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DETERMINATION OF MIR-372 AND CDC42 GENE EXPRESSION LEVELS IN THE SERUM OF PEOPLE WITH LUNG CANCER

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Abstract – *Objective:* *Despite advances in early diagnosis of lung cancer, most lung cancers are diagnosed in the advanced stages of the disease. Disorders of microRNA expression are often associated with the onset and progression of diseases such as lung cancer. MicroRNAs in plasma or serum as biomarkers are effective in early detection and screening for lung cancer. The cdc42 gene has also been overexpressed in several types of cancer, includin¬¬g lung cancer and this gene is involved in processes such as cell growth and metastasis. Therefore, the expression of cdc42 and miR-372 gene expression may be used to predict metastasis and early diagnosis.*

Patients and Methods: *To conduct the present study, 200 serum samples (100 serum samples of healthy individuals and 100 serum samples of people with NSCLC) were collected from people referring to Masih Daneshvari Hospital in Tehran. Individual and clinical information of all patients were prepared by questionnaires. Then, plasma separation, RNA extraction, cDNA synthesis, and primer design were performed, and Real Time PCR method was used to qualitatively evaluate the expression of Cdc42 gene and miR-372 expression changes.*

Results: *The data showed that serum expression of cdc42 gene and miR-372 in the first to third stages did not differ significantly from healthy serum samples. However, in the serum of the fourth stage of metastasis, the expression of cdc42 and miR-372 genes, had a significant increase ($p < 0.05$) compared to healthy samples, 3.4 ($p=0.032$) and 7.3 ($p=0.022$) times, respectively.*

Conclusions: *Based on the results of this study, it is possible to predict the stage of metastasis in lung cancer by examining the expression of Cdc42 gene and miR-372 in serum.*

KEYWORDS: *Lung Neoplasms, Serum, Biomarkers, Tumor, cdc42 gene, miR-372.*

INTRODUCTION

The molecular basis of lung cancer is complex and heterogeneous. Lung cancer progresses through a multi-stage process involving several genetic and epigenetic changes, particularly the activation of advanced growth pathways and inhibition of tumor suppressor pathways[1].

Lung cancer is one of the leading causes of death in both men and women in developed and

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developing countries and it causes more than 1.5 million deaths a year[2]. In Iran, the prevalence of lung cancer is 4.9-7.2 per 100,000 people, which is the second leading cause of death in men and the third leading cause of death in women[3].

Smoking has been associated with a 70% increase in mortality in men and a lower increase in mortality in women[4].

There are two main types of lung cancer, including small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which account for 15% and 85% of all lung cancers, respectively. NSCLC is mainly divided into three categories: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma[5-7].

The (Cell division cycle 42) Cdc42 gene is located on the short arm of chromosome 1 (1p36.12)[8].

The Cdc42 gene is a member of the Rho GTPase family that is involved in cellular motility, proliferation, survival, invasion, and metastasis of human cancer cells. Increasing Cdc42 expression plays a role in increasing cell proliferation and migration. Decreased expression of the Cdc42 gene also induces apoptosis. Studies have shown that extinguished Cdc42 has the ability to reverse metastasis and the growth of colon, bladder and lung cancer cells in humans. Frequent changes in the expression of Cdc42 were detected in NSCLC, but the mechanism or mechanisms underlying the changes in expression were not fully defined. Cdc42 is the main transmitter of the propagation signal to regulate the G 1 / S phase of the cell cycle[9].

MicroRNAs or miRNAs are a group of small RNA molecules, single-stranded, protected, non-coding protein and 25-18 nucleotides which are involved in the partial connection of complementary sequences within the target 3´UTR (untranslated region 3´) mRNAs[10].

MiRNAs are released as a result of cell death into the bloodstream or may be actively secreted by cells. They may play a pathogenic role in the disease process by acting as oncogenes or tumor suppressor genes[11].

MiR-372 regulates cell cycle, apoptosis, invasion and proliferation in many types of human cancers and it also increases the rate of metastasis[12,13].

Improper expression of miRNAs promotes or inhibits tumor metastasis. Identifying miRNAs and better understanding their complex functions in tumors will provide prognostic and diagnostic biomarkers, as well as therapeutic goals for clinical use[14].

Despite the completeness of chemotherapy, radiation therapy, surgery, and targeted molecular therapies performed in the clinic, the survival rate of patients with advanced pulmonary adenocarcinoma is low, so the pathogenesis mechanisms of pulmonary adenocarcinoma should be investigated[15]. Given that adenocarcinoma accounts for 40% of NSCLC and is more prevalent in individuals, this study was performed on adenocarcinoma[10]. Also, due to the high prevalence and mortality rate, cdc42 and miR-372 genes can be used as biomarkers for early detection and because of the ability to access the serum and the ability to identify genes in the serum, in this study, the expression of miR-372 and the gene Cdc42 in the serum of people with lung cancer were examined.

MATERIALS AND METHODS

To conduct this research, random sampling was performed, and first patient consent was obtained. This research was performed in a descriptive-analytical manner. The cdc42 and miR-372 gene expression were then quantified in healthy individuals and people with lung cancer. The study sample consisted of 50 serum samples of healthy individuals and 50 serum samples of cancer patients from NSCLC patients who were collected from Masih Daneshvari Hospital in Tehran. Patient samples were collected from people with the first to fourth stages of cancer.

Questionnaire design

To record individual and clinical information, first consent was obtained from individuals to enter the present study, then a questionnaire was designed. In the questionnaire, patient information including name and surname, date of reference, age, sex, occupation, smoking (type and duration), duration of illness, place of residence, type of tumor, tumor stage, family history of the patient, history of other disease, primary tumor, and tumor after treatment, were collected.

Blood sampling and plasma separation

From the subjects, 5 ml of blood was prepared, and plasma was separated using refrigerated centrifuge (Hanil, Chungcheongnam-do, Republic of Korea) at 4°C for 15 min at 1900 rpm. The resulting plasmas were poured into RNase free microtubes and stored in the 80°C (JAL-Iran) freezer until the experiments were performed.

Plasma miRNA extraction, quantitative and qualitative study of extracted RNA, cDNA synthesis

RNA was extracted from plasma using a German Qiagen extraction kit (Qiagen, Hilden, Germa-

TABLE 1. Primers Used in Real Time PCR.

Primer name	*Sequence*	*Amplicon*
cdc42 F	5'-GATGGTGCTGTTGGTAAA-3'	127 bp
cdc42 R	5'-GAGTATATGGTTCTCCACC-3'	127bp
miR-372 F	5'-GCCCGCAAAGTGCTGCGACAT-3'	121 bp
miR-372 R	5'-CCAGTGCAGGGTCCGAGGT-3'	121 bp
U6 F	5'-GCTTCGGCAGCACATATAC-3'	137 bp
U6 r	5'-ATTCCGTTTCTGGGAGGG-3'	137bp

ny). Then, with the spectrophotometric (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis (Iran-Padideh Nojen pars) methods, the quantity and quality of extracted RNA were examined. Using cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) from RNA samples, cDNA (complementary DNA) synthesis was performed. NCBI and Allele ID software were used to design the primers. The sequence of primers used is given in Table 1. In this study, house keeping Gene was the U6 gene.

Measuring the efficiency of primers

The performance of the primers was checked, and a standard curve was drawn for each. To determine the efficiency of primers, five dilutions were first prepared from cDNAs. Then, for these dilutions, together with each of the primers, the Real time PCR reaction was repeated twice, separately. Finally, the standard curve for each primer was plotted based on the Ct values obtained against the dilutions used. Using the slope of the resulting curve (Slope) and the relation $E= 10^{(-1/slope)} - 1$, the efficiency of the reaction (E) was calculated for each primer.

Real time PCR

Reproduction for cdc42 gene and miR-372 expression measurements was performed relatively by Real time PCR based on standard method. Exicycler™ 96 bioneer (Daedeok-gu, Daejeon, Republic of Korea) fixed type as well as Eva Green paint were used to perform the Real time PCR reaction. To perform the reaction, samples were taken to a final volume of 20 µl and according to the Universal RT microRNA qPCR kit protocol made by Exiqon-Denmark (Eva Green Master Mix 10 µl, l5 µ cDNA (20 ng / µl), 12 µ specific primers (10 pmol) and 3µl Sterilized distilled water) was prepared. The Real time PCR reaction was performed with the same heat program for cdc42 gene, miR-372 and reference gene proliferation. The conditions and temperature of each cycle included four stages of incubation at 95°C for 5 minutes, 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Finally, the raw data were extracted from the device and the expression was measured using the ΔΔct Method. The gene expression was then evaluated using REST software.

In the Real time PCR method, the control group consisted of two groups: 1- U6, which is the reference cell line and is considered as positive control, that is, people with lung cancer. 2- Negative control, meaning healthy people who do not have lung cancer.

RESULTS

Patient information

Demographic and clinical information of patients was extracted and recorded from the patients' files and the electronic system of the hospital's Cancer Department. Seventy out of 100 patients with lung cancer were male and 30 (30%) were female. Of these, 72 (72%) smoked and 28 (28%) did not smoke. In terms of having a history of cancer among family members, out of 50 patients with lung cancer, 56 (56%) have a history of cancer among members. They had no family and 44 people (44%) had a family history of cancer. 46 (46%) of the 100 patients were in the age range of 70-61 years.

Evaluate the quality of RNAs extracted from blood serum

With the Agarose gel electrophoresis method, the quality of the RNAs was checked and the presence of ribosomal 18S and 28S bands

TABLE 2. *p*-value for cdc42 gene and miR-372.

Gene	*p-value*
Cdc42	$p<0.05$, $p=0.032$
MiR372	$p<0.05$, $p=0.022$

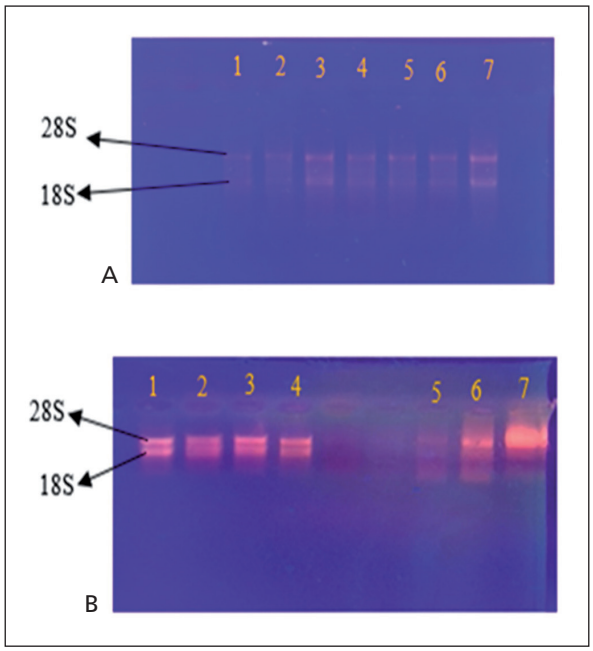

Fig. 1. Agarose gel electrophoresis to evaluate the quality of the extracted RNA No. 1-6: Examples, No. 7: Marker. *A*, miR-372. *B*, The cdc42 gene.

showed that the extracted RNAs were of good quality. These bands are shown in Figure 1 for some samples.

Using nanodrapes, the RNA concentration was obtained, and its quantity was investigated. The adsorption ratio of 260 to 280 determines the purity of nucleic acids. In this study, the adsorption ratio from 260 to 280 was between 1.8 and 2, and the quality of the extracted RNAs was confirmed.

Check the performance of primers

The performance of the primers used in this study was obtained by cDNA dilution method of the samples, which is shown in Figure 2.

According to Figure 2, the primer efficiency for miR-372 and cdc42 gene is 99%.

Check the accuracy of cDNA and Conventional PCR

After determining the efficiency of the primers used, the conventional PCR step was performed to check the size of the PCR products as well as their specificity. Figure 3 shows the result.

Using the Q-RT PCR method, the expression of Cdc42 gene and miR-372 was measured and repeated three times for each sample. REST software was used to analyze the results.

The rate of expression of miR-372 and the cdc42 gene studied by stage of cancer

According to Figure 4, the expression of cdc42 gene and miR-372 in the serum of people with the first to third stages of the disease did not differ significantly from the serum of normal individuals. The expression levels of the cdc42 and miR-372 genes increased significantly by 3.4 (p=0.032) and 7.3 (p=0.022) times (p<0.05) compared to the serum of healthy individuals.

DISCUSSION

Cancer includes all types of malignant tumors, which in medicine are more commonly known as neoplasms[16].

Genetic factors or factors that disrupt cell activity are likely to cause problems in the cell nucleus and contribute to cancer. These include radioactive materials, chemicals and toxins, or excessive radiation such as sunlight[17].

Lung cancer begins when lung cells grow out of control. Statistics show that the incidence of lung cancer is 15% in men and 14% in women[18].

The main reasons for the choice of lung cancer in this study were the detection of cancer in advanced stages, increased prevalence, mortality and metastasis.

MicroRNAs are present in a stable form in body fluids (e.g., blood, plasma, serum, or sputum). They have the ability to be used as targeted therapeutic molecules. Another advantage of using microRNAs is the repeatability of the test[2,19].

Due to the fact that it is easier to get serum from people and it is a non-invasive method. Also, because serum is a new way to make kits, it's easier to transfer and separate biomarkers from the serum, so the serum was used in this study.

The cdc42 gene belongs to the large Ras family, and excessive cdc42 expression is associated with carcinogenesis and the progression of many types of human tumors[20].

The expression of miR-372 and cdc42 gene was measured using Real time PCR method. The findings of this study showed that the expression of miR-372 and cdc42 gene in the first to third stages of cancer serum samples were not significantly different. However, in the serum samples of the fourth stage of the disease, the expression of miR-372 and cdc42 gene had a significant increase of 7.3 (p=0.022) and 3.4 (p=0.032) times, respectively, compared to normal samples (p<0.05). Therefore, according to

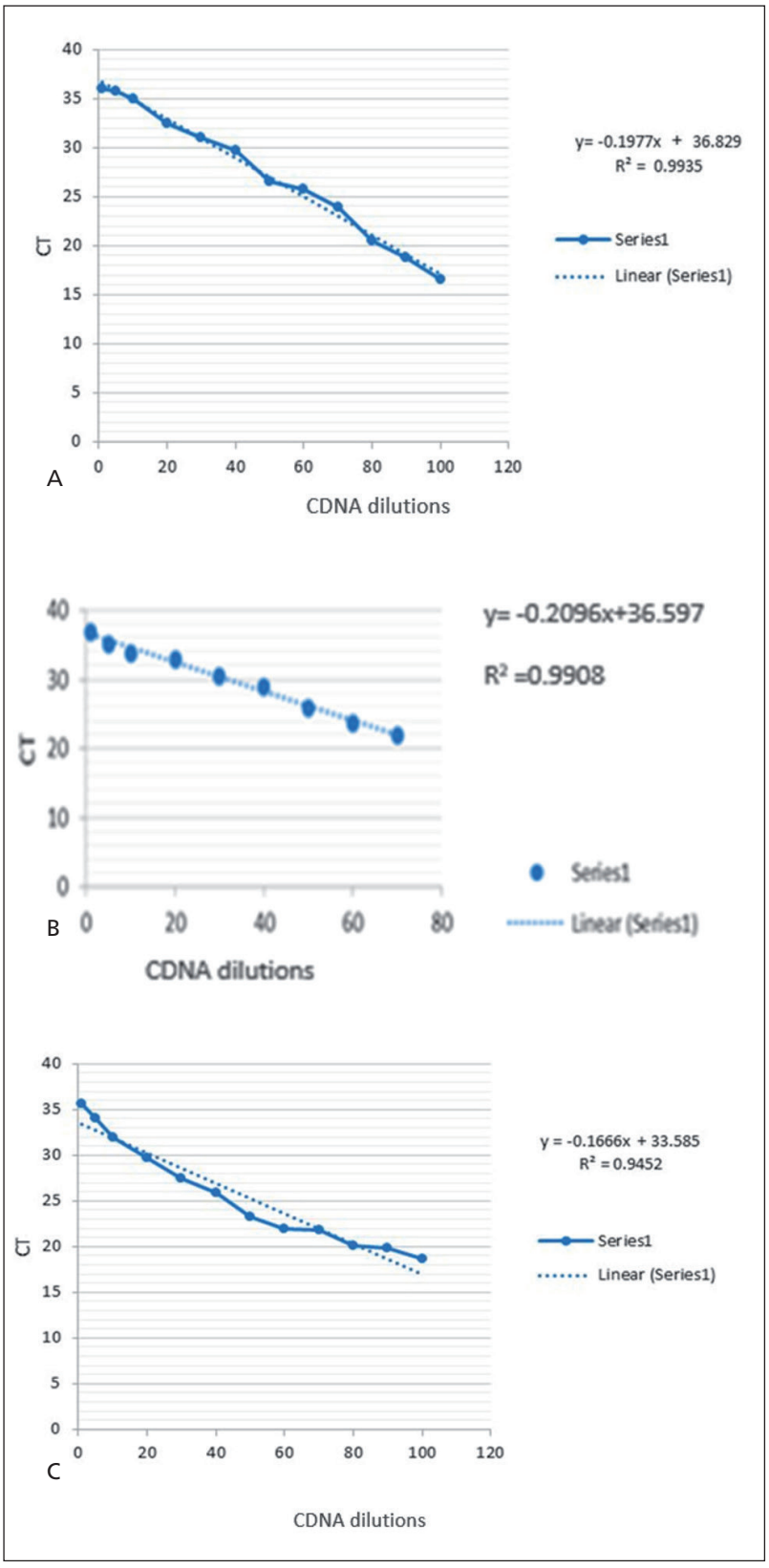

Fig. 2. Evaluation of the efficiency of specific primers used for: *A*, The efficiency of the miR-372 specific primer: 99%. *B*, The efficiency of the cdc42 gene specific primer: 99%. *C*, The efficiency of the U6 gene specific primer: 94%.

the results of this study and if these results are confirmed in more samples, it can be concluded that the study of miR-372 expression in serum may be used to predict the stage of metastasis in lung cancer. The results of this study also showed that increasing cdc42 gene expression prevents cell proliferation and promotes disease progression.

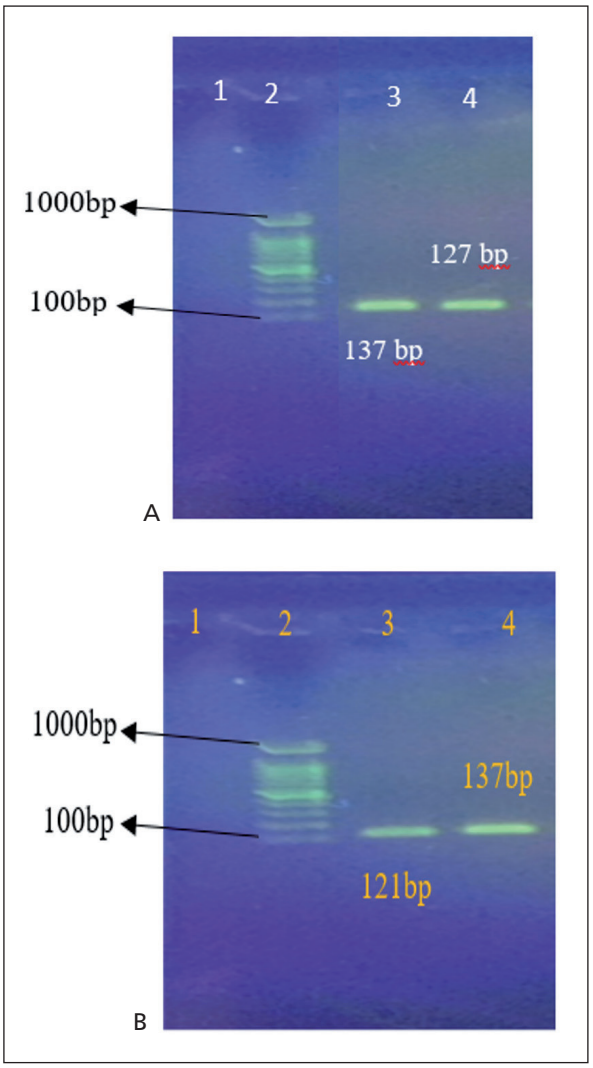

Fig. 3. Conventional PCR with specific primers. *A*, PCR product for U6 and cdc42 genes. Column 1: Negative control, Column 2: Marker 100 bp, Column 3: PCR product for U6, Column 4: The cdc42 PCR product. *B*, PCR product for miR-372 and U6 gene. Column 1: Negative control, Column 2: Marker 100 bp Column 3: The miR-372 PCR product, Column 4: PCR product for U6.

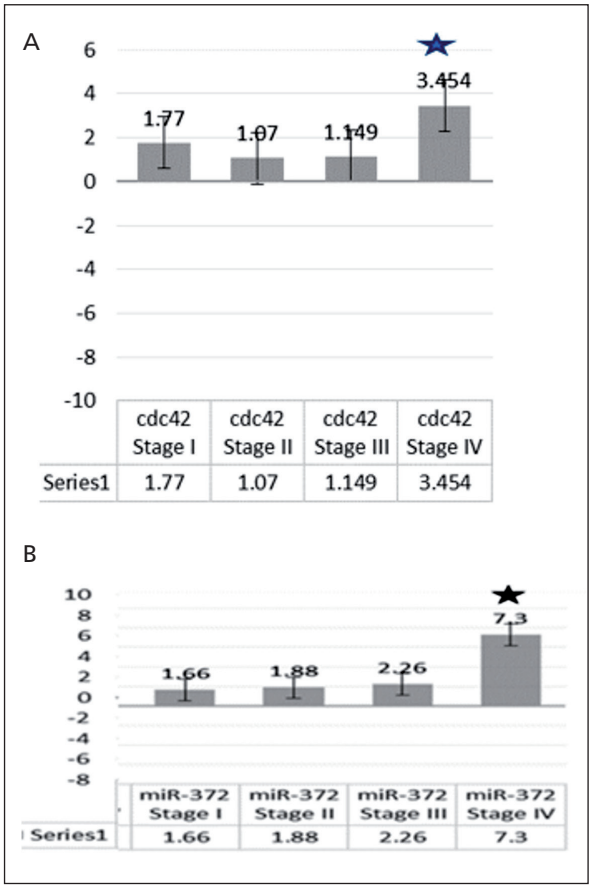

Fig. 4. The expression of (*A*) miR-372 and (*B*) cdc42 gene according to the cancer stage.

According to the demographic information of people with lung cancer, it was found that there is a significant relationship between sex and smoking with lung cancer ($p<0.05$). There is also no significant difference between the history of lung cancer among family members and the incidence of lung cancer. It is noteworthy that the disease is more common in old age.

According to research, high levels of cdc42 may be detrimental to the survival of patients with melanoma and have a positive relationship with prognostic markers. According to the study, which was based on the elimination of cdc42 in mice, a tumor suppressor role for cdc42 was discovered, indicating that cdc42 loss in the liver causes chronic liver damage and the development of liver cell cancer. Also, targeting the cdc42 gene in hematopoietic stem cells and bone marrow hematopoietic cells leads to loss of hematopoietic stem cell extinction and excessive proliferation of blood ancestral cells[21]. In our study, the results showed that the expression of cdc42 gene in the fourth stage of the disease, which is the metastatic phase, had a significant increase ($p<0.05$, $p=0.032$).

One group of miRNAs, including five miRNAs (miR221, let-7a, miR137, miR372, and miR182), has been linked to overall survival and disease progression in patients with primary NSCLC[22]. In the present study, miR-372 expression was significantly increased only in serum samples of the fourth stage of the disease and in patients with metastatic lung cancer ($p<0.05$, $p=0.022$).

According to reports, cdc42 has been overexpressed in colorectal cancer, and cdc42 has an oncogenic role in colorectal cancer by regulating the transcription of cancer-related pathways. Most results support the use of specific cdc42 inhibitors to treat invasive colorectal cancers[23]. In the present study, increasing cdc42 gene expression contributed to the progression of the disease.

Studies have shown that serum levels of miR-372 after surgery in patients with primary colorectal cancer are significantly reduced compared to pre-operative values. These findings suggest that serum miR-372 could be a non-destructive biomarker for early detection and prognosis of colorectal cancer[24]. The expression of miR-21, miR-210, and miR-372 in saliva (sputum) may be a very sensitive and specific method for diagnosing NSCLC and as a potential complementary screening tool[25]. The results of the present study show that the expression of miR-372 increases with the progression of the disease, but in our study, only in the fourth stage, a significant increase ($p<0.05$, $p=0.022$) was obtained.

Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1) causes migration and invasion of breast cancer cells and reduces the expression of the cdc42 gene[26]. Research has also shown that G-protein-coupled receptor kinase interacting protein 1 (GIT1) is involved in cellular activation, which is a fundamental process during tissue development and cancer progression. GIT1 stimulates migration and invasion in NSCLC cells by altering Rac family small GTPase 1 (Rac1) / Cdc42 activity[27]. In our study, the expression of the Cdc42 gene in the fourth stage of the disease, which is the metastatic phase, had a significant increase ($p<0.05$, $p=0.032$) and increased the metastasis process.

In gastric cancer, miR-372/373 are TNFα-induced protein 1 (TNFAIP1) targets and promote gastric carcinogenesis. TNFAIP1 appears to be involved in DNA synthesis and apoptosis[28]. In the present study, it was found that miR-372 expression is involved in the metastasis process.

The cdc42 gene regulates the cell cycle and differentiation in lung cancer cells, and reducing the expression of the cdc42 gene can disrupt the progression of the cell cycle[9]. However, in the present study, increasing the expression of cdc42 gene in the fourth stage of the disease has led to the progression of the disease.

Elevated levels of miR-372 in the sputum, along with other miRNAs (miR-21, miR-143, miR-155, miR-210), are biomarkers used for early detection of NSCLC adenocarcinoma[29]. However, the present study showed that miR-372 can be detected during metastasis.

Cdc42 has been overexpressed in patients with primary lung cancer, and excessive expression of Cdc42 plays an important role in the progression and metastasis of lung cancer cells. Their results also show that inhibiting the expression of the cdc42 gene in laboratory conditions and in living organisms inhibits the migration and invasion of lung cancer cells. Therefore, cdc42 may become an important molecule in the treatment of cancer, and its expression is directly related to tumor stage, lymph node metastasis, and patient survival[20]. The results of our research also showed that excessive expression of Cdc42 plays an important role in the progression and metastasis of lung cancer cells.

Studies have shown that miR-372 plays an anti-oncogenic role in cervical cancer by controlling cell growth and cell cycle progression by low expression of CDK2 and cyclin A1 cycles[30]. In the present study, miR-372 had a significant increase ($p<0.05$, $p=0.022$) in patients with stage IV metastatic disease.

Disabling cdc42 induces apoptosis and stops the G1 phase of the cell cycle in gastric cancer cells31. In this study, a significant increase in the cdc42 gene ($p<0.05$, $p=0.032$) in the metastatic stage of lung cancer played a role in the increase in metastasis.

Expression of miR-137, miR-182, and miR-372 increases the invasiveness of lung cancer cells[32]. In our study, it was shown that increasing the expression of miR-372 increases the process of metastasis and disease progression.

Studies have shown that miR-372 expression in ovarian carcinoma was significantly lower compared to normal ovarian tissue and benign tumors. In addition, overexpression of miR-372 significantly inhibits cell proliferation and increases cell apoptosis. The findings show that miR-372 plays an important role in inhibiting tumor growth and is a valuable target for the treatment of ovarian cancer[33].

Studies have shown that the expression of miR-372 in breast cancer tissues and human cell lines is significantly reduced compared to breast tissue cell lines. In addition, the results of functional assays showed that miR-372 inhibits cell proliferation and increases apoptosis in MCF-7 in the human breast cancer cell line. E2F1 was identified as a direct functional target of miR-372 in breast cancer. As a result, the findings suggest that miR-372 may have the potential to act as a new molecule for the diagnosis and treatment of breast cancer patients[34]. CdcC42 induces invasiveness and metastatic activity by breast cancer cells[35]. Studies have shown that CDC42-v2 is expressed at lower levels in ovarian cancer cell lines and ovarian tumor tissue than in normal control cells and tissues. In addition, CDC42-v2 has an inhibitory effect on ovarian tumor cell growth, colony formation, and invasion[36].

According to studies and easy access to serum samples, it is also easy to identify genes and microRNAs in body fluids due to the stability of microRNAs in body fluids and their non-invasiveness. Therefore, the use of serum cdc42 gene and miR-372 is recommended for early detection and screening. Therefore, the expression of cdc42 gene and miR-372 should be investigated in more samples and its role as serum biomarkers should be determined.

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Conflict of interest:
The authors declare no conflict of interest.

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