

Original Article

## The tumor suppressor effect of miRNA 200-c-3p on A549 non-small cell lung cancer cell line is associated with down-regulation of programmed cell death ligand 1; an immune-check point mechanism

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### Abstract

MiRNA 200-c-3p has varying functions in different tumor types, whether tumor suppression or promotion. Comprehensive assessment of its function in non-small cell lung cancer (NSCLC) together with its effect on antitumor immune response have not been declared before. We aimed to explore the effect of replacement and suppression of miRNA 200-c-3p on (NSCLC) and its impact on immune checkpoint function and subsequently antitumor immunity. MiRNA 200-c-3p mimic/inhibitor was transfected into the A549 (NSCLC). A 549 (NSCLC) viability was done by trypan blue staining and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Flowcytometric analysis was done for apoptosis detection. Real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis were used to study its effect on relative gene expression and relative protein level of programmed cell death ligand 1 (PD-L1). Finally, co-culture with isolated and activated T cells was performed. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. Decreased cell viability, increased apoptosis, reduced PD-L1 relative gene expression and its relative protein level, together with enhanced T cell cytotoxicity towards tumor cells were detected after miRNA 200-c-3p mimic transfection of A549 NSCLC cell line. However, these results were reversed in miRNA 200-c-3p suppression. MiRNA 200-c-3p had a tumor suppressive effect in non-small cell lung cancer cells which might be through down regulation of PD-L1 relative gene expression, and it may be used as a new target to improve immune checkpoint dysfunction.

**Keywords:** miRNA 200-c-3p, PD-L1, T cells, non-small cell lung cancer (NSCLC), immune-check point, immunotherapy

### 1. Introduction

Lung cancer is the most prevalent and deadly cancer worldwide [1]. The age-standardized rate (ASR) of lung cancer in Egypt is 7.6 [2]. Lung cancer incidence among Arabs is alarmingly increasing. It is expected that by 2030 there will be 1.8-fold increase in cancer incidence in the Eastern Mediterranean Region and Gulf states [3]. Eighty-five percent of patients with lung cancer have a group of microscopic subtypes collectively known as non-small cell lung cancer (NSCLC); while the remaining 15% are small cell lung cancer (SCLC)[4, 5]. NSCLC is more resistant to chemotherapy in comparison to SCLC [6]. Unfortunately, treatment of lung cancer is still unsatisfactory.

Despite accelerating improvement of multiple therapeutic approaches, the 5-year overall survival rate for patients with lung cancer is still poor [7].

Immunotherapy which is the treatment modality that evokes the immune system to eliminate cancerous cells, has joined the therapeutic arsenal of conventional therapies for lung cancer [8]. Immune checkpoints are present on T cells, antigen-presenting cells (APCs) as well as cancerous cells. They are responsible for modulating the homeostasis of both the co-stimulatory and the co-inhibitory signals which are critical to immune tolerance [9]. Thus, immune checkpoint therapy targeting co-inhibitory pathways in T cells can enhance anticancer T cell cyto-

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toxicity-mediated immune response. Currently, one of the well-studied immune checkpoints is programmed death-1 (PD-1)/PD-ligand 1 (PD-L1). PD-1 is located on the surface of T-cells, whereas PD-L1 is found on tumor cells. The interaction of PD-L1 and its receptor PD-1 results in cancer cells evasion from immune destruction [10–12].

Of note, several miRNAs suppress the expression of PD-L1 such as miRNA 34, miRNA 197, and miRNA 200 family [13–15]. As known, micro RNAs (miRNAs) are a group of single-stranded non-coding RNAs that are small in size ranging from 20–22 nucleotides. They are highly conserved evolutionally among species [16, 17]. The miRNA 200 family consists of two clusters of miRNAs. The first cluster contains miRNA 200a, miRNA 200b and miRNA 429. The second cluster contains the following pre-miRNAs; miRNA 200c and miRNA 141. [18, 19].

The miRNA 200-c-3p is the most widely studied strand of the miRNA 200-c family member and it is more biologically active than the miRNA 200-c-5p strand. It was found that miRNA 200-c-3p can act as a positive or negative regulator of epithelial-mesenchymal transition (EMT) in different cells and tissue types [20–22]. MiRNA 200-c-3p can modulate tumor proliferation, invasion and metastasis by acting differentially on the EMT process [23–25]. Due to its differential expression and actions in various tissue and cells, miRNA 200-c-3p is reported as being either a tumor-suppressive or an oncogenic miRNA in many cancer types [24, 25].

Different modalities and combinational therapies regarding immune checkpoint inhibitors are being implemented nowadays, but there is a risk of developing multiple adverse effects which might be serious. Immune-related adverse events (IRAEs) are common with occurrence rate reaching up to 70% with anti-PD-1/PD-L1 therapy [26, 27]. So, there are increasing efforts to investigate other ways to establish immune check point deregulation that might evoke less adverse effects.

The present study aimed to evaluate the effect of miRNA 200-c-3p mimic/inhibitor transfection in A549 NS-CLC cell line as a future target to immunotherapy.

## 2. Materials and Methods

### 2.1. Study design

The study protocol was approved by the Faculty of Medicine Ain Shams University review board and research ethics committee. The study was conducted on non-small cell lung cancer cell line A549 in Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Ain Sham University. The experimental design included 7 study groups, namely: A549 untreated cells, A549 Mock cells (cells transfected with transfection reagent only), A549 cells transfected with miRNA 200-c-3p mimic, A549 cells transfected with miRNA 200-c-3p inhibitor, A549 cells transfected with miRNA mimic-positive control, A549 cells transfected with miRNA inhibitor-positive control, and A549 cells transfected with miRNA mimic/inhibitor-negative control. Viability, proliferative ability and apoptosis in non-small cell lung cancer were evaluated. The molecular effect of miRNA-200c-3p on the expression of PD-L1 relative gene expression and relative protein level was measured. Finally, the effect of miRNA 200-c-3p mimic/inhibitor on T cell anti-tumor cytotoxicity was investigated.

### 2.2. Cell culture growth and maintenance

The non-small lung cancer A549 cell line was purchased from NAWAH SCIENTIFIC Company, Cairo, Egypt. The cell line was cultured in 100ml Dulbecco's Modified Eagle medium (DMEM) (Catalog No. 11960044, ThermoFisher Scientific, USA) with 10% Fetal Bovine serum (FBS) (Catalog No. 26140079, ThermoFisher Scientific) and 1% penicillin /streptomycin. The cells were grown in vitro as adherent cells in T25 flask, incubated for 48 hours at 37°C and 5% CO<sub>2</sub>.

### 2.3. Cell transfection

The miRNA 200-c-3p inhibitor (Anti-hsa-miRNA 200-c-3p miRNA Inhibitor, Catalog No: AM17000, ) was purchased from ThermoFisher Scientific. MiRNA 200-c-3p mimic (Syn-hsa-miR-200-c-3p miScript miRNA Mimic, Catalog No: MSY0000617), miRNA mimic- positive control (Syn-hsa-miR-1 miScript miRNA Mimic, Catalog No. MSY0000416), miRNA inhibitor- positive control (Anti-hsa miR-1 miScript miRNA Inhibitor, Catalog No. MIN0000416 and Syn-hsa-miR-1 miScript miRNA Mimic Catalog No. MSY0000416) and miRNA mimic/inhibitor- negative control (AllStars Negative Control siRNA, Catalog No. 1027280) were all ordered from QIAGEN, Hilden, Germany. Transfection was performed using the 24-well plates. MiRNA mimic-positive control was used to confirm that the experimental system was working as expected and the mimic was efficiently transfected and caused downregulation of the target gene. MiRNA mimic/ inhibitor -negative control indicates whether the results are nonspecific or specific to the miRNA 200-c-3p mimic/ inhibitor (i.e., Comparison of results from the negative control with results from the miRNA 200-c-3p mimic/inhibitor was used to confirm that the observed results were specific to the miRNA 200-c-3p mimic/inhibitor). MiRNA inhibitor-positive control excludes that the obtained results are due to the effect of other miRNAs (other than miRNA 200-c-3p) that have the same target gene.

In this protocol, one day before transfection,  $1.6 \times 10^5$  cells were seeded per well in a 24-well plate and 500  $\mu$ l of DMEM culture medium containing serum and antibiotics were added. The cells were incubated under normal growth conditions (typically at 37°C and 5% CO<sub>2</sub>). MiRNA 200-c-3p mimic (0.15  $\mu$ l), miRNA 200-c-3p inhibitor (1.5  $\mu$ l), mimic positive- control (0.15  $\mu$ l), inhibitor-positive control (0.15  $\mu$ l) and negative controls (1.5  $\mu$ l) were diluted each with 100  $\mu$ l culture medium without serum followed by the addition of 1.5  $\mu$ l HiPerFect Transfection Reagent for each reaction (Catalog No: 301704, QIAGEN) to form a miRNA/HiPerfect transfection complexes. After complex formation (incubation for 10 min at room temperature 20°C), the complex mixtures were added to the A549 cells in the wells drop-wise[28].

### 2.4. Cell viability by trypan blue assay

Total cell count was recorded by a hemocytometer (Catalog No. 22-600-107, ThermoFisher Scientific), followed by trypan blue staining. The stained and unstained cells were counted. Blue-stained cells are non-viable and unstained cells are viable. The percentage of viable cells was calculated using the following equation: (No. of viable cells/ Total No. of cells) x 100 = % viability[29].

### 2.5. Cell apoptosis detection by flow cytometry (accor-

**ding to manufacturer's instructions.)**

The assay was done using the Annexin V apoptosis detection kit (Catalog No: BMS500FI-100, ThermoFisher Scientific). The harvested cells were washed in phosphate-buffered saline (PBS) and then re-suspended in binding buffer at  $5 \times 10^6$  cells/ mL. Five  $\mu$ L of fluorochrome-conjugated Annexin V were added to 100  $\mu$ L of the cell suspension and incubated for 15 minutes at room temperature. Then cells were washed and re-suspended in binding buffer. Five  $\mu$ L of Propidium Iodide staining solution were added to the cells, and incubated for 15 minutes on ice in the dark. Stained cells were analyzed by flow cytometry within 4 hours. Alive cells are negative for both Annexin V and Propidium iodide, early apoptotic cells are Annexin V positive and Propidium iodide negative, while late apoptotic/necrotic cells are positive for both Annexin V and Propidium iodide.

**2.6. Assessment of A549 cells proliferative activity by (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) MTT assay**

Cell proliferative activity was performed using the Vybrant® MTT Cell Proliferation Assay Kit (Catalog No. M6494, ThermoFisher Scientific). The A549 transfected cells ( $8 \times 10^3$  cells per well) were seeded in 96 well culture plate and incubated at 37°C with 5% CO<sub>2</sub> for 72 hours. Twenty  $\mu$ L of MTT solution were added to each well and the plates were incubated at 37°C and 5% CO<sub>2</sub> for four hours. Then, 100  $\mu$ L of Sodium dodecyl-sulfate-hydrochloric acid (SDS-HCL) was added to the wells. Cell proliferative activity was determined by measuring the optical density at 570 nm on a spectrophotometer (ELx 800; Bio-Tek Instruments Inc., Winooski, VT, USA). Results were expressed in optical density, where levels of optical density are directly proportional to proliferative activity[29].

**2.7. Western immunoblot analysis for detection of PD-L1 relative protein level**

The A549 cells were lysed with the radioimmunoprecipitation assay lysis buffer (RIPA) (Catalog No. 89900, ThermoFisher Scientific). Then, the lysates were centrifuged at 14,000×g for 15 minutes, and the supernatants were harvested for protein assay. After being quantified with bicinchoninic acid (BCA) protein assay kit (Catalog No. 23227, ThermoFisher Scientific), the protein samples were separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Catalog No. 88018, Thermo Scientific membranes). The membranes were blocked with Tris-buffered saline supplemented with 3% BSA, 0.1% Tween® 20 then incubated with primary antibodies at 4°C overnight. The PD-L1 monoclonal antibody (Catalog No. 14-5983-82) and the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Loading Control Antibody (Catalog No. MA5-15738) used as reference protein were all purchased from ThermoFisher Scientific. After binding to secondary antibodies (Biotin- XX goat anti-rabbit IgG (H+L) Catalog No.1305936, ThermoFisher Scientific), the “Qdot R 625 streptavidin conjugate” (Catalog No. W10142, ThermoFisher Scientific) was added on the membrane and was left over night on a rocking platform, then the substrate was discarded. Calculation of protein concentration relative to the internal control protein (GAPDH) was performed using the Image J software. The levels of the targeted pro-

teins were quantified through densitometry[30].

**2.8. Estimation of PD-L1 relative gene expression by real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (according to manufacturer's instructions)**

Total RNA was extracted from A549 cells using miScript RNA Mini kit (Catalog. No. 217004, QIAGEN) according to the manufacturer's protocol. The samples were considered with good RNA quality when RNA: Protein ratio (260:280 ratio) was approximately 1.8. Reverse transcription was performed using miScript II RT Kit (Catalog No. 218160, QIAGEN) following the manufacturer's protocol. The undiluted complementary DNA (cDNA) was transferred to -20°C until later use in RT-qPCR that was performed using QuantiTect SYBR Green PCR kit (Catalog No. 2041413, QIAGEN). QuantiTect Primer Assays for PD-L1 (Catalog No. 249900, ID: QT00056322) and  $\beta$ -actin as a house keeping gene (Catalog No. 249900, ID: QT00095431) both purchased from QIAGEN. Three  $\mu$ L of cDNA was used in a total reaction volume of 20  $\mu$ L. Initial activation was done at 95°C for 15 minutes followed three-steps cycling: denaturation for 15 seconds at 94°C, annealing for 30 seconds at 55°C, and extension for 30 seconds at 70°C, for 40 cycles. Data acquisition was performed during the extension step. The fold change (FC) of PD-L1 gene expression level was normalized to internal control ( $\beta$ -actin) and relative to calibrator (mRNA from untreated cells). The fold change was calculated using the equation  $2^{-\Delta\Delta Ct}$  (Livak & Schmittgen, 2001).

**2.9. Co-culture of A549 transfected cells with activated CD8<sup>+</sup> T cells (according to manufacturer's instructions)**

T cells were isolated from peripheral blood samples, activated and co-cultivated with A549 cells which have been previously treated with miRNA 200-c-3p mimic and inhibitor.

**2.9.1. Polymorph nuclear cells (PMNCs) isolation**

Blood sample was diluted with phosphate-buffered saline (PBS) solution (1:1 dilution). Blood was layered over a Ficoll (Catalog No. 45-001-750, ThermoFisher Scientific) medium, and spun at 833 xg (2125 rpm for 20 min, at 20°C). Cells at the interphase were carefully collected and washed and re-suspended in isolation buffer (PBS which is Ca<sup>2+</sup> and Mg<sup>2+</sup> free supplemented with 0.1% BSA and 2 mM EDTA).

**2.9.2. Isolation of CD8<sup>+</sup> T cells from PMNCs**

CD8<sup>+</sup>T cells were isolated from PMNCs using Dynabeads® Untouched™ Human CD8<sup>+</sup> T Cells (Catalog No. 11348D, ThermoFisher Scientific). 100  $\mu$ L Antibody Mix was added to PMNCs in isolation buffer and incubated for 20 min at 8°C. cells were washed in isolation buffer then 500  $\mu$ L pre-washed Dynabeads were added and incubated for 15 min at 25°C. The tube was placed in magnet (DynaMag™-5, Catalog No. 12303D, ThermoFisher Scientific) for 2 minutes then the supernatant containing the untouched human CD8<sup>+</sup> T cells was collected.

**2.9.3. T cell activation**

The Dynabeads® Human T-Activator CD3/CD28 (Catalog No. 11131D, ThermoFisher Scientific) was used for activation of T cells. Purified T cells ( $8 \times 10^4$ ) in 200

$\mu$ L OpTmizer™ T Cell Expansion serum-free medium (Catalog No. 0080022SA. ThermoFisher Scientific) were placed in a 96-well tissue culture plate then 2  $\mu$ L of pre-washed and re-suspended Dynabeads were added to obtain a bead-to-cell ratio of 1:1. Then cells were incubated in a humidified CO<sub>2</sub> incubator at 37°C for 48 hours. The activated T cells were harvested and directly used.

**2.9.4. Co-culture of A549 transfected cells with activated CD8<sup>+</sup> T cells**

A549 cells transfected with miRNA 200-c-3p mimic and inhibitor were co-cultured with activated CD3/CD28 positive CD8<sup>+</sup> T cells for 72 hours in DMEM media supplemented with 11% penicillin /streptomycin in 96 well plates. The cultured cells were incubated at 37°C and 5% CO<sub>2</sub> for 24 hours then MTT assay was performed as described before.

**2.10. Statistical analysis**

All analyses from experiments were performed using Statistical Package for the Social Sciences (SPSS software version 20). The data were expressed as mean  $\pm$  standard deviation (SD). Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparison test, where  $p < 0.05$  was considered significant.

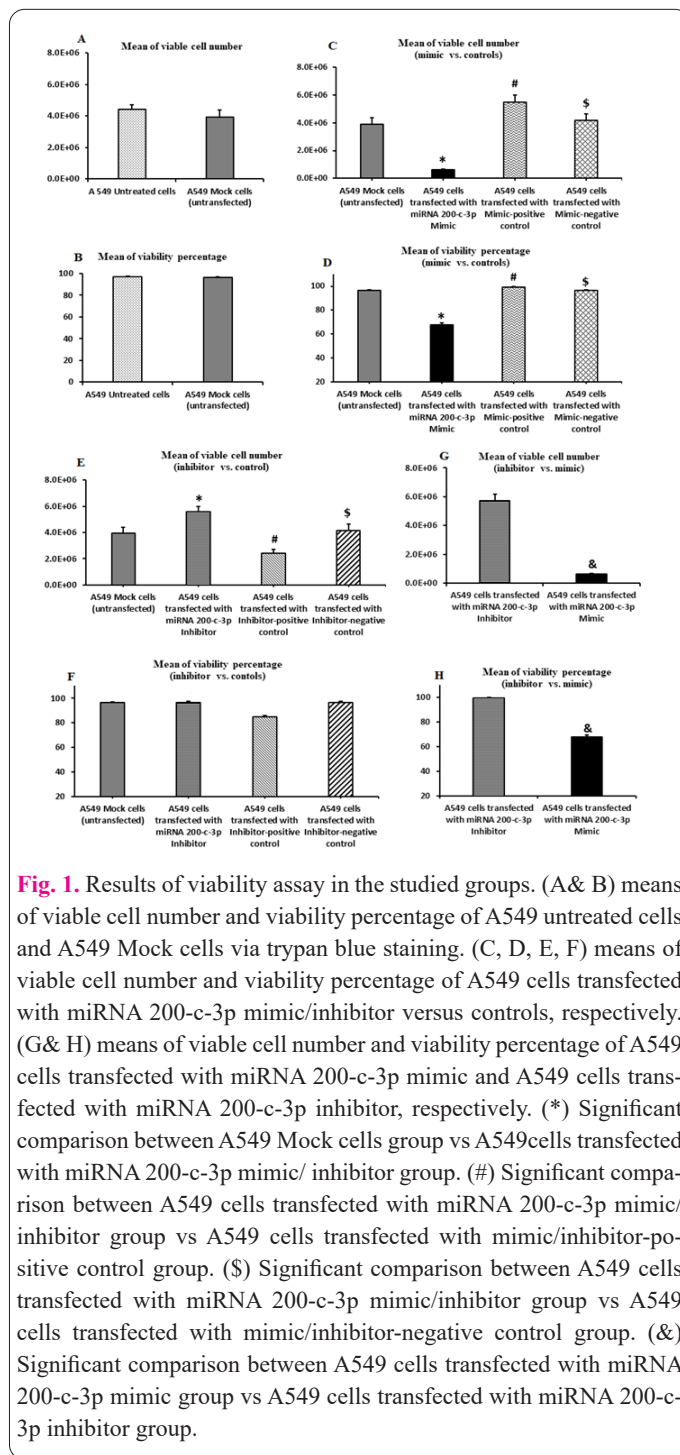
**3. Results**

We compared A549 untreated cells to A549 Mock cells for all parameters studied. There were no significant differences between the two groups (Figure 1 A, B), (Figure 2 A, B), (Figure 3 A, C, E, G) and (Figure 4 lanes 3, 4).

**3.1. Effect of miRNA 200-c-3p mimic and inhibitor on A549 cell viability**

Trypan blue–viability assay revealed non-significant difference in the mean values of viable cell number and viability percentage among A549 Mock cells, A549 cells transfected with miRNA mimic-positive control and A549 cells transfected with miRNA mimic-negative control ( $P > 0.05$ ) as shown in (Figure 1 C, D) which denotes effective transfection. Transfection with miRNA 200-c-3p mimic reduced the viable cell number and viability percentage compared to A549 Mock cells, A549 cells transfected with miRNA mimic-positive control and A549 cells transfected with miRNA mimic-negative control ( $P < 0.05$ ) as shown in (Figure 1 C, D).

On the other hand, cells transfected with miRNA 200-c-3p inhibitor resulted in an increase in the mean of viable cell number compared to A549 Mock cells, A549 cells transfected with miRNA inhibitor-positive control and A549 cells transfected with miRNA inhibitor-negative control ( $P < 0.05$ ), (Figure 1 E). While there was no significant difference in the viability percentage compared to A549 Mock cells, A549 cells transfected with miRNA inhibitor-positive control and A549 cells transfected with miRNA inhibitor-negative control ( $P > 0.05$ ) as shown in (Figure 1 F); since that miRNA 200-c-3p inhibitor increased the total cell count of the A549 cells with concomitant increase in the viable cells so that the viability percentage remained unchanged. There was high significant difference in viable cell number and viability percentage between A549 cells transfected with miRNA 200-c-3p mimic and A549 cells transfected with miRNA 200-c-3p inhibitor ( $P < 0.01$ ) as



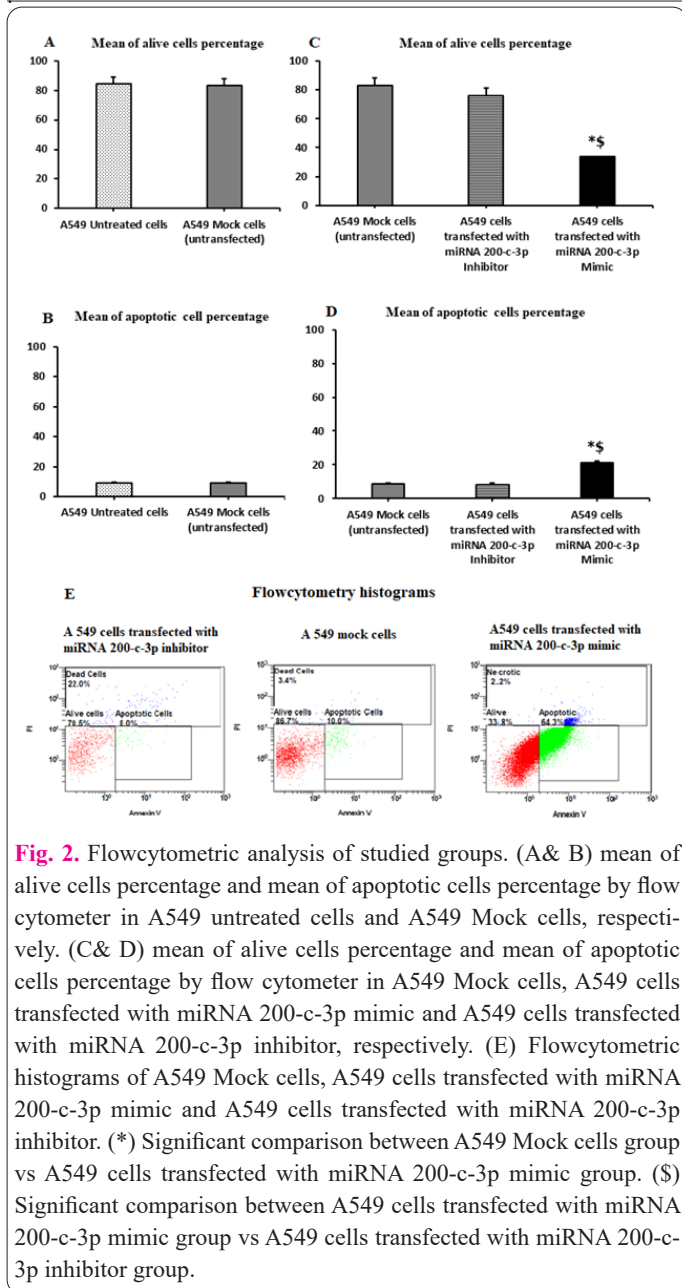
**Fig. 1.** Results of viability assay in the studied groups. (A& B) means of viable cell number and viability percentage of A549 untreated cells and A549 Mock cells via trypan blue staining. (C, D, E, F) means of viable cell number and viability percentage of A549 cells transfected with miRNA 200-c-3p mimic/inhibitor versus controls, respectively. (G& H) means of viable cell number and viability percentage of A549 cells transfected with miRNA 200-c-3p mimic and A549 cells transfected with miRNA 200-c-3p inhibitor, respectively. (\*) Significant comparison between A549 Mock cells group vs A549cells transfected with miRNA 200-c-3p mimic/ inhibitor group. (#) Significant comparison between A549 cells transfected with miRNA 200-c-3p mimic/ inhibitor group vs A549 cells transfected with mimic/inhibitor-positive control group. (\$) Significant comparison between A549 cells transfected with miRNA 200-c-3p mimic/inhibitor group vs A549 cells transfected with mimic/inhibitor-negative control group. (&) Significant comparison between A549 cells transfected with miRNA 200-c-3p mimic group vs A549 cells transfected with miRNA 200-c-3p inhibitor group.

shown in (Figure 1 G, H).

While performing flowcytometric analysis it was noted that the viability percentage of A549 cells transfected with miRNA 200-c-3p mimic was significantly reduced compared to A549 Mock cells ( $P < 0.05$ ) as shown in (Figure 2 C). However, the viability percentage of A549 cells transfected with miRNA 200-c-3p inhibitor had no significant difference when compared to A549 Mock cells ( $P > 0.05$ ). There was a significant difference in viability percentage between A549 cells transfected with miRNA 200-c-3p mimic and A549 cells transfected with miRNA 200-c-3p inhibitor ( $P < 0.01$ ) as shown in (Figure 2 C).

**3.2. Effect of miRNA 200-c-3p mimic and inhibitor on A549 cell apoptosis**

As shown in (Figure 2 D, E), A549 cells transfected with miRNA 200-c-3p mimic had significantly elevated



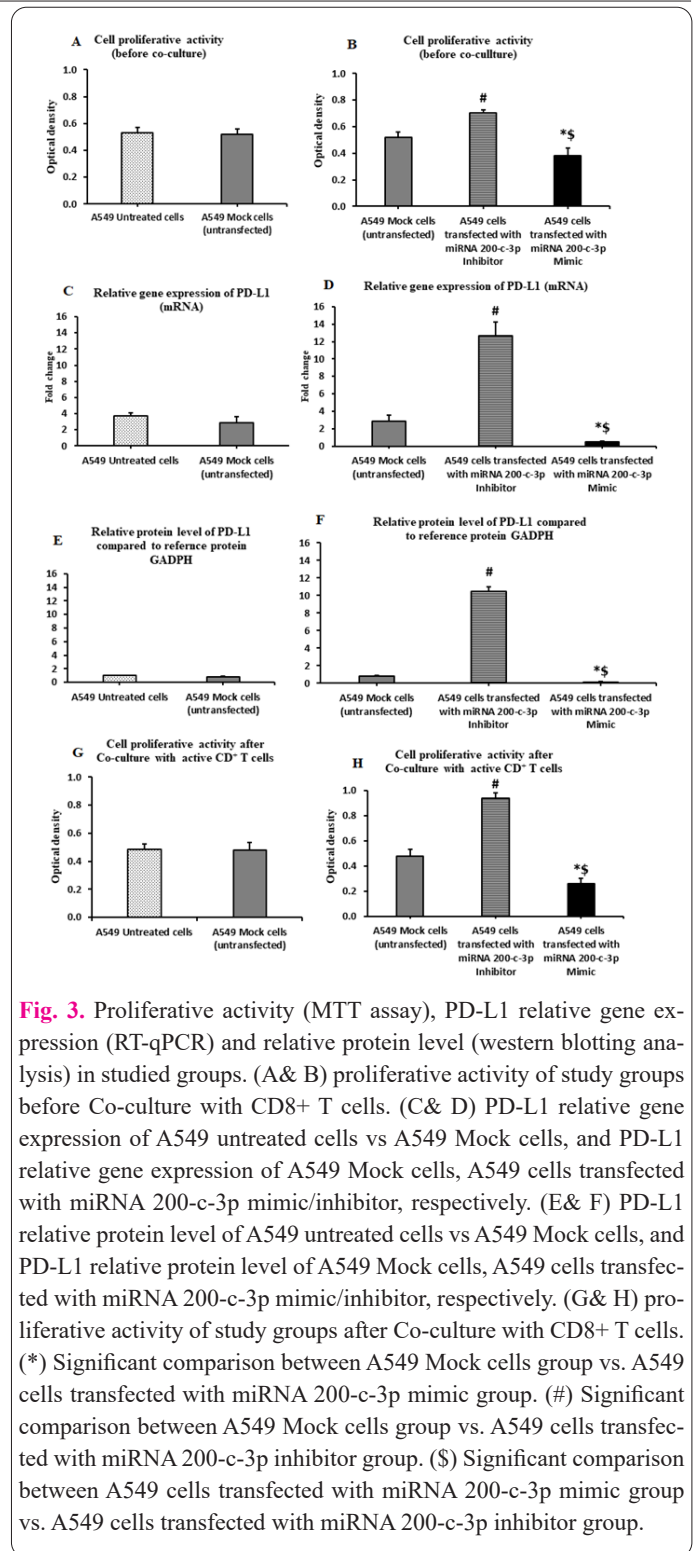
ted apoptotic percentage compared to A549 Mock cells ( $P < 0.01$ ). No significant difference was recorded in A549 cells transfected with miRNA 200-c-3p inhibitor compared to A549 Mock cells ( $P > 0.05$ ). There was a significant difference between A549 cells transfected with miRNA 200-c-3p mimic and A549 cells transfected with miRNA 200-c-3p inhibitor in apoptotic percentage ( $P < 0.05$ ).

### 3.3. Effect of miRNA 200-c-3p mimic/inhibitor on A549 cell proliferative activity

Our study revealed that transfection with miRNA 200-c-3p mimic hindered the proliferative activity of the A549 cells when compared to A549 Mock cells ( $P < 0.05$ ), while transfection with miRNA 200-c-3p inhibitor significantly promoted A549 cells proliferative activity when compared to A549 Mock cells ( $P < 0.05$ ) as shown in (Figure 3 B).

### 3.4. Effect of miRNA 200-c-3p replacement and suppression on PD-L1 relative gene expression and relative protein level

A549 cells transfected with miRNA 200-c-3p mimic had significantly downregulated level of PD-L1 gene when



compared to A549 Mock cells ( $P < 0.05$ ), While A549 cells transfected with miRNA 200-c-3p inhibitor showed significant upregulation in the expression of PD-L1 gene when compared to A549 Mock cells ( $P < 0.05$ ) as shown in (Figure 3 D). Regarding the effect of miRNA 200-c-3p replacement and suppression on PD-L1 relative protein level, it was found that A549 cells transfected with miRNA 200-c-3p mimic had significantly lower PD-L1 relative protein level when to compared to A549 Mock cells ( $P < 0.05$ ), while A549 cells transfected with miRNA 200-c-3p inhibitor had markedly increased relative level of PD-L1 protein when compared to A549 Mock cells ( $P < 0.05$ ) as shown in (Figure 3 F) and (Figure 4).

### 3.5. Co-culture of A549 transfected cells (with either miRNA 200-c-3p mimic or inhibitor) with isolated and activated CD8<sup>+</sup> T cells

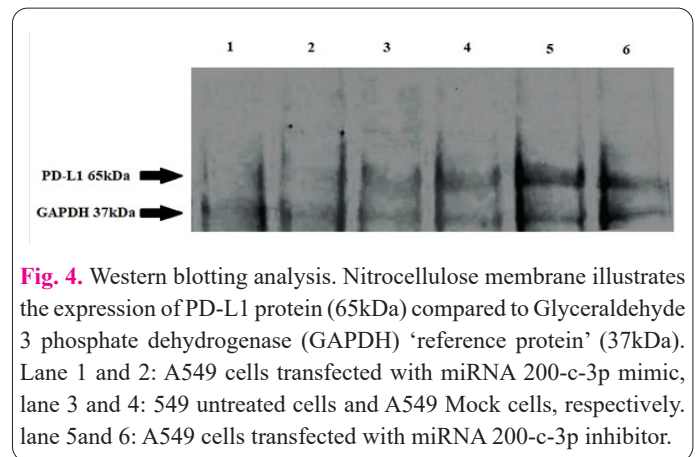
After T cell isolation and activation, T cells were co-cultured with A549 cells transfected miRNA 200-c-3p mimic or inhibitor followed by MTT assay to detect the effect of miRNA 200-c-3p replacement and suppression on the immune recognition of the A549 cells. In this study, co-culture with A549 cells transfected with miRNA 200-c-3p mimic showed significant increase in the immune recognition by the T cells when compared to the A549 Mock cells ( $P < 0.05$ ), which was evidenced by markedly reduced proliferative activity of the A549 cells. On the other hand, co-culture with A549 cells transfected with miRNA 200-c-3p inhibitor exhibited lesser immune recognition when compared to the Mock cells ( $P < 0.05$ ), which was evidenced by increased proliferative activity of the A549 cells transfected with miRNA 200-c-3p inhibitor which exceeded the proliferative ability of the Mock cells as shown in (Figure 3 H).

### 4. Discussion

Based on the current knowledge of how miRNAs affect tumor cell behavior, tumor initiation, progression and dissemination, therapies targeting miRNAs are proposed. MiRNA mimics or miRNA-expressing vectors are used in settings where there is down-regulation of the target miRNA, and the aim is to restore its level. On the other hand, anti-miRNAs or miRNA inhibitors (modified antisense nucleotides) are used in settings where there is up-regulation of the target miRNA, and the aim is to abolish this increased level. So, miRNA mimics and inhibitors are being used to restore balanced gene networks in lung cancer cell lines and xenograft animal models [14].

The objectives of this study were to evaluate the effects of miRNA 200-c-3p replacement and suppression on the viability, proliferative ability, and apoptosis in NSCLC. Moreover, we investigated the impact of miRNA 200-c-3p replacement and suppression on the expression of PD-L1 gene and protein level and consequently its impact on the T cell-mediated antitumor cytotoxicity.

In the salient study, miRNA 200-c-3p mimic transfected cells showed marked significant reduction in the absolute number of viable cells and percentage of cell viability compared to A549 Mock (untransfected) cells. For validation, A549 cells transfected with miRNA 200-c-3p inhibitor showed a significant increase in the absolute number of viable cells; while the percentage of cell viability remained unchanged compared to the A549 Mock cells. Reported contradictory results, as miRNA 200-c-3p suppression resulted in decreased cell viability in pituitary adenoma [31]. In line with our findings, Yu et al., demonstrated a significant reduction in cell viability obtained with miRNA 200-c-3p mimic transfection and significantly enhanced viability when cells were transfected with the inhibitor on murine BV-2 cell line [32]. Recently, Dong et al., found an inverse relation between miRNA 200-c-3p expression level and cell viability in their study on endometriosis where they reported that miRNA 200-c-3p over-expression led to decreased viability while suppression of miRNA 200-c-3p resulted in enhanced viability [33]. However, Y. Wang et al., demonstrated an inverse relation between miRNA 200-c-3p inhibitor and cell viability where miRNA 200-c-3p inhibitor was found to have anti-



**Fig. 4.** Western blotting analysis. Nitrocellulose membrane illustrates the expression of PD-L1 protein (65kDa) compared to Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) 'reference protein' (37kDa). Lane 1 and 2: A549 cells transfected with miRNA 200-c-3p mimic, lane 3 and 4: 549 untreated cells and A549 Mock cells, respectively. lane 5 and 6: A549 cells transfected with miRNA 200-c-3p inhibitor.

oncogenic effect on gastric cell carcinoma BGC-823 and gastric mucosal epithelial cell GES-1 cell lines [24].

In the current study, a significant reduction in the proliferative activity was observed in cells transfected with miRNA 200-c-3p mimic compared to A549 Mock cells, a result reflects the anti-oncogenic effect of miRNA 200-c-3p on A549 cells. On the other hand, a marked increase in the proliferative activity in cells transfected with miRNA 200-c-3p inhibitor was noticed. These findings are consistent with previous research indicating that miRNA 200-c-3p has an inhibitory effect on various cancer cells. For instance, miRNA 200-c inhibited cell proliferation in human bladder cancer and endometriosis [33, 34]. Such beneficial effect was explained by down regulation of ERK1/2 as mentioned by Katoch et al [35]. Both Liu [36] and Anastasiadou [23] have recorded results in concordance with the aforementioned studies and our study about the relation between miRNA 200-c-3p expression and cell proliferation in rat renal artery endothelial cell and epithelial ovarian cancer cells, respectively. While others demonstrated that miRNA 200-c had oncogenic effects as it increased the proliferation rate which favors tumor progression in endometrial carcinoma cells [37], colorectal cancer [38] and human papillary thyroid carcinoma cell lines TPC-1 and K1 [39].

Our results revealed that cells transfected with miRNA 200-c-3p mimic exhibited marked increase in apoptotic cells compared to A549 Mock cells; while the reverse was noticed in cells transfected with miRNA 200-c-3p inhibitor. These findings were in accordance with studies performed in cancer colon [40] and Fanconi anemia pathway functionally deficient lung cancers [41]. The anti-apoptotic effect of miRNA 200-c-3p may be mediated by down-regulation of phosphor-AKT (ser473) that induces anti-apoptotic proteins like Bcl-x1 and represses pro-apoptotic proteins like Bim [35]. In contrast, C.-H. Chen et al., reported that silencing of miRNA 200-c expression promoted apoptosis in pituitary tumors while miRNA 200-c replacement was associated with decreased apoptosis [31].

Tumor cells develop many mechanisms and strategies to avoid recognition and destruction by the circulating immune cells. Established tumors are thought to evolve by selecting tumor clones that can evade being recognized by the immune system, a process known as immunoediting [42].

Tumor cells can directly evade immune recognition by down regulating features that make them vulnerable such as tumor antigens or MHC class I, or indirectly by taking advantage of negative feedback mechanisms that the body

has evolved to prevent immunopathology as immune checkpoints like PD-1/PD-L1 pair [43].

As known, PD-1/PD-L1 signaling pathway leads to attenuation of the T-cell response, induces apoptosis of tumor-specific T cells, and promotes differentiation of CD4<sup>+</sup> T cells into Tregs all of which lead to tumor cell resistance [44]. So, the disruption of the PD-1/PD-L1 interaction is beneficial to restore the anti-tumor T cell cytotoxicity.

In a very recent study conducted by Cercek et al., dostarlimab, an anti-PD-1 monoclonal antibody was used in locally advanced rectal cancer. Despite achieving promising results, it still led to immune-related adverse effects due to the relatively long duration of anti-body administration (6 months)[45].

As per our study, the relative gene expression of PD-L1 and relative protein level was significantly reduced upon cell transfection with miRNA 200-c-3p mimic, and the opposite was observed in the case of cell transfection with miRNA 200-c-3p inhibitor. Similarly, the negative impact of miRNA 200-c-3p on PD-L1 was noted in breast cancer [46], cancer colon [47], gastric cancer [48], and ovarian cancer [23].

In the current study, our main goal was to evaluate the impact of PD-L1 level, after transfection with miRNA-200c-3p mimic and inhibitor, on the T cell-mediated anti-tumor cytotoxicity. Co-culture of A549 cells transfected with miRNA 200-c-3p mimic with activated T cells was accompanied by marked enhancement of T cell cytotoxicity towards the tumor cells that was evidenced by lowered proliferative ability compared to A549 Mock cells co-cultured with activated T-cells. The reverse was noticed upon co-culture of A549 cells transfected with miRNA 200-c-3p inhibitor with activated T cells. In the same context, it was reported that down-regulation of PD-L1 effectively improved T cytotoxicity and led to immunogenic cell death of melanoma cells [49]. Further, T cell activation and cytotoxicity were evident against high-grade serous ovarian cancer after treatment with biphasic anti-PD-1/PD-L1 antibody and monophasic anti-PD-L1 antibody [50]. Additionally, Chen et al, demonstrated that tumor-released exosomes expressing PD-L1 led to T cell exhaustion during metastasis progression and upon treatment with PD-L1 blockade, T cell activation and cytotoxicity were restored in pulmonary metastasis model with melanoma cells [51]. Moreover, Nguyen et al., in their study on murine colon carcinoma MC-38 cell line, introduced miRNA 200-c loaded nanoparticles which led to down regulation of PD-L1 and enhanced T cell activation and cytotoxicity towards the tumor cells [52].

## 5. Conclusion

We performed an assessment of miRNA 200-c-3p mimic/inhibitor effects in non-small cell lung cancer. MiRNA 200-c-3p mimic transfection led to decreased cell viability, proliferation, and increased apoptosis. Additionally, it was associated with decreased PD-L1 relative gene expression and relative protein level. These results shed light to the anti-oncogenic effect of miRNA 200-c-3p. Also, miRNA 200-c-3p mimic transfection improved the T cell anti-tumor cytotoxicity. Conversely, miRNA 200-c-3p inhibitor transfection led to opposite results.

## Conflict of interests

The authors declare there are no conflict of interests.

## Consent for publications

The authors read and approved the final manuscript for publication.

## Ethics approval and consent to participate

No human or animals were used in the present research.

## Informed consent

No patients were included in this research.

## Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Authors' contribution

**Sara Ezzat:** Conceptualization, Methodology, Investigation, Data curation, Writing - Original Draft, Writing-Review & Editing. **Magda I Mohamad:** Software, Validation, Formal analysis, Visualization. **Manal Basyouni Ahmed:** Resources, Writing - Review & Editing, Supervision. **Sara Elsayed Abdelrahman:** Methodology, Software, Data curation. **Nesma Hussein Abdel hay:** Writing - Original Draft, Validation, Resources. **Nashwa El-Khazragy:** Conceptualization, Methodology, Formal analysis, Resources. **Maha I Ismail:** Validation, Supervision, Project administration, Funding acquisition.

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