Background: Lung cancer is one of the most common malignant tumors with poor survival, which is usually diagnosed at advanced stages. In recent years, increasing evidence has revealed that circulating miRNAs exhibit great potential in screening and early detection of various types of cancers including lung cancer. miR-21 is one of the most highly expressed members of the microRNA family in many human cancer types.

Methods: The authors analyzed miR-21 expression in matched tumor and normal tissues and plasma in 17 patients affected with non-small cell lung cancer. Samples were collected from the NSCLC patients before surgery, radiotherapy or chemotherapy. Expression levels of tissue miR-21 were assessed, using TaqMan RT-PCR assay. Expression levels of plasma miR-21 were assessed, using LNA™ RT microRNA PCR primer set and SYBR green qRT-PCR assay.

Results: miR-21 expression was higher in 9 out of 17 patients’ plasma samples. No change in miR-21 expression was observed in 8 plasma samples. In lung tissues, ten patients showed up-regulation of miR-21 and six patients were down-regulated. One patient had no change in miR-21 expression level in tissue samples. The expression level of mir-21 in tissue and plasma was concordant in 6 patients.

Conclusion: It seems that the level of miR-21 in plasma samples of NSCLC patients as a marker for screening needs more investigation.

Keywords: Non-small cell lung cancer, miR-21, plasma, tissue
INTRODUCTION:

Lung cancer is one of the most prevalent malignant tumors worldwide. It is the leading cause of cancer-related death in Europe and North America. 3050 new cases of lung cancer were observed in 2008 in Iran. Histologically, lung cancer is divided into two subtypes: non-small cell lung cancer (NSCLC), responsible for 85% of incidences, and small cell lung cancer (SCLC) comprising 15% of cases. In spite of different treatment modalities, the 5-year survival rate of NSCLC patients is still 15%-20%. Unfortunately, diagnosis of NSCLC at early stages is not often possible because of nonspecific clinical signs and symptoms and a shortage of diagnostic tools. Therefore, diagnosis of the disease in more than two-thirds of cases is made in late stages of the disease. Clinical outcomes, therefore, can be potentially improved by a screening of individuals at a higher risk of developing lung cancer. According to the research conducted at the National Lung Cancer Screening Trial, the mortality can be reduced by approximately 20% through annual low-dose computerized tomographic screening. There is an emerging need for noninvasive tools for screening and early detection of lung cancer to improve the prognosis. A wide range of tumor markers has been considered for detection of lung cancer. Among these markers, microRNAs are promising, which are present in tissues and body liquids.

MicroRNAs (miRNAs) as noncoding RNA molecules with 18-25 nucleotides regulate the expression of numerous genes. miRNAs contribute to critical processes in differentiation, proliferation, apoptosis, and metabolism at the cellular level, which are ultimately connected to cancer progression. A large body of literature indicates the potential of miRNA as a useful biomarker for the diagnosis and prognosis of cancers. Moreover, a number of recent investigations have shown that miRNAs in body fluids, including serum and plasma, have the potential to be considered as diagnostic and prognostic as non-invasive markers.

Because circulating miRNAs are small and may bind to proteins such as the Argonaut-2 protein and high-density lipoproteins, they are resistant to endogenous ribonuclease activity. Alternatively, miRNAs may be bundled by secretory particles, including apoptotic bodies and exosomes in plasma/serum. Therefore miRNAs are considered to play a pivotal role as a biomarker for diagnosis and prognosis in lung cancer. The up-regulation of circulating miRNAs takes place in the plasma of tumor-bearing patients. Hence, the assessment of circulating miRNAs takes place in the plasma of tumor-bearing patients. The up-regulation of circulating miRNAs takes place in the plasma of tumor-bearing patients. Therefore, clinical outcomes, therefore, can be potentially improved by a screening of individuals at a higher risk of developing lung cancer. Among these markers, microRNAs are promising, which are present in tissues and body liquids.

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(PTEN), thereby, stimulating oncogenesis and progression of different carcinomas. The present study aimed to compare the level of miR-21 as a marker in tissue and plasma of NSCLC patients for the diagnosis of NSCLC.

**METHODS:**

**Tissue samples:** Primary lung tumor tissues (n=17, roughly 3×3×3 mm volume) and paired adjacent normal tissues were sampled from patients who were under surgical removal for NSCLC before radiotherapy or chemotherapy at Masih Daneshvari and Atieh Hospitals (Tehran, Iran) from September 2011 to December 2016. The freshly collected tissue samples were transferred to liquid nitrogen tank and stored at −80 °C.

**Plasma samples:** Peripheral blood samples were obtained from the same NSCLC patients, and normal samples were selected from healthy volunteers (n= 50) who were assigned as the control group, and whose age, gender, and smoking habits matched those of NSCLC patients. Prior to surgery and/or adjuvant therapy, blood samples were collected in tubes containing EDTA at the time of initial consultation. Plasma was separated from the blood sample within two h of collection by centrifugation in 12000rpm for 15min at 4°C. The whole process for plasma extraction was based on platelet poor plasma protocol. Plasma samples were poured into fresh tubes and kept at −80 °C for further examination.

All patients and controls signed an informed consent form prior to surgical resection and collection of blood samples. The Ethics Committee (sbmul.REC1394.112) at Shahid Beheshti University of medical science approved the research protocol. Standard diagnostic techniques pathologically examined all the samples. A revised AJCC/UICC 7th edition TNM classification schema was used to stage the patients. Tumor size was categorized to T1≤3 cm, T2>3 and ≤7 cm, and T3>7 cm.

**RNA extraction and cDNA synthesis**

**Tissue samples:** miRNeasy Kit (Qiagen, USA) was used to extract total RNA, including miRNA, from tumors and the adjacent normal tissues (15–35 mg) based on manufacturer’s instructions. Using a NanoPhotometer, the extracted RNA was analyzed in terms of concentration and purity (the ratio of absorbance at 260 and 280 nm). The cDNA for TaqMan RT-PCR was synthesized for each miRNA using specific stem-loop primers instead of oligodT primers by two-step RT-PCR Kit (Vivantis, USA).

**Plasma:** Total RNA was isolated from plasma (100 µl), using MiRNeasy Serum/Plasma kit (QIAGEN Co. Cat#217184). First strand cDNA was synthesized, using a universal cDNA synthesis kit II (EXIQON Co. cat#203301) according to manufacturer’s instruction. It is noteworthy that UniSp5 and cel-mir-39-3p spike-ins with the lowest concentrations among RNA spike-ins were applied in order to determine the accuracy and efficiency of RNA isolation and cDNA synthesis. Thus, UniSp5 and cel-mir-39-3p spike-ins primer sets were prepared from EXIQON (Cat #203955 and #203952). All the cDNA samples were analyzed through qRT-PCR according to the protocol specified for this spike-ins by the manufacturer. The SYBR Green RT-PCR reactions were carried out in MicroAmp Optical 96-well plate, using StepOne v.2.2.2 software (Applied Biosystems, USA) based on the instructions provided. The thermal-cycling conditions were 95 °C for 10 min, then 45 cycles at 95 °C for 10 sec and 60 °C for 10 sec.

**qRT-PCR**

**Tissue samples:** quantitative RT-PCR Specific TaqMan probes, forward primers, and universal reverse primer were designed by the authors, using Allele ID 6.0 and Oligo7 software According to a previous report. Duplicate TaqMan RT-PCR reactions were carried out in MicroAmp Optical 96-well plate via SDS.
v.1.0.1 software (ABI System 7300, Applied Biosystems, USA). Briefly, each response consisted of 5μl TaqMan master mix, 1μl specific forward and universal reverse primers (10pmol), 1pmol specific probe, and 2μl (800 ng) of cDNA for a total reaction mixture of 12μl. Each analysis contained a non-template control (NTC). Thermal cycling conditions were 95 °C for 10 min for initial denaturation, then 45 cycles at 95 °C for 15 sec and 60 °C for 1 min. cDNA dilutions were made sequentially to specify the dynamic range and amplification efficiency of miR-21 via plotting corresponding Ct values versus log concentrations of cDNA. The reference gene used was RNU6b (RNA, U6 small nuclear 2).

**Plasma samples:** qRT-PCR was carried out by ExiLENT SYBR® Green master mix, with similar reactions to the spike-ins thermal-cycling condition. Afterward, the method of comparative ΔCt was calculated for miR-21. The SYBR Green RT-PCR reactions were accomplished according to manufacturer’s instruction. Each assay was provided with a Non-Template Control (NTC). The thermal-cycling conditions were 95 °C for 10 min, then 45 cycles at 95 °C for 10 sec and 60 °C for 10 sec. miR-24-3p in the plasma samples was selected as a reference gene by the use of NormFinder as previously reported4.

**Data analysis**

The comparative ΔCT method was employed to calculate relative expression of miR-2113, which is represented as fold changes. Using 2-ΔΔCt equation, the fold change equal or more than 2-fold in miR-21 expression level was considered to be significant.

**RESULTS:**

In this study, 17 NSCLC tumor and normal adjacent tissue samples and their plasma samples, and fifty plasma samples from healthy normal matched controls were collected. There were six (35%) female and 11 (65%) male patients with an average age of 58 years, consisting of eight (47%), six (35%), and three (18%) patients in stages I, II, and III, respectively. Four (23.5%) out of 17 and 13 (76.5%) patients had T1 and T2 tumor sizes, respectively. In total, two (11.8 %) patients had positive, and 15 (88.2 %) patients showed negative lymph node.

**Expression of miR-21 in tissue samples**

Examination of tissue samples revealed up-regulation of miR-21 in 10 out of 17 tumor tissue samples (58.8%) compared to normal tissues. Similarly, levels of miR-21 significantly decreased in six (35.3%) out of 17 tumor tissue samples compared to normal tissues. No significant change was found in the expression level of miR-21 in one patient. In addition, four out of six down-regulated miR-21 are adenocarcinoma and two of them SCCs which were detected in 17 tissue samples. Based on the histological classification, miR-21 expression was increased in seven out of 12 adenocarcinoma samples and only one sample showed no significant change in the expression level of miR-21 compared to normal tissues. Moreover, up-regulation of miR-21 expression was observed in three out of five SCC samples. The results are shown in Figure 1.

**Expression of miR-21 in plasma samples**

The miR-21 expression level displayed no significant down-regulation in NSCLC plasma samples in comparison with normal ones. Up-regulation of miR-21, however, was observed in nine out of 17 NSCLC plasma samples (53%) whereas eight samples (47%) exhibited no significant changes compared to normal plasma samples. In addition, five out of nine up-regulated
miR-21 were adenocarcinoma and 4 of them SCCs which were detected in 17 plasma samples. It was further found that six, two, and one of the plasma samples (among nine up-regulated miR-21) were in stages I, II, and III, respectively. Only one out of six plasma samples from females had an increase in miR-21 expression compared to normal plasma. Furthermore, two out of six plasma samples from non-smoker patients displayed up-regulation of miR-21 expression. The results are shown in Figure 1.

### Table 1. The clinicopathological parameters of the NSCLC patients

<table>
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<th>no.</th>
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<th>Sex</th>
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<th>Subtype</th>
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<td>T2</td>
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<tr>
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<tr>
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<tr>
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</tbody>
</table>

**Expression of miR-21 in plasma and tissue samples**

From 10 up-regulated miR-21 in the tissue samples, six patients exhibited up-regulation of miR-21 in plasma whereas no change was found in four patients. Considering six down-regulated miR-21 in tissue samples, four patients showed no changes and two patients revealed up-regulation of miR-21 in plasma. Moreover, miR-21 expression level of tissue samples did not change in one patient but it was up-regulated in the
plasma sample.

**DISCUSSION:**

Early detection of lung cancer plays an essential role in raising overall survival of patients. It is highly necessary to investigate biological and molecular features in order to determine specific markers for lung cancer development and define novel potential therapeutic targets. Identification of circulating cancer-specific miRNA profiles has now become a developing field of particular interest.

The current study focused on the correlation of miR-21 expression levels between tissue and plasma samples of NSCLC. The miR-21 expression levels in tissue and paired plasma samples were compared in 17 NSCLC patients. A correlation was found among 35%

![miR-21 expression levels](image)

**Figure 1.** shows fold change of miR-21 expression in plasma and tissue samples compared to normal.
of samples with up-regulation in both tissue and plasma samples. The role of miR-21 in tumor pathogenesis and during all other stages of carcinogenesis has been emphasized in numerous investigations. According to Tang et al., lung cancer patients can be distinguished from healthy smokers by higher plasma miR-21 and miR-155 expression levels with 69.4% sensitivity and 78.3% specificity. Yin et al. (2015) identified aberrant expressions of miR-21 in tissue and blood samples of different malignancies, indicating the potential of this molecule as a diagnostic and prognostic biomarker. NSCLC diagnosis has markedly developed through direct evaluation of tissue gene biomarkers, though, their applications are limited by the aggressiveness and undesirability of the diagnostic practices.

The expression of miR-21 was down-regulated in tissue but up-regulated in plasma samples from two out of 17 patients, which might have been caused by other diseases. As stated above, increased miR-21 level has been documented in a considerable number of diseases. From one point of view, increased miR-21 expression is accompanied by weak immune responses, including asthma, psoriasis, cancer, and importantly chronic bacterial or viral infections. Conversely, increased miR-21 expression has also been reported in diseases fueled by chronic inflammation, including colitis, atherosclerosis, type 2 diabetes, and SLE. In such cases, it would be advantageous that a regulatory response is triggered via miR-21; however, the inflammatory environment of diseased tissues has not revealed such a response.

The expression level of miR-21 in different cancers has been studied. According to The authors’ recent report, the expression of miR-21 was up-regulated in 53.3% of cancer tissues compared to matched adjacent normal tissues. In 2012, the investigation of Solomides et al. showed that Expression of miR-21 was significantly increased in tissue but some samples were down-regulated in miR-21 compared to normal samples. In the present study, miR-21 was up-regulated in tissue but not in plasma samples from four out of 17 patients. Similarly, in a study, Heegaard et al. found that the expressions of miR-146b, miR-221, let-7a, miR-155, miR-17-5p, miR-27a, and miR-106a significantly declined in sera of NSCLC patients, whereas significant elevations were detected in miR-29c levels. Surprisingly, the plasma samples of the same patients showed no significant differences with controls. It was concluded that discordant miRNA level in plasma and tissue might be caused by an unknown reason because miR-21 was not released from tumor tissue into the blood. The discordance in tissue and plasma levels of miR-21 in the study also reflects the small number of samples. Therefore, the results should be confirmed by further investigations on larger populations.

REFERENCES:

The Evaluation of mir21- Level in Lung tissue...


