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RESEARCH ARTICLE

# Altered expression of miRNA-155 and miRNA-21 and higher numbers of T cells in the peripheral blood of early diagnosed lung cancer patients

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## ABSTRACT

**Background:** MicroRNAs (miRNAs) play a significant role in shaping immune response. The expression profile of miRNAs, however, has been found to be affected by the presence of tumor, but whether the incidence of non-small cell lung cancer (NSCLC) alters the expression profile of miRNAs has not been studied. **Objective**: The current study aims to investigate the expression of miRNAs in the peripheral blood mononuclear cells (PBMCs) from patients with NSCLC as well as to analyze the numbers of helper $CD4^+$ and cytotoxic $CD8^+$ T lymphocytes in PBMCs of the same patients as compared to healthy controls. **Methods:** PBMCs were prepared from blood harvested from early-diagnosed patients with NSCLC (n=10). The gene expression of miRNAs in PBMCs was measured using microarray and the phenotypic analysis of helper $CD4^+$ T-cells and cytotoxic $CD8^+$ T-cells in PBMCs was determined by flow cytometry. **Results:** A significant upregulation of miRNA-21 (2-fold) and downregulation of miRNA-155 (-1.5-fold) was observed in the PBMCs of NSCLC patients as compared to healthy controls. The relative and absolute numbers of $CD4^+$ and $CD8^+$ T-cells were significantly elevated in the PBMCs of NSCLC patients. **Conclusion**: Our results indicate that both expression levels of miRNA-21 and miRNA-155 in PBMCs and the numbers of $CD4^+$ and $CD8^+$ T-cells are altered by the presence of NSCLC.

**Keywords:** Blood, Leukocytes, miRNA, T-cells, Non-Small Cell Lung Cancer

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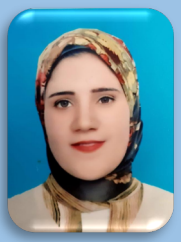

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## INTRODUCTION

MicroRNAs (miRNAs) are a class of short, evolutionarily conserved, endogenous, small non-coding RNAs that target protein-coding mRNAs at the post-transcriptional level and result in target mRNA degradation or translational inhibition (Macfarlane and Murphy 2010; Solomon and Soria 2016). Circulating miRNA levels in cancer patients are proportional to both the stage of cancer and the response to treatment (Zhang et al., 2007). As such, miRNA play important roles in cancer development and progression.

Non-small cell lung cancer (NSCLC) is characterized by high metastatic potential. Despite that, various therapeutic regimens and new diagnostic methods are developed toward NSCLC; the challenge of drug resistance of this cancer results in poor prognosis. Rapid tumor growth, recurrence, and metastasis are one of the known the principal factors that to contribute to poor therapeutic outcomes in NSCLC patients (Cappuzzo and West 2019). Also, the current understanding of the biologic

causes of treatment failure in patients with lung cancer is limited and the treatment of refractory or relapsed patients remains a major challenge (Dai et al., 2019). Thus, investigation of the cellular and molecular mechanisms of NSCLC pathogenesis is of paramount significance to advance the development of new and effective therapeutic modalities for NSCLC.

Multiple studies have indicated that miRNAs may provide an innovative approach, at least in part, as biomarkers for cancer treatment (Devaraj and Natarajan 2011). Among these miRNAs, miRNA-21 and miRNA-155 have been found to have an important effect in the occurrence and development of lung cancer. For instance, miRNA-155 has been found to promote the growth and development of tumor cells by regulating a variety of cancer-promoting pathways; with high expression in a variety of malignancies by direct inhibiting the programmed cell death (Gao et al., 2014; Liu et al., 2017). Also, it has considerable regulatory effect in the proliferation, differentiation and growth of T cells (Liu et al., 2015; Xue et al., 2016). Additionally, miRNA-21 is a type of oncogene and has abnormal high expression in many malignant tumors (Ribas et al., 2012) .

The quality and quantity of the immune response inside the tumor microenvironment play significant roles in the tumor progression and aggressiveness (Hong et al., 2016; Pages et al., 2005). T-cells are one of the important factors in the cellular immune response playing a crucial role in the control of cancer (Yi et al., 2015). In particular, $CD8^+$ lymphocytes, which represent 80% of tumor-infiltrating lymphocytes in NSCLC (Das et al., 2013; Kataki et al., 2002), are the effector arm of adaptive immunity producing protection against tumor progression. Infiltrating CD8+ T cells and CD4+ T cells in NSCLC may collaborate to suppress cancer progression and their presence together seems to be an independent suitable prognostic factor in this disease. Despite the previously reported strong association between cancer-related immune response and the clinical outcomes of cancer patients, the role of immunological markers as predictors of NSCLC prognosis is less defined. In the current study, we have examined the relationship between the frequencies of $CD8^+$ and $CD4^+$ T-cells and the expression levels of miR-21 and miR-155 in peripheral blood mononuclear cells isolated from NSCLC patients.

## MATERIALS AND METHODS

### Patients and blood sampling

NSCLC peripheral blood (PB) samples (5 ml) were collected from 10 healthy volunteer subjects and 10 NSCLC males (n=7) and females (n=3) patients with age ranged between 47 57 years. The patients were recruited from Chest Department, Tanta University Hospital, Tanta, Egypt between May 2017 and September 2018. The patients were recently diagnosed with non-small cell lung cancer; they did not receive prior chemotherapy or radiation therapy included. The patients who received any kind of chemotherapy, surgery or radiation therapy previous to sample collection were excluded from the study. Additionally, patients with concomitant asthma, COPD, bronchiectasis, upper/lower respiratory tract infection in the preceding 4 weeks, active pulmonary tuberculosis, and associated cancer besides lung cancer were excluded from the study. The experimental protocols were approved by the Ethics Committee of Tanta University Hospital, Egypt. All patients delivered written informed approval prior to their enrollment in the present study. More detailed information of patients and controls are shown in Table 1.

### Affymetrix Microarray Analysis

Briefly, total RNAs were extracted with TRIZOL (Invitrogen) in combination with the RNeasy Mini Kit from Qiagen, quantified with Nanodrop Spectrophotometer. Affymetrix microarray analyses were done at Microarray Core Facility at Center of Excellence in Cancer Research, Tanta University, Egypt. For complete miRNA profiling, blood samples were prepared using Flash Tag Biotin HSR RNA Labeling Kit as recommended by the manufacturer's protocol. The samples were hybridized on Gene Chip miRNA 3.0 Arrays (Affymetrix) for 16 h at 48 °C. The arrays were washed to remove nonspecifically bound nucleic acids and stained on Fluidics Station 450 (Affymetrix) and then scanned on Gene-Chip Scanner 3000 7G system (Affymetrix). Cell files were imported into Chromosome Analysis Suite 1.2 (Affymetrix, Inc) to generate copy number from the raw intensity. Gene Chip miRNA 3.0 cell files were analyzed with Affymetrix Expression Console

(Version 1.3.1) and Transcriptome analysis console (TAC - version 3.0).

**Flow Cytometric Analysis**

Briefly, 100 µL fresh peripheral blood samples were stained with monoclonal antibodies (mAbs; obtained from BD Biosciences, CA, USA) against CD4 and CD8 receptors using the manufacturer's protocol. Flow cytometry analysis was done using the BD FACSCanto II Flow Cytometer at the Flow Cytometry Core Facility at Center of Excellence in Cancer Research, Tanta University Teaching Hospital, Tanta University, Egypt. Data were analyzed using the software program FlowJo.

**Statistical Analysis**

Statistical analysis was performed using the Student's *t*-test. Log-rank nonparametric analysis using Graph Pad Prism (Graph Pad Software, Inc.). All P values were two-sided, with P< 0.05 considered significant. Cumulative survival was calculated using a Kaplan-Meier curve. Cell files were analyzed with the Genotyping console for quality control analysis. Samples with QC call above 80% were admitted into the analysis.

## RESULTS

**Expression analysis of miRNA-155 and miRNA-21 in peripheral blood of NSCLC patients**

The expression of miRNA-155 was significantly down-regulated in NSCLC patients before the induction of chemotherapy as compared to healthy control volunteers "CTRL" (0.04 ± 0.01 versus 1.14 ± 0.05, p< 0.01**) as shown in Figure 1A. However, the expression level of miRNA-21 was up-regulated when compared with healthy control volunteers (0.12 ± 0.016 vs. 0.005 ± 0.012, p< 0.01**) as shown in Figure 1B.

**Analysis of leukocyte populations in the peripheral blood of NSCLC patients**

In the present study, we firstly investigated the flow cytometry analysis of different subpopulations of leukocytes, including lymphocytes, monocytes and granulocytes in the peripheral blood mononuclear cells (PBMCs) of NSCLC patients and compared it to healthy control volunteers. We gated on each population and counted the events. As shown in Figure 2, there was a remarkable decrease in the percentage of lymphocytes, monocytes, and granulocytes in PBMCs of NSCLC patients as compared to those of control.

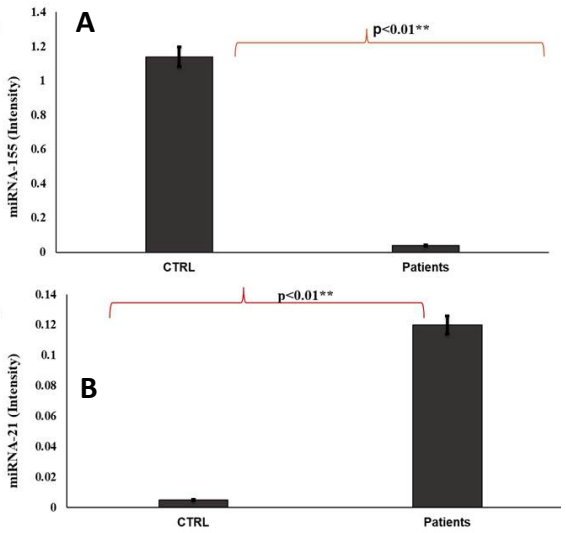


**Figure 1. (A)** Expression of miRNA-155 and **(B)** miRNA-21 in NSCLC patients before treatment as compared to healthy control volunteer using microarray.

In PBMCs of NSCLC patients, the absolute number and relative percentage of leukocytes were (5,350, 55.7%), for lymphocyte were (1500, 15.2%), for monocytes were (10, 2%) and for granulocytes were (2100, 39.3 %). These values were lower when compared to those of healthy controls; leukocytes (6880, 91.2%), lymphocytes (2060, 30.2%), monocytes (20, 3.5%) and granulocytes (4120, 60.5%) as shown in Figure 3A and B.

**Immunophenotypic analysis of $CD4^+$ and $CD8^+$ T cells in PBMCs**

The phenotypic analysis strategy of T-cells was presented in Figure 4A. From the forward and side scatters, we first gated on the lymphocyte population (P1) and then analyzed the $CD4^+$ and $CD8^+$ T cell subpopulations from P1. The results showed that the relative numbers of $CD4^+$ and $CD8^+$ T cells were highly increased in PBMCs of NSCLC patients when compared to healthy control volunteers (Fig. 4B). The statistical analyses of the relative and absolute numbers of these 2 subpopulations in NSCLC patients versus control are summarized in Figure 5A and B which showed significant increases as compared to those of control volunteers.

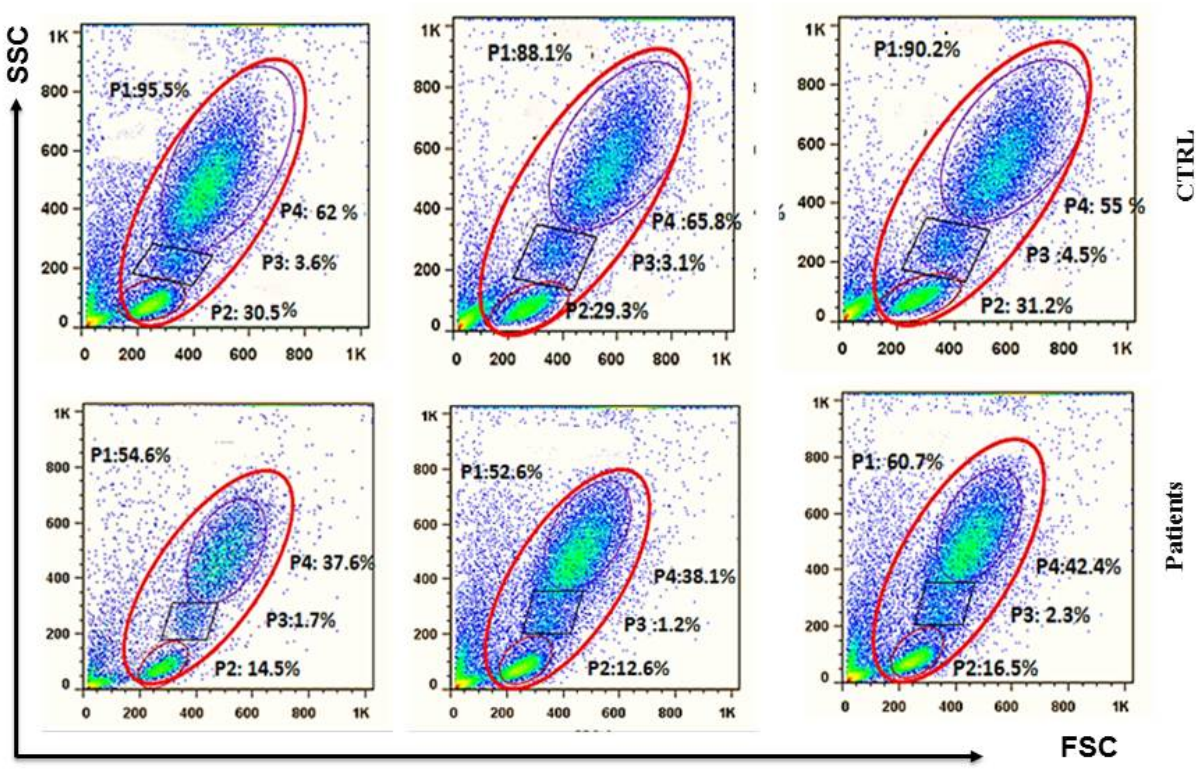


**Figure 2**. Representative data from flow cytometry analysis of the expression of peripheral leukocytes (P1), Lymphocytes (P2), Monocytes (P3), and Granulocytes (P4) in NSCLC patients against those of control**.**

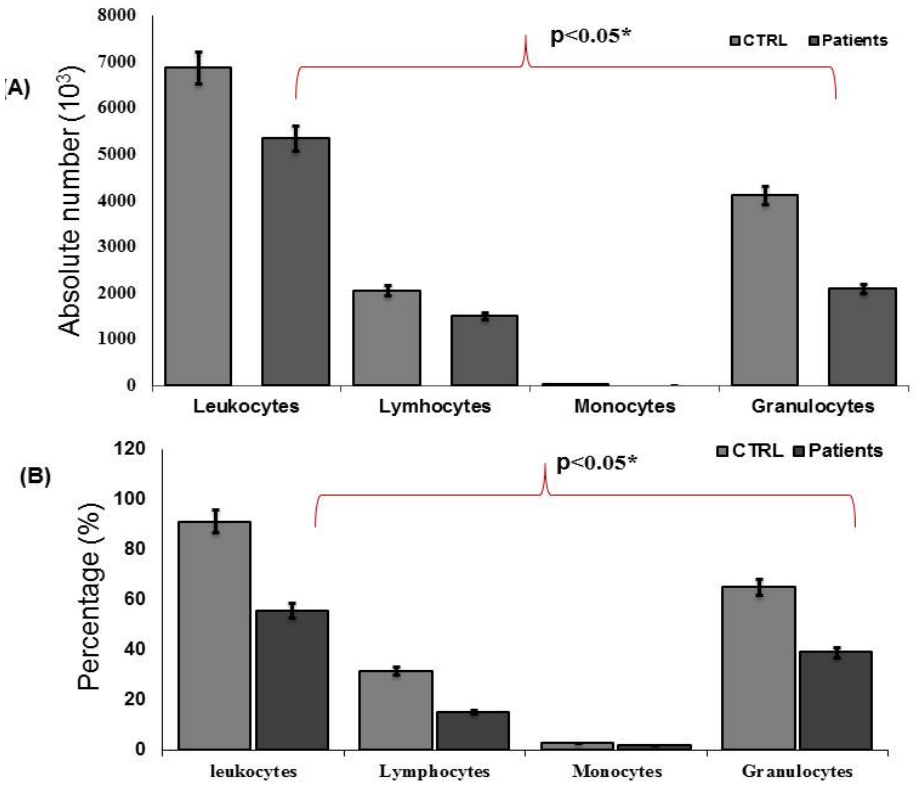


**Figure 3. (A)** Absolute number and relative percentage of peripheral leukocytes and their different subpopulations in NSCLC patients compared to healthy control volunteers, (**B)** relative percentage of peripheral leukocytes and their different subpopulations in NSCLC patients against control.

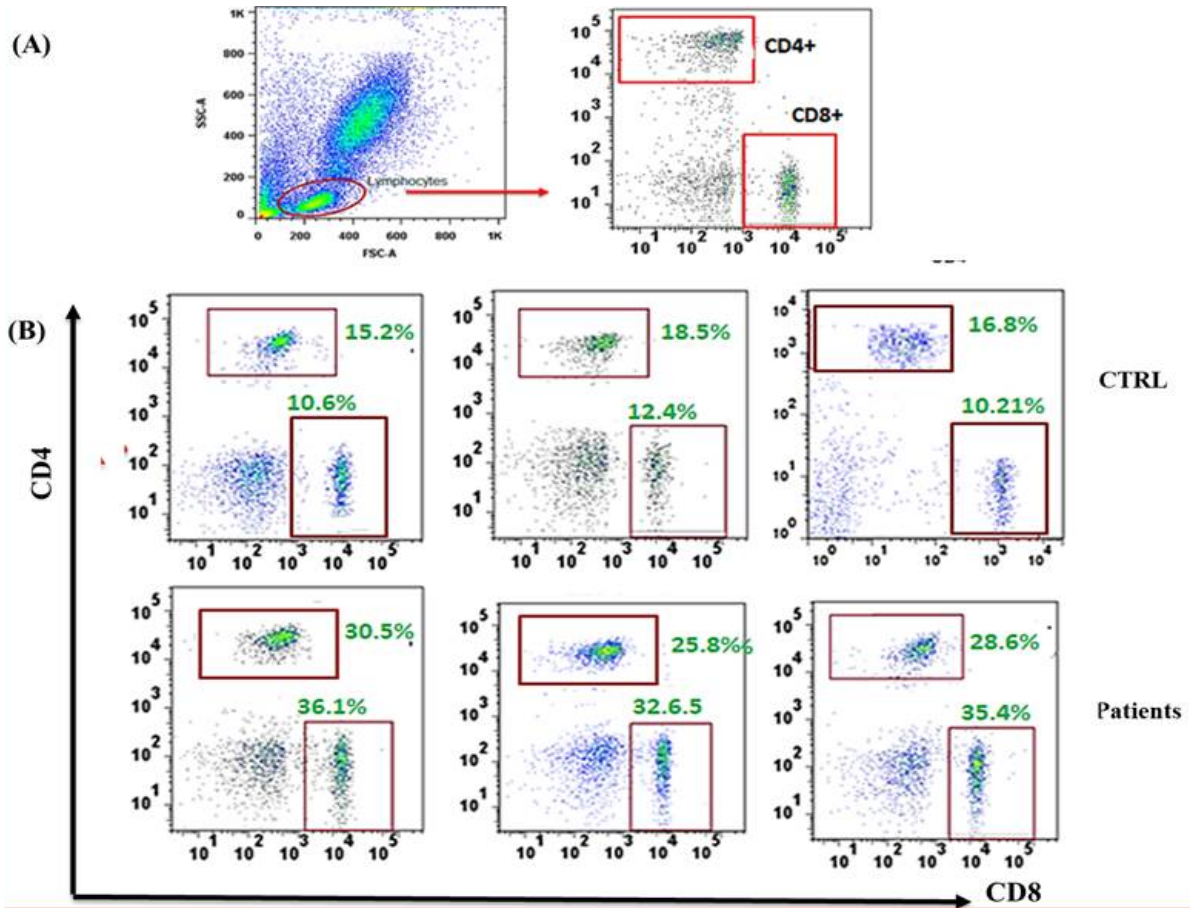


**Figure 4.** A representative flow cytometric analysis of $CD4^+$ and $CD8^+$ T-cells in NSCLC patients. **(A)** Gating strategy of $CD4^+$ and $CD8^+$ T-Cells, **(B)** percentage of $CD4^+$and $CD8^+$ T-Cells in NSCLC patients and healthy control volunteers.

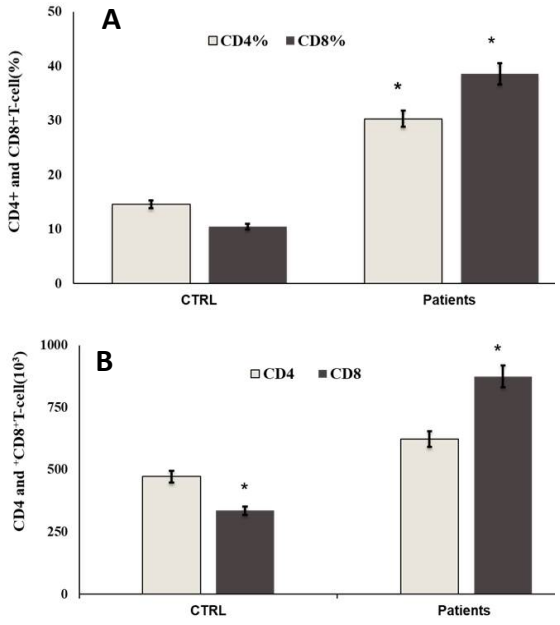


**Figure 5. (A)** The relative percentage of $CD4^+$ and $CD8^+$ T-cells, **(B)** and their absolute number in PBMCs of NSCLC patients compared to healthy control volunteers and before treatment.

## DISCUSSION

miRNAs have the ability to target various genes including various important signaling pathways that play critical roles in cancer development and progression (Blenkiron and Miska 2007). Several studies have documented that miRNAs are frequently aberrantly expressed in NSCLC (Yang et al., 2018), where their differential expression may be closely related to NSCLC genesis and development (Iqbal et al., 2018). Thus, miRNA expression levels in NSCLC may provide a crucial role in the pathogenesis of NSCLC. As such, studying the alteration in miRNA expression profile in this aggressive malignant tumor seems to be an attractive and effective therapeutic approach for patients.

The present study demonstrates that significant low expression of miRNA-155 and marked high expression of miRNA-21 in NSCLC patients. This data is in line with those of Li et al., (2017) who reported high expression of miRNA-21 in NSCLC

patients. Similar to our finding, Fortunato et al., (2016) also reported downregulation of miRNA-155 considering it as predictor of poor prognosis in NSCLC patients. Taken together, the alteration in the expression of both miR-21 and miR-155 indicate to their important effect in the development of lung cancer.

Importantly, the current study also demonstrates that the numbers of both $CD8^+$ and $CD4^+$ T cells is significantly elevated in NSCLC patients in comparison to controls. Importantly, the ratio of $CD4^+/CD8^+$ T cells was altered as compared to healthy controls. This data is consistent with the findings of Hiraoka et al., (2006) who revealed synergistic high infiltration of both CD4+ and CD8+ T cell in cancer, which usually indicate to a stronger immune reaction against cancer and demonstrate a significantly good prognosis. These data indicate that $CD8^+$ T cells could represent key effector cells for anti-tumor immunity affecting immune response against lung cancer and might have prognostic significance.

In summary, both expression levels of miRNA-155 and miRNA-21 and numbers of $CD4^+$ -cells and $CD8^+$ T-cells are altered in NSCLC patients. These miRNAs may be used as predictors of NSCLC progression. However, further studies are needed with more number of patients to confirm these findings.

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