of miR-144 in lung cancer tissues was detected by quantitative real time polymerase chain reaction (qPCR). Stable transfected A549 lung cancer cell lines were established by transfecting A549 cells with lentiviral vector-mediated miR-144. A blank control group was set up. Effects of miR-144 on cellular proliferation were detected by CCK-8 assay and the impact of miR-144 on cloning of lung cancer cells was observed. The TUNEL method was used to identify cell apoptosis. An experiment of subcutaneous xenografts of lung cancer in mice was performed for observation of the impact of miR-144 on xenograft tumor size. Results: miR-144 expression in lung cancer tissues was markedly lower than in normal adjacent lung tissues (P<0.001). Expression of miR-144 in A549 cells, which were transfected by lentiviral vector-mediated miR-144, was substantially higher than that in normal adjacent lung tissues (P<0.001). Moreover, stable transfected cell lines were successfully screened. Compared with blank control and Lenti-NC groups, proliferative activity and colony formation rates of A549 cells in the Lenti-miR-144 group were significantly lower (both P<0.001), the apoptosis rate of A549 cells was significantly higher (both P<0.001), and the size of xenograft tumors was significantly lower at 1 week, 2 weeks, 3 weeks, and 4 weeks, respectively (all P<0.001). Conclusion: Overexpression of miR-144 can significantly suppress the growth of lung cancer cells. This may be achieved by suppression of the proliferative activity and colony formation ability of lung cancer cells, as well as the promotion of apoptosis.

Keywords: miR-144, lung cancer, cellular proliferation, colony formation, apoptosis

Introduction

Lung cancer is one of the most common reported malignancies worldwide. Morbidity and mortality rates of lung cancer are high and pose an increasingly high trend, threatening the life and health of patients [1, 2]. One study reported that non-small cell lung cancer (NSCLC) accounted for about 80%-90% of lung cancers [3]. Most patients with lung cancer are in the advanced stage when they are diagnosed, with low survival rates. Therefore, it is necessary to find a biomarker with high specificity and sensitivity in identifying early lung cancer. Currently, it is generally considered that the occurrence of lung cancer is affected by various factors. Its pathogenesis is complicated and unclear [4]. Previous research has focused on tumor suppressor genes, tumor susceptibility genes, cell signaling pathways, and apoptosis [5-7]. Recently, microRNA has become one of the hot biological molecules in tumor research. Multiple studies have demonstrated a close association of microRNAs with proliferation, metastasis, invasion, differentiation, and apoptosis of lung cancer [8, 9]. Observations have revealed that overexpression of miR-126 in lung cancer cell lines significantly suppressed expression of Crk protein, resulting in decreased migration, adhesion, and invasion of lung cancer cells [10]. miR-31 inhibits proliferation of lung cancer cells and tumor tissue formation *in vivo* [11]. miR-17-92 promotes metastasis and invasion of lung cancer by reducing expression of PTEN

[12]. Hence, microRNAs play a critical role in the development of lung cancer.

Recent evidence has shown that miR-144 plays distinct and complex roles in different tumors [13, 14]. One study reported that miR-144 was low-expressed in colorectal cancer and elevated expression of miR-144 could suppress invasion of colorectal cancer cells [15]. In contrast, miR-144 was high-expressed in nasopharyngeal cancer and high expression of miR-144 can promote invasion of nasopharyngeal cancer cells [16]. In thyroid cancer, miR-144 targets ZEB1 and other proteins to suppress invasion of cancer cells [17]. Of note, expression of miR-144 has complex regulatory effects on the biological behavior of tumor cells. However, there are few reports concerning the function of miR-144 in the onset and development of lung cancer. A549 cell lines were first established by Giard in 1972 by transplanting and culturing of lung cancer tissue from a NSCLC patient [18]. In most NSCLC studies, the cell line A549 has been selected as the representative [19]. Therefore, in this study, for the purpose of clarifying the effects of miR-144 on the biological behavior of lung cancer cells, lung cancer cells A549, which are adenocarcinomic human alveolar basal epithelial cells, were included as subjects. miR-144 expression in lung cancer tissues was detected and A549 cells were transfected with miR-144 medicated by lentivirus vectors to explore the effects of miR-144 on proliferation, cloning, apoptosis, and other biological behaviors of lung cancer cells.

Materials and methods

Cell lines

A549 human lung cancer cell lines were obtained from the American Type Culture Collection (ATCC, USA). Cells were seeded in DMEM medium containing 10% fetal bovine serum (FBS) and routinely cultured in an incubator with 5% CO_2 at 37°C. Cells in the logarithmic growth phase were selected for further experimentation.

Tissue specimens

Clinical specimens collected in this study were approved by the Ethics Committee of Huai'an Affiliated Hospital of Xuzhou Medical University and The Second People's Hospital of Huai'an. All patients provided written informed consent. Samples of fresh lung cancer tissues used in this experiment were taken from lung cancer patients that underwent radical therapy of lung cancer. None of them had received radiotherapy or chemotherapy before the operation. All cases were pathologically confirmed as NSCLC. A total of 30 specimens, including lung cancer tissues and normal adjacent lung tissues, were collected for the present study.

Experimental animals

BALB/C nude mice with thymus defects (4 to 6 weeks old, irrespective of gender, and weighing 18 to 21 g) were purchased from Shanghai Slike Experimental Animal Co., Ltd. (Shanghai, China) and raised under SPF conditions.

Reagents and instruments

High-glucose DMEM cell culture medium and FBS were purchased from Gibico, USA. TaqMan MicroRNA Reverse Transcription Kits and detection kits were purchased from Applied Biosystems, USA. miR-144 lentivirus overexpression vectors were synthesized by Shanghai GenePharma Co., Ltd, China. TRIzol Reagent and Lipofectamine TM 2000 Transfection reagent were purchased from Invitrogen Company, USA. CCK-8 kit and crystal violet were purchased from Sigma, USA. TUNEL kits were purchased from BD Biosciences, USA. An inverted microscope was purchased from Nikon, Japan, and a Real-time PCR analyzer was purchased from Applied Biosystems, USA.

miR-144 expression detected by qPCR

Total miRNA was extracted from lung cancer tissues and A549 cell lines, following manufacturer instructions for the mirVana microRNA isolation kit. MicroRNA was reverse transcribed into cDNA with the use of a TaqMan microRNA reverse transcription kit. U6 internal reference was applied as control. Primer sequences were as follows: miR-144, forward, 5'-TCCGATCATGTAG TAGATAT TGACAT-3', and reverse, 5'-GTGCAGGTCCGAGGT-3'; U6, forward, 5'-CTCGCTTCGGCAGCACA-3', and reverse, 5'-AACGCTTCACGAATTT GCGT-3. The reaction system (20 μL) included 2 μL of cDNA, 0.6 μL of forward primer, 0.6 μL of reverse primer, 10 μL of SYBR Green Realtime PCR Master Mix, and 6.8 μL of

ddH_2O. They were centrifuged and put into an Applied Biosystems 7300 quantitative PCR instrument for amplification. Reaction conditions were pre-denaturation at 95°C for 3 minutes, denaturation at 95°C for 20 seconds, and annealing/extension at 62°C for 40 seconds, followed by 40 cycles. Taking U6 as the internal reference, relative expression of miR-144 was calculated by the $2^{-\Delta\Delta}$Ct method.

Cell culture and construction of transfected stable cell lines

A549 cells were cultured in DMEM medium containing 10% FBS and cultivated in an incubator with 5% CO_2 at 37°C. Cells in the logarithmic growth phase were harvested for experiments. Cells were randomized into three groups: the blank control group, lentivirus NC infection group (Lenti-NC group, A549 cells were transfected by empty vector (lentivirus)), and miR-144 lentivirus overexpression group (Lenti-miR-144 group, Lenti-miR-144 virus solution was added after cell adherence. After 6 hours of transfection, the virus solution was removed by washing the cells with PBS solution. The medium was then replaced with a normal culture medium. The lentiviral vector transfected stable cell lines were established after 2 weeks of purinomycin screening).

Proliferative activity of A549 cell lines tested by CCK-8

Human A549 lung cancer cell lines of each group were seeded in 96-well plates at a density of 5 * 10^4/mL. The culture medium was added. Cells were cultivated in an incubator with 5% CO_2 at 37°C for 72 hours. After 10 μL of LCCK-8 was added into each well, the cells were incubated for another 4 hours. The optical density (OD) value of each well was read at 450 nm using an enzyme-linked immunosorbent assay. The mean OD of 5 wells was calculated.

Colony formation assay

A549 cells of each group in the logarithmic phase were seeded into 6-well cell plates at a density of 100 cells per well and incubated in a cell incubator with 5% CO_2 at 37°C for 2 weeks. Subsequently, the medium was removed. The cells were fixed with 4% paraformaldehyde for 15 minutes. After washing, they were stained with 0.1% crystal violet for 10 minutes. After the cells were rinsed with double distilled water to become colorless and dried at room temperature, clones were counted. Colony formation rate (%) = Number of clones/Number of inoculum cells * 100%.

Apoptosis of A549 lung cancer cells tested by TUNEL

Human A549 lung cancer cells of each group were seeded in 12-well plates at a density of 3 * 10^4/mL. After 72 hours of culturing, the cells were fixed with 4% neutral formalin for 10 minutes, hydrated with 100%, 95% and 70% gradient alcohol for 5 minutes, respectively, and washed with PBS for 10 minutes. Subsequently, TUNEL staining was performed, according to manufacturer instructions. Stained A549 lung cancer cells of each group were visualized and counted in 5 randomly selected fields of view under an inverted microscope. Apoptosis rates of the cells were then calculated. Apoptosis rate = Number of apoptotic cells/total number of cells * 100%.

Experiment of subcutaneous xenograft tumors in nude mice

Human A549 lung cancer cell lines of each group were collected. Cell concentrations were adjusted to 5 * 10^6/mL. Cell suspension (0.2 mL) was inoculated subcutaneously on the tail side of the back of nude mice. The nude mice in each group were observed for their daily activities after inoculation. Maximum and minimum diameters of the tumors were measured using a vernier caliper once a week. Changes in the subcutaneous tumor size of each group were observed.

Statistical analysis

All data were analyzed with the use of SPSS statistical software, version 19.0. Measurement data are expressed as mean ± standard deviation. Pairwise comparisons across the groups were performed by t-tests, while comparisons among the three groups were made by one-way ANOVA. The size of xenograft tumors at different time points was compared using repeated measures ANOVA, followed by a post-hoc Bonferroni's test. Categorical data were analyzed by Chi-squared test or Fisher's exact probability test. *P*-values less than $P<0.05$ indicate statistical significance.

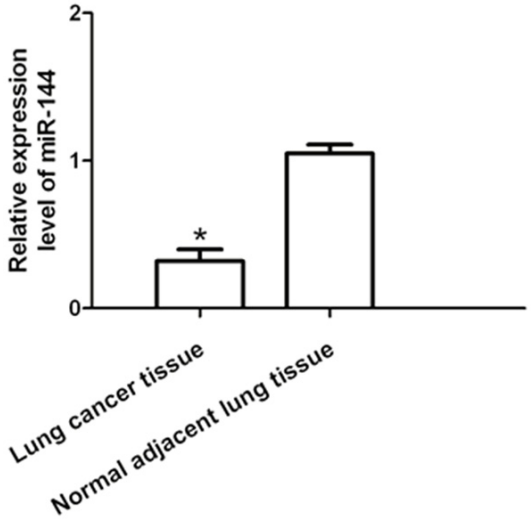

Figure 1. Relative expression of miR-144 in lung cancer tissues. *P<0.001 for comparison with normal adjacent lung tissues.

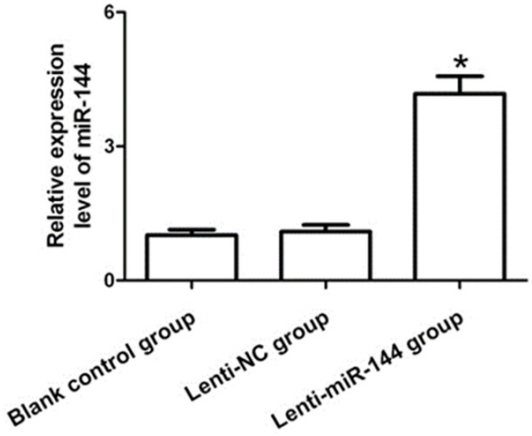

Figure 2. Relative expression of miR-144 in A549 lung cancer cells in each group. *P<0.001 for comparison with the blank control and Lenti-NC groups.

Results

miR-144 expression in lung cancer tissues

qPCR found that expression of miR-144 was 0.32±0.08 in lung cancer tissues, markedly lower than that in normal adjacent lung tissues (P<0.001; **Figure 1**).

Expression of miR-144 in transfected cells A549 with lentiviral vectors

miR-144 expression of A549 cells in the Lenti-miR-144 group was significantly higher, compared to that in the blank control group and Lenti-NC group (both P<0.001). However, miR-144 expression was insignificantly different between the blank control group and Lenti-NC group (P>0.05; **Figure 2**).

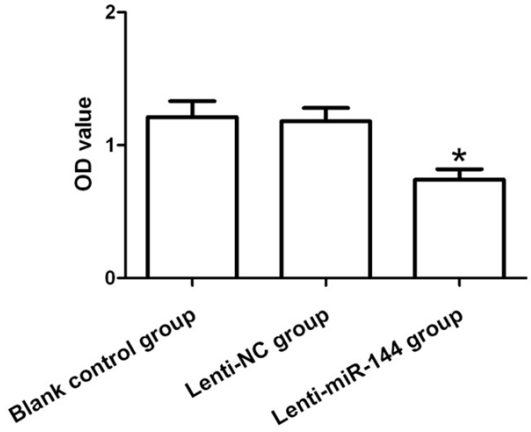

Figure 3. Proliferative activity of A549 lung cancer cells of each group after 72-hour culturing, tested by CCK-8 assay. *P<0.001 for comparison with the blank control and Lenti-NC groups.

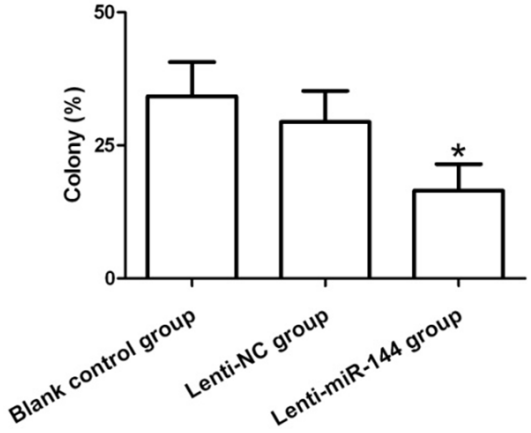

Figure 4. Comparison of colony formation rates of A549 cells in each group. *P<0.001 for comparison with the blank controls and Lenti-NC groups.

Proliferative activity of lung cancer A549 cells of each group

After 72 hours of cell culturing, findings of CCK-8 assays indicated that OD values of the blank control and Lenti-NC groups were (1.21±0.12) and (1.18±0.10), respectively. There were no significant between-group differences (P>0.05). The OD value in the Lenti-miR-144 group was 0.74±0.08, substantially lower than blank control and Lenti-NC groups (both P<0.001; **Figure 3**).

Table 1. Comparison of xenograft tumor size of each group (mm^3)

Testing factor	Group	Case (n)	1 w	2 w	3 w	4 w
Xenograft tumor size	Blank control group	10	61.43±10.82	156.76±35.19	330.64±63.57	645.08±81.52
	Lenti-NC group	10	69.12±12.35	165.93±40.45	361.28±70.06	711.43±89.65
	Lenti-miR-144 group	10	24.65±6.28*	50.73±11.29*	98.74±39.22*	187.16±46.38*
P value			<0.001	<0.001	<0.001	<0.001

Note: *P<0.001 for comparison with the blank control and Lenti-miR-144 groups.

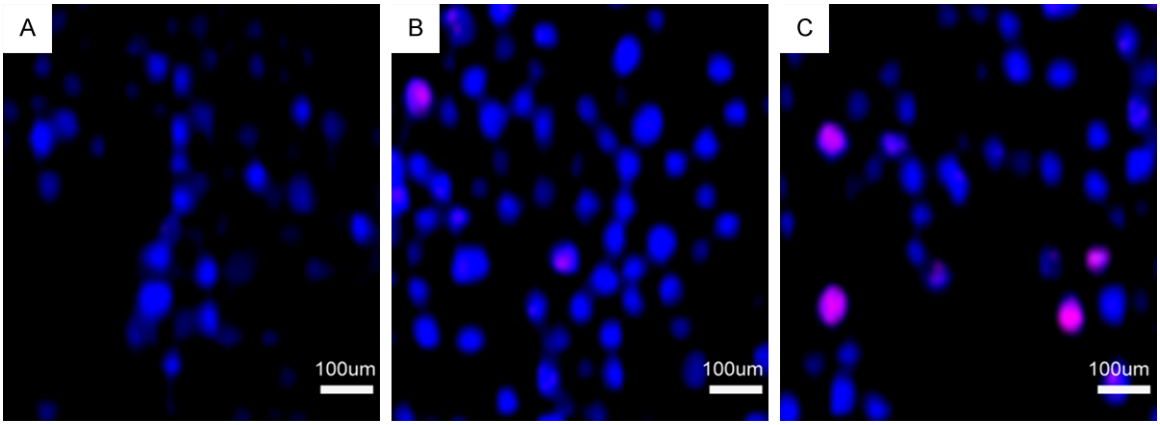

Figure 5. Apoptosis of A549 lung cancer cells in each group detected by TUNEL. A: blank control group; B: Lenti-NC group; C: Lenti-miR-144 group.

Colony formation of A549 cells in each group

The colony formation rate (29.38±5.84%) in the Lenti-NC group was insignificantly different than that (34.21±6.43%) in the blank control group (P>0.05). However, the colony formation rate (16.52±4.96%) in the Lenti-miR-144 group was significantly lower than those in blank control and Lenti-NC groups (both P< 0.001; **Figure 4**).

Apoptosis of A549 cells in each group

After 72 hours of cell culturing, TUNEL assay showed that apoptotic rates of A549 lung cancer cells in blank control and Lenti-NC groups were (2.27±0.14)% and (3.68±0.27)%, respectively. There were insignificant differences between the two groups (P>0.05). The apoptosis rate (12.45±0.82%) of the Lenti-miR-144 group was significantly higher than those of blank control and Lenti-NC groups (both P< 0.001; **Table 1** and **Figures 5**, **6**).

Xenograft tumor size in each group

None of the nude mice in the three groups were agitated, depressed, or showed abnormal emotions. No nude mice died. Repeated measures ANOVA suggested that there were significant differences in the size of xenograft tumors at 1 week, 2 weeks, 3 weeks and 4 weeks among the three groups (all *P<0.001). The size of xenograft tumors in the Lenti-miRi-144 group was significantly smaller than that of blank control and Lenti-NC groups at 1 week, 2 weeks, 3 weeks, and 4 weeks, respectively (all *P<0.001). No between-group differences were seen, however, between the blank control group and Lenti-NC group (P>0.05; **Table 1**).

Discussion

Lung cancer is the most frequent primary malignant tumor of the lung. Patients with lung cancer have poor prognosis despite receiving surgeries, radiotherapy and chemotherapy, and other comprehensive treatment. Lung cancer is characterized by high malignancy, aberrant proliferation, and high recurrence rates, presenting a great challenge to patients. Studies have demonstrated that lung cancer is like other malignant tumors. Onset and development include a pathological process that reflects the co-work of epigenetics and genetics (two major mechanisms) and involves multiple genes and accumulated multiple-stage variations [20].

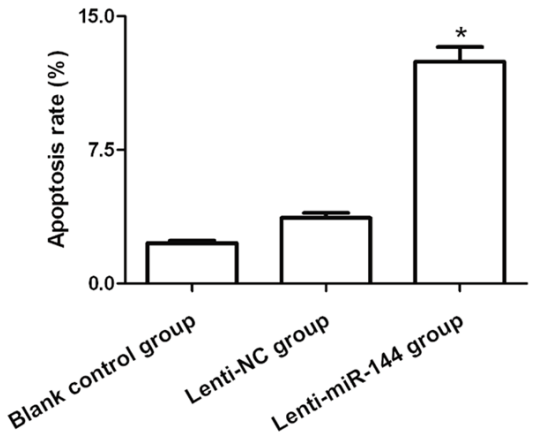

Figure 6. Apoptosis rates of A549 lung cancer cells in each group. *P<0.001 for comparison with the blank control and Lenti-NC groups.

Failure in treating lung cancer has been generally attributed to tumor recurrence. Therefore, how to suppress the biological behavior of lung cancer cells effectively is one of the key factors in the management of lung cancer.

MiRNAs are a class of endogenous non-coding small RNAs, highly conserved in evolution. MiRNAs regulate the expression of target genes by binding complete or incomplete base pairing to the 3' untranslated regions (3'-UTR) of target mRNAs and inhibiting translation or degradation of target mRNAs, thereby regulating cellular proliferation, migration, differentiation, growth and development, and other vital activities. They are involved in the pathophysiological processes of a range of diseases [21, 22]. Studies have reported that aberrant expression of miRNAs is closely associated with the biological behavior of tumor cells, such as proliferation, migration, invasion, and apoptosis, playing a key role in the occurrence and development of tumors [23-25]. Recent literature has indicated the downregulation of miR-26a expression in lung cancer and of miR-143 in colon cancer [26, 27]. These new findings of miRNAs bring new insight and targets for diagnosis and treatment of lung cancer. Recently, research on regulation of tumor growth by miRNA, which is mediated by lentiviral overexpression vectors, has made some progress in animal experiments [28]. In this study, lentiviral vector-mediated transfection of miR-144 was selected to interfere with the biological behavior of lung cancer cells, with an aim of providing an experimental basis for the treatment of lung cancer.

miR-144 regulates a sea of physiological and pathological processes in the body and plays an anti-oncogene role in a variety of tumors. miR-144 inhibits proliferation and migration of tumor cells, promoting apoptosis. However, its role in lung cancer is relatively unknown. One study reported that, as a tumor suppressor gene, miR-144 showed a low expression in breast cancer. Overexpression of miR-144 could inhibit proliferation, invasion, and metastasis of tumor cells [29]. In this study, expression of miR-144 in the samples of lung cancer tissue was tested by qPCR. Findings indicated that miR-144 expression was significantly lower in lung cancer tissues than in normal adjacent lung tissues, suggesting that miR-144 may be used as a predictor for severity of malignancies and act as a tumor suppressor gene in lung cancer. Guo et al. reported that, as a tumor suppressor gene, miR-144 could target EZH2 and regulate Wnt signaling pathways to inhibit proliferation of bladder cancer cells [30]. Cao et al. also stated that miR-144 played an inhibitory role in the onset and development of liver cancer. miR-144 was low-expressed in liver cancer tissues. After miR-144 expression returned to normal levels, miR-144 significantly suppressed proliferation, migration, and colony formation of liver cells [31]. In addition, lentiviral overexpression vectors harboring miR-144 were transfected into A549 lung cancer cell lines. Results showed that miR-144 expression increased significantly after transfection of overexpressed miR-144 lentiviral vectors. Overexpressed miR-144 significantly suppressed proliferation, cloning, and apoptosis of A549 lung cancer cell lines. Finally, animal experiments revealed miR-144 inhibits growth of xenografts of lung cancer, implying that miR-144 also plays a role of tumor suppressor gene in A549 lung cancer cells. Lv et al. confirmed that overexpression of miR-144 could inhibit proliferation and migration of liver lung cells [32]. Tao et al. also proved that miR-144 suppressed growth of cervical cancer cells, consistent with findings of the present study [33].

In conclusion, low expression of miR-144 in lung cancer tissues could significantly inhibit proliferation, cloning, and apoptosis of lung cancer cells, while upregulation of miR-144 expression may become a new target for treatment of lung cancer. However, the mechanisms of miR-144 in lung cancer remain unknown. Additional studies are required in the future.

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Disclosure of conflict of interest

None.

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