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Comparative investigation of miRNA422a serum expression level in healthy and metastatic lung cancer Patients by Real Time PCR method

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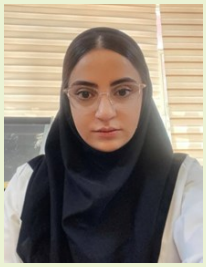

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Abstract

Introduction: MiRNAs are a family of small non-coding RNAs that play an important role in regulating the expression of various oncogenes or tumor suppressor genes. These miRNAs play roles in almost all biological phenomena, including cell cycle regulation, cell growth, apoptosis, cell differentiation, and stress response. MiRNA422a is one of these miRNAs that different roles in various types of cancers. Ths study aimed to compare the expression of miRNA422a in healthy individuals and patients with lung cancer by Real time PCR.

Methods: This prospective study analyzed serum samples of 40 patients with lung cancer, and serum samples of 40 healthy individuals. Patient samples consisted of individuals who were at all stages, stages one, two, three, and four. In this study, miRNA was extracted from serum with Trizol solution. Then, using a cDNA synthesis kit, cDNA was constructed, and using the Rael Time PCR method, the expression of this miRNA was investigated.

Results: The results showed that there was no significant difference between miRNA422a serum expression in stages 1, 2, and 3 between healthy and patient, but in step 4, there was a significant difference between miRNA serum expression between healthy and patient ($p < 0.05$). In patients, who were in stage four, the level of miRNA422a serum expression increased significantly, which could be used to detect metastatic patients with lung cancer.

Conclusion: This increase can be used as a biomarker for the detection of metastatic lung cancer and its treatment.

Keywords: lung cancer, Real Time PCR, miRNA422a

1. Introduction

Lung cancer (LC) is a frequently occurring type of cancer mortality in males [1], with treatment varying based on type and stage via surgery, chemotherapy, or radiation [2]. The 5-year survival rate of lung cancer is 16.8% and less than 5% for those with metastatic disease. LC is the leading cause of cancer death in Iran. In 2018, it was estimated that approximately 28,000 people died from the disease [3]. The risk factors for LC in Iran are similar to those in other countries, including smoking, exposure to secondhand smoke, and exposure to hazardous air pollutants. In addition, Iran has a high prevalence of air pollution, which is believed to be a contributing factor to the high incidence of lung cancer [4, 5].

The classification of LC has been established with a categorization into two main subtypes, namely small-cell lung cancer (SCLC), and non-small-cell lung cancer (NSCLC). The most common type of LC is NSCLC, which accounts for approximately 75% of all lung cancer cases[6].

miRNA422a-3p is a type of microRNA (miRNA) that is found in humans and other species. It is a member of the miR-422 family, which is involved in the regulation of cancer-related pathways. In particular, miRNA422a-3p has been linked to the regulation of cell cycle progression, apoptosis, and the development of drug resistance in cancer cells. Studies have also suggested that miRNA422a-3p may be involved in the regulation of angiogenesis and inflammation.

in lung cancer, and that its expression is associated with better prognosis in patients. Furthermore, miRNA422a can suppress the growth and metastasis of lung cancer cells. In addition, miRNA422a is involved in the regulation of several pathways associated with lung cancer, such as the PI3K/AKT/mTOR pathway. Thus, miRNA422a may be a potential therapeutic target for the treatment of lung cancer.

The present study aimed to identify individual blood miRNAs that could be used as potential biomarkers for early diagnosis of lung cancer.

2. Materials and methods

2.1. Study population

Forty patients with newly diagnosed NSCLC (57.9 ± 9.5 years old, mean ± SD) were recruited at the Masih Daneshvari Hospital (Tehran, Iran) between April 2010 and September 2012. Histology and clinical parameters confirmed the presence of lung cancer, and patients were not on any treatment and had no history of other cancers or inflammatory diseases. Age- and sex-matched controls (n=40) were also recruited following a general health check and a negative history of cancer and inflammatory diseases.

2.2 Preparation and RNA isolation

The TRIzol (Invitrogen) was used to isolate RNA from serum samples. Briefly, serum containing TRIzol was treated by chloroform (Merck, Germany), and after isopropanol (Merck, Germany) sedimentation and ethanol washing, total RNA was diluted in sterile DEPC-treated water. The isolated RNA was quantified and assayed for purity using a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, U.S.A.) and then stored at –80 °C. The concentration and absorption spectrum of RNA was measured spectrophotometrically (NanoDrop® ND-2000) at 260 nm in ng/µl. The purity of RNA was verified based on A260/A280 and A260/A230 absorbance ratios. Samples with a ratio of A260/A280 in the range of 1.8–2.2 and a ratio of A260/A230 in the range of 1.7–2.9 were used to determine selected miRNAs.

2.3 Reverse transcription

Extracted RNA was reverse transcribed using the miRCURY LNA Universal RT microRNA cDNA Synthesis Kit (miRCURY LNA RT Kit-QIAGEN, MD, USA) according to the manufacturer's instructions.

2.4 Quantitative PCR

miR-422a was detected by real-time PCR assays by using the SYBR Green Master Mix kit (QIAGEN, MD, USA). Quantitative PCR was performed in ABI 7500 Fast Real-Time PCR system (Applied Biosystems) with the following cycle: 95°C for 10 min, followed by 40 cycles of 95°C for30 s, 60°C for 30s and 72°C for 30s. The cycle threshold (Ct) values were calculated with the SDS 2.0.1 software (AppliedBiosystems). The average expression levels of miRNAs in serum were normalized with U6 snRNA using the 2^{-DDCt} method. The DCt was calculated by subtracting the Ct values of U6 snRNA from the Ct values of the miRNAs of interest. The DDCt was then calculated by subtracting DCt of the surrounding normal lung serum or the average expression of healthy volunteers from DCt of lung cancer patients. Fold changes were calculated by the equation 2^{-DDCt}, and a cut-off value of 2^{-DDCt} [2.00 was considered positive for overexpression].

2.5 statistical analysis

Statistical analysis was performed using SPSS 19 software (SPSS Inc., Chicago, IL, USA) and the results were analyzed by one-way ANOVA. The expression level of target genes between the treated samples and control group was measured by Tukey's HSD *post hoc* test. Data were presented as mean ± standard deviation (SD) and $p < .05$ was considered statistically significant.

3.Results

3.1 The level of miRNA422a and U6 gene expression in the stage of a disease

Real-time PCR was applied to evaluate the expression levels of microRNAs in the two groups (Figure 1) and the clinical stages of NSCLC. In stage one disease, there was no significant difference between miRNA422a and U6 genes (P=0.330) (Figure 1A).

). There was no significant difference between the miRNA422a and U6 genes in the two disease stages (P=0.208)) (Figure 1 B). There was no significant difference between the serum expression of miRNA422a and U6 genes in people who were in the third stage of this cancer (P=0.141) (Figure 1C).

). In people who were in the fourth stage of this disease, there was a significant difference between the serum expression levels of miRNA422a and U6 genes (P=0.000) (Figure 1D).

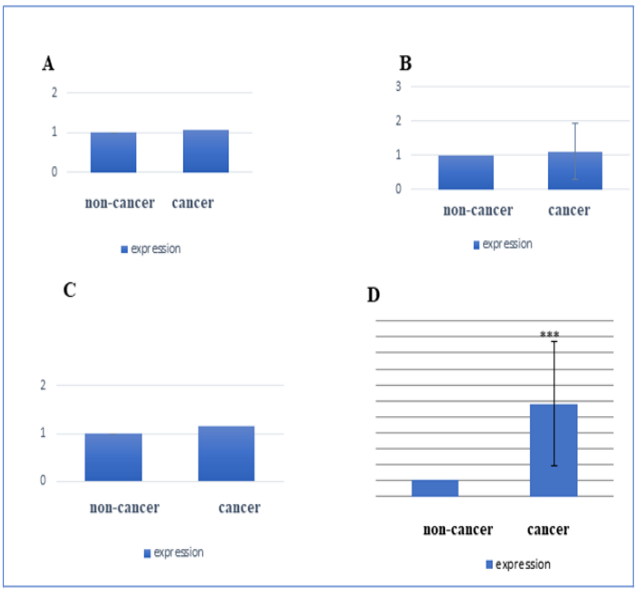

Figure. 1. Serum expression of miRNA422a and U6 gene in different stages of lung cancer. Expression of miR422a in stage one (a), stage two (b) stage three (c), and stage four in NSCLC patients and non-cancerous groups was compared (P<0.05).

3.2 Distribution of the population in terms of gender and family history

There was a significant difference in the distribution of the studied population in terms of gender in men and women, and the prevalence is higher in men with lung cancer ($p<0.05$) (Figure 2A). but In terms of family history, there was no significant difference between the two groups in the distribution of the population ($p < 0.05$).

4. Discussion

Cancer refers to a set of diseases that arise from the uncontrolled proliferation of cells. Cancer cells are separated from the normal mechanisms of cell division and growth. The exact cause of this phenomenon is unclear, but it is possible that genetic factors or things that disrupt the activity of cells, cause problems in the cell nucleus and play a role in causing cancer. In a healthy organism, there is always a balance between the rate of cell division, natural cell death, and differentiation [7]. Cancer includes all types of malignant tumors, which are more commonly known as neoplasms in medicine. When one of the cells of the body undergoes abnormal growth due to various factors, it eventually causes the abnormal growth of other cells as well. This process eventually leads to the production of a tumor that disables that part and spreads to other parts as well. There is a possibility of cancer at different ages, but with increasing age, the probability of getting cancer increases[8].

One of the most common cancers is lung cancer. Lung cancer is a type of disease characterized by the uncontrolled growth of cells in the lung tissue. If the disease is not treated, the cell growth can spread outside the lung to nearby tissues or other organs in a process called metastasis. Most cancers that start from the lung, called primary lung cancers, are carcinomas that originate from the epithelial tissue [43]. The main types of lung cancer are small cell lung cancer (SCLC), also called squamous cell carcinoma, and non-small cell lung cancer (NSCLC) [9].

The results showed that there is no significant difference between the serum expression of miRNA422a gene of U6 in stages one (P=0.330), two (P=0.208), and three (P=0.141) of the disease, but in stage four there is a significant difference between the serum expression of this gene and U6 were present (P=0.000). That is, only those who were in stage four of the disease increased the expression of this miRNA.

Diagnosis methods in these patients should be done step by step, but unfortunately, it cannot be detected at the stage where the cells are multiplying indiscriminately and irregularly. When lung cancer is diagnosed, it has reached a stage in terms of growth that can show itself in the CT scan. The main reasons for choosing lung cancer in this research are the late diagnosis of the disease, and the increasing prevalence, mortality, and metastasis of this disease.

Micro ribonucleic acids (miRNA) are non-coding ribonucleic acids that are evolutionarily protected and have a length of 20-24 nucleotides. MiRNAs control the expression of genes after transcription by degrading mRNA or inhibiting their translation. These molecular structures participate in the control of physiological and pathological cellular processes, and many of them can act as oncogenes or tumor suppressors. There is very little information about the factors that affect the expression of miRNAs. However, by inhibiting disease-causing miRNAs (oncogenes) and creating necessary and functional miRNAs (tumor suppressors), these regulatory small RNAs can be used therapeutically in cancer. For this reason, the identification of miRNAs and their target molecules has provided a bright horizon for understanding the pathways that lead to cancer. Therefore, these compounds can be used as potential biomarkers in the diagnosis, prognosis, and treatment of cancer[10].

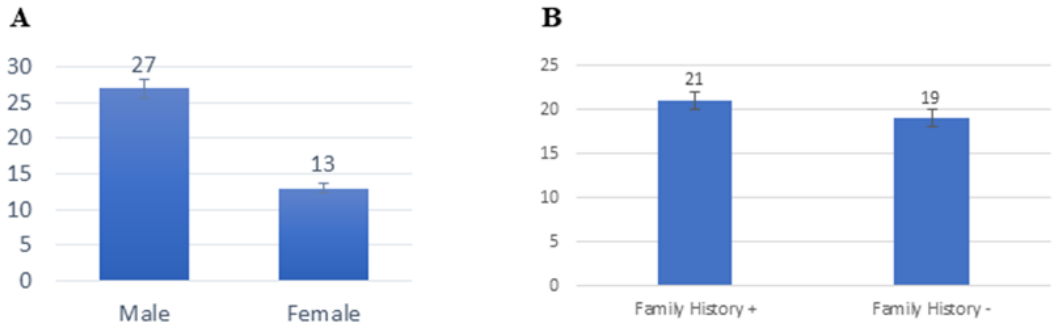

Figure2. The distribution of the population in terms of gender(A) and smoking(B).

Also, more research is needed to clarify the regulatory mechanisms of miRNA biogenesis and their role in cancer. Identifying the target molecules of miRNAs and investigating their molecular interference effects in message transmission pathways will help to better understand the mechanism of cancer. One of these miRNAs is miR422a, whose expression level varies in different cancers. Song KH and colleagues concluded in 2010 that the sequences recognized by miRNA122a and miRNA422a were located in the 3′-UTR region of human CYP7A1 mRNA. Naturally, the increase in CYP7A1 mRNA expression in the body inhibits these miRNAs[45]. Reports by Mao et al. in 2012 suggest that miRNA422a causes genome instability, tumorigenesis, and colon cancer by reducing the expression of MutLα by suppressing the expression of MLH1 through binding to the ′3UTR-MLH1 region [11].

Faltejskova et al reported in 2012 that dysfunction of miRNA378, miRNA375, miRNA422a, miRNA215 and miRNA135B in colorectal cancer patients played an important role in the pathogenesis of this disease [12]. In 2014, Jin Zhang and his colleagues investigated the function of miRNA422a in liver cancer, they found that increased expression of miRNA422a in HCC tumor cells significantly inhibited cell proliferation and migration in vitro. In 2016, Faltejskova P and colleagues did a lot of research to determine the expression level of miRNA422a in colorectal cancer tissue (CRC) and investigate the prognostic value and function of miRNA422a in CRC cancer. They observed that the decrease in the expression of this miRNA is an independent factor for the development of colon cancer[13]. Zheng GX1 and colleagues 2016 investigated the expression level of miRNA422a in colorectal cancer. Another role of miRNA422a is to inhibit the proliferation and invasion of glioma by targeting IGF1 and IGF1R. Glioma is a common type of malignant brain tumor that can aggressively metastasize. Recent evidence has shown that non-coding RNAs, including miRNAs, play an important role in the development of glioma pathophysiology [14].

The results of this research show the oncogenic role of miRNA422a in lung cancer. Also, because this miRNA was increased only in the four stages of the disease, the role of this miRNA in metastasis can be understood.

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