RESEARCH ARTICLE

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The Effect of MicroRNA-409-3p for Treatment and Response to Tumor Proliferation of Lung Cancer Cell Lines (In Vitro)

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Abstract

Objective: Monitoring the result of miR 409 3p and its response to tumor proliferation and its mechanism of action on some types of lung cancer in vitro (A549 cell line). **Methods:** Two A549 cell line group negative control group with oligonucleotide cultured under conventional conditions and transfected with positive control nucleotides. Experiments Based on the control group, chemically synthesized miR 409 3p mimics were used in the positive group with liposome transfection to construct A549 cells with high miR-409-3p expression. **Result:** miR4093p expression was estimated using the qPCR method in the two groups after 48 h. Later, the miR-409-3p expression in A549 cells obviously increased significantly with a positive attitude in the positive control group that was transfected by miR-409-3p (mimics) ($P<0.20$). As a result of this investigation, a significant increase in the percentage of total cell apoptosis was significantly increased in the positive group compared to the control group (22.68% ± 4.62%), (7.79%±1.94%) respectively, ($P<0.05$). However, in terms of the G1 phase, the rate is obviously low compared to the control group (40.22%±5.36%); (56.08%±5.21%) ($P<0.05$). endogenous ELF2 was considerably reduced after overexpression of the miR-409-3p mimic ($P<0.05$). **Conclusion:** miR-409-3p may prevent non-small cell lung cancer (NSCLC) by affecting ELF2 transcription and other cellular regulators to regulate A549 cell division and induce apoptosis.

Keywords: A549 cell line- microRNAs- miR-409-3p- non-small cell lung cancer (NSCLC)- proliferation- ELF2

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Introduction

Research in the field of cancer treatment increased significantly in finding a drug or an effective way to stop tumors with no side effects (Azhar et al., 2021), miRNA which is a new class of small non-coding RNA molecules that pair with the 3′ non-coding region of target mRNA, resulting in the deterioration of the mutated mRNA or inhibition of translation, thus controlling and increasing the level of some other people (Fu et al., 2020; Josson et al., 2014), Cancer is usually caused by mutation, and this mutation causes damage to the cellular gene and depresses the tumor protected gene, such as P53, with an increase in other mutagenic proteins (A Al-Hassany et al., 2021; Fu et al., 2020). It is a miRNA with a tumor suppressor effect discovered in recent years and has been found in different types of tumors, such as gastric cancer , bladder and prostate cancer (Yuan et al., 2019; Zhang et al., 2020). Down-regulation of expression targets a variety of genes to regulate tumor proliferation, invasion, and spreading metastases (Bagherian et al., 2021). In the cell, there are several regulatory proteins such as BAX and Bcl2, cyclin D, and P53; P53 works as a tumor suppressor protein regulated by the theTP53 gene, this protein involves controlling cell DNA and preventing cancer development by either apoptosis or modifying and renewing the mutated gene (Alkuraishy et al., 2017;Yudhani et al., 2019) . In the case of DNA mutation P53 forced to increase the expression of the BAX protein, which eventually leads to apoptosis of mutated cells, Bcl2 in the antiapoptotic protein works by suppressing the effect of BAX and increasing the incidence of cancer of several studies such as this regulatory protein with cancer (protection of propagation) but any defect in cellular DNA can cause a mutation in p53 and BAX expression by decreasing its expression, and this will increase the incidence of cancer (Figure 1), however, limited articles describe this effect of miR 409 3p in this occurrence and propagation of NSCLC (Al-hassany et al., 2021). Influence of target genes on lung cancer and how it works and its effect on the level of the P53 and BAX protein (Alkuraishy HM; et al., 2017; Alzobaidy et al., 2021).

The Aim of this study was to monitoring the result of miR 409 3p and its response to tumor proliferation and its mechanism of action on some types of lung cancer in vitro (A549 cell line

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Materials and Methods

Materials

cell line A549 (Cell Resource Center, Iraqi medical and genetic center, Baghdad, this type of cells is one of the best human non-small cell lung cancer cell lines used).

Reagent

DMEM medium (HyClone company); fetal calf serum (FCS) (Hangzhou Sijiqing Bioengineering Co., Ltd.) miR-409-3p as in (Habibzadeh et al., 2022).

Mock and negative control nucleotides (Shanghai Gema Pharmaceutical Technology Co., Ltd.); Luciferase expression vector (Ambion Co.); Dual luciferase reporter gene analysis kit (Promega Co.); -Aldrich Company); Flow flow apoptosis detection kit (Nanjing Keygen Biotechnology Development Co., Ltd.); Mouse anti-human ELF2, rabbit anti-human β-actin (CST Company); Lipoibctamine2000 transfection reagent, RIPA3 lysate, Invitrogne Trizol kit and by RT-qPCR ("real-time quantitative reverse transcription polymerase chain reaction kit") ; concentration of protein assay method such as (Bradford kits) and ECL chemiluminescence-based immunodetection kit (Shanghai Biyuntian Biotechnology Co., Ltd.).

Methods

Cell grouping and treatment

DMEM medium composed of approximately 10% fetal serum, penicillin-streptomycin 100 units per ml was used, cells were placed in humidity and incubated conditions at 37 ° for a day before transfection, and after which cells were seeded in 6 wells at a concentration of about 1×10^5 cells/well, and when cell confluence reached 70%-80%, 100 nmol / L MicroRNA-409-3p mimicking this group will be a positive control. The second (- control) was transfected into cells with lipofectamine in a 10 μL system After 6-8 h, it was replaced with cell culture medium containing serum and double antibody. The expression of the target gene was detected after 48 h. (Khan et al., 2019; Yudhani et al., 2019).

RT-qPCR to identify miR 409 3p expression: Total ribonucleic acid (RNA) and extraction using the Trizol technique and Prime Script RT

The mature miRNA converted to cDNA (by reverse transcription) then the quantity evaluated by the ABI7500 quantitative fluorescence PCR instrument. We used the SYBR-Green method to estimate cell expression (MicroRNA-409 3p). Moreover, final sequences of the primer have been as described in the following: upstream of miR-409-3p: 5'-GCGAATGTTGCTCGTGGA-3', Among them, 5'GTGCAGGGTCCGAGGT-3 '. The internal reference U6 upstream: 5 ′ AAGAGCCCTGTGGTCG 3 ′, downstream: 5 ′ CATTTCAAAGCACTTCCCCT 3 ′. The relative expression of $^{\Delta}$miR-409-3p was calculated using the $2^{-\Delta\Delta Ct}$ method. CtmiR-409-3p-CtU6) experiment-(CtmiR-409-3p-CtU6) control group (Khan et al., 2019;Liu et al., 2022).

Western blot detection of protein expression: RIPA cleavage method

The concentration of total cell protein was measured using the Bradford technique,10 μg total cell protein was separated by SDS polyacrylamide condensation electrophoresis, after western polyvinylidene fluoride Western blot membrane was used, proteins were transferred to this membrane , blocked with 5% bovine serum albumin (BSA) for approximately 1 hour at 25°C (room temperature) on a shaker, and mouse anti-human ELF2 (1:1,000) and BCL2 were added, respectively. (1:1,000), BAX (1:1,000),

CyclinD1 (1:1,000), rabbit anti-human β-actin (1:1,000) was placed at 25°C at room temperature for 1 hour and then the horseradish peroxidase labeled secondary antibody was added Goat anti-rabbit secondary antibody (1:2,000) then we were incubated for a second time at room temperature for one hour prior to detection by chemiluminescence (ECL) (Naji et al., 2022).

To detect cell proliferation, the MTT test was used: Cells were harvested 48 hours after transfection

In relative density, about 3500 cells/well A594 cells have been seeded in 96-well plates, then fresh MTT solution diluted in buffer was added, then this sample was incubated, the Colorimetric assay carried out for each 24, 48, 72, and 96 hours after inoculation. This colorimetric assay calculated the absorbance value (A) at wave length 570 nm was measured to compare the proliferation of cells in each group (Nazarian et al., 2019).

The use of a flow cytometer for the detection and counting of cell cycle and apoptosis

Take A549 cells 48 hours after transfection, adjust cells to 1×10^6 cells/mL, perform apoptosis staining according to the instructions of the apoptosis detection kit, and use flow cytometry (BD FACS Calibur) to detect.

Target gene prediction

To predict target genes, use the target gene prediction websites TargetScan and miRanda, and look for genes with 3 'UTR binding sites to miR-409-3p (Nazarian et al., 2019).

Vector construction and detection of dual fluorescent gene reporter: According to the target

Synthetic primers from the ELF2 'UTR gene sequences 3' were considered and the amplified fragment contained the predicted oncogene ELF2 combined with miR-409-3p.

The conserved sequence was amplified by PCR using 293T cell DNA as a template (Wan et al., 2014).

The PCR product and the luciferase expression vector pMIR-REPOR were digested with restriction endonuclease and then ligated, transformed into DH5α competent cells of DH5, and the recombinant plasmid was prepared with the name pMIR-REPOR-ELF2-WT; ELF2 and ELF2 were constructed by a similar method. A549 cells were normally cultured in DMEM, surrounded with 10% fetal calf serum at normal cell growth temperature and stable carbon dioxide approximately 37°C and 5% carbon dioxide (Wan et al., 2014).

The liposome lipofectamine 2000 method was used for transfection and the operation was performed according to the instructions. The dual luciferase reporter was used. Gene analysis kit to detect fluorescent signal (Thangavelu et al., 2022).

Statistical analyses

After collecting the evaluation of the result, the second step is to assess the standard deviation typically found (x ± s) in these two groups using SPSS 16.0. statistical program. Data were also calculated using the t-test for the two groups (Zhang et al., 2020).

Results

Changes in miR 409 3p expression

The express of transfected cells with miR 409 3p mimic approximately (4.46% ± 1.98%), This result was significantly more than the negative control oligonucleotides 0.52%±0.06% of the acid group (P<0.05).

Effect of miR 409 3p overexpression on A549 cell spread

After increasing miR-409-3p expression, the cell growth and production rate was higher than that of the control grouphis effect show in (Table 1).

*The effect of increased expression of miR-409-3p on apoptosis of NSCLC A549 cell*s

The level of apoptosis has increased significantly as a result of this overexpression of miR-409-3p (P<0.05) (Table 1). This overexpression results in an increase in the level of BAX, which is one of the important regulatory proteins that worked as apoptosis or antitumor (tumor suppressor gene) the in positive controlled cells increasing the level of P53 will increase the level of BAX that eventually leads to apoptosis, as shown in (Figure 2), however, the level of BAX suppresser level (Bcl-2) decreased significantly in the Bcl2 protein that acts as an anti-apoptotic regulator in miR-409-3p mimic treated cells

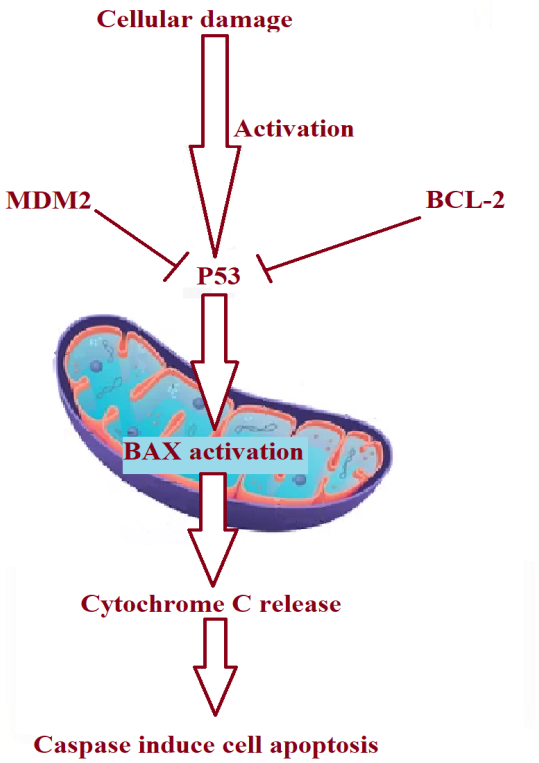

Figure 1. Shows the Role of P53-BAX-BCl2 in the Apoptosis Pathway

(P<0.05) (Table 2, Figure 1) (Feng et al., 2021).

Western blot analysis and Cyclin-D1 level

Effect of overexpression of miR-409-3p on the NSCLC A549 cell line cycle / stage (P<0.05) (Table 3), and the level of cyclin cyclinD1 was significantly reduced (P<0.05) (Figure 3,4).

Overexpression of MicroRNA 409 3p

In the ELF2 oncogene The prediction of the impact target gene shows that the ELF2 oncogene is miR-409-3p.

Table 1. Effect of MiR-409-3p Overexpression on A549 Cell Cancer and Propagation in the Cancer Cell Line (x±s, A value, n=96)

Group	24 H	48 H	72 H	96 hours
Contrast oligonucleotides	0.24±0.08	0.36±0.11	0.79±0.2	1.08±0.23
miR-409-3p Mimic	0.22±0.09	0.30±0.11	0.48±0.13	0.79±0.19*

*P<0.05 compared to contrast oligonucleotides , H-hours

Table 2. Apoptosis Ratio of miR-409-3P Expression in A549 Cells (x±s, %, n=96)

Group	Early stage	Late period	Apoptosis
Contrast oligonucleotide's	4.11±1.94	3.67±2.09**	7.79±1.94**
miR-409-P3 mimic	7.35±2.89	15.31±6.54	22.68±4.62

*P<0.05, **P<0.01 compared to contrast oligonucleotide

Table 3. Effect of Overexpression of miR-409-3p on the Expression of Cyclin D1, Bax, Bcl-2 (x±s, A value, n=96)

Contrast oligonucleotides	3.47± 0.82	2.84±0.23	5.26±1.02
miR-409-3P mimics	1.31±0.55*	7.19±1.53**	2.82±0.73*

*P<0.05, **P<0.01

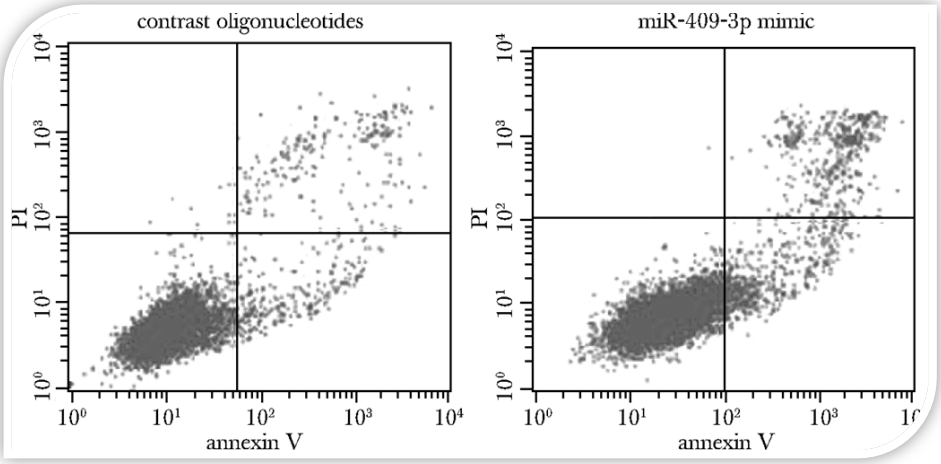

Figure 2. Effect of miR-409-3p Overexpression on Apoptosis of A549 Cells

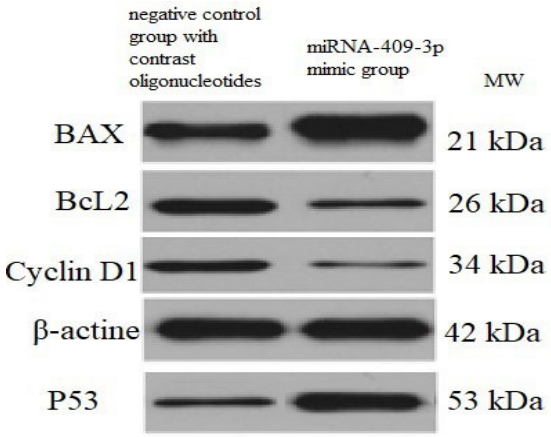

Figure 3. Western Blot Analysis of Cyclin D1, BAX, Bcl-2, and p53 Protein in Both Groups-

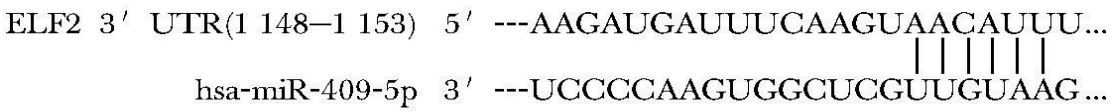

Figure 4. Software Prediction of the miR-409-3p Target Gene ELF2 Sequence

Dual-luciferin reporter assay to verify that miR-409-3p targets ELF2.

When the wild-type plasmid pMIR-REPOR-ELF2-WT and miR-409-3p were co-transfected to 293T cells, the relative luciferase activity was 0.51 When the mutant plasmid pMIR-REPOR-ELF2-Mut and miR-409-3p were transfected to 293T cells, the results were similar to those of the control group. The relative activity of luciferase was 1.07 ± 0.01. The results illustrate that miR-409-3p could suppress the level of ELF2 (Filardi et al., 2022; Al-Kuraishy et al., 2022).

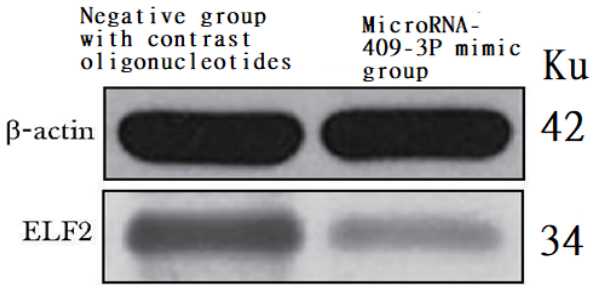

Figure 5. Effect of miR-409-3P Overexpression in ELF2

Table 4. Level of Expression of the ELF2 Protein Expression Level of ELF2 by Over-expression miR-409-3p (x±s A value n=96).

Group	ELF21	B- actin
Contrast oligonucleotide's	4.35±2.15	5.37±6.32
miR-409-3P mimics	2.18±1.96*	5.84±2.51*

*P<0.05, **P<0.01 compared to contrast oligonucleotide

Discussion

miR4093p is a miRNA with a tumor suppressor effect. The expression of miR-409-3p is significantly decreased in tumor cell lines, however, if we induce overexpression of miR-409-3p, it can inhibit and suppress cancer by decreasing the expression level of the finger protein (Al-Kuraishy et al., 2022;Al-Hussaniy et al., 2021).

However, several studies also show that miR-409-3p is highly expressed in bone metastases of prostate tumors and is linked to "progression-free" survival in life in some cases (Al-hussaniy et al., 2022b). Overexpression of miR-409-3p in prostate cancer in vitro promotes tumor growth and bone metastasis and decreases the expression of this microRNA associated with a poor prognosis (Akeel Naji, 2021; Cao et al., 2016). However, miR-409-3p plays an important role in the development of NSCLC.

The role of miR-409-3p in transfected cells

Cells transfected with miR-409-3p mimics showed a significant increase in MicroRNA expression compared to the negative control oligonucleotide group (P<0.20). Furthermore, as a result of this, there is a significant suppression of proliferation ability, indicating that miR-409-3p has the effect of inhibiting tumor growth (Ursu et al., 2020; Rashid et al., 2018). Additionally, flow cytometry along with supplement V/PI double staining showed that after increasing the level of miR-409-3p it significantly suppresses cancer cells, Compared to cells transfected with the control group, the proportion of total cell apoptosis also increased. The above results and literature reports indicate that miR-409-3p mRNA play a significant role in the tumor suppressor gene in (NSCLC). miR-409-3p can inhibit the growth of NSCLC non-small cell lung cancer by regulating tumor cell proliferation and apoptosis. In this investigation, ELF2 was also predicted as the target of miR-409-3p from hundreds of potential target genes (Al-hussaniy et al., 2021; Al-hussaniy et al., 2022 a).

Furthermore, overexpression of ELF2 can also directly promote tumor cell proliferation, indicating that ELF2 plays the role of an oncogene in the process of tumorigenesis and development. The protein expression level was significantly reduced, suggesting that ELF2 is the target gene for miR-409-3p and may inhibit cell proliferation by controlling the transcription level of ELF2. miR-409-3p can suppress the proliferation of (NSCLC) A549 cells and promote their programmed cellular death (Abdulameer et al., 2022). This mechanism is related to the regulation of the expression of the ELF2 oncogene and increases the expression of BAX and P53.

Author Contribution Statement

All work is done by Hany Akeel Al-Hussaniy.

Acknowledgments

Approval

If it was approved by any scientific Body.

Ethical Declaration

This research receives ethical approval number 202112 from Iraqi Medical Research Center - Baghdad - Iraq.

Study Registration

not required

Conflict of Interest

The authors declare that there is no conflict of interest.

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