Expression of miRNA1, miRNA133, miRNA191, and miRNA24, as Good Biomarkers, in Non-Small Cell Lung Cancer Using Real-Time PCR Method

Mehdi Kazempour Dizaji1, Behrooz Farzanegan2*, Naghmeh Bahrami3,4, Zahra Khoshnam5, Mohammad Fathi6, Hossein Dargahi7, Saviz Pejhan7, Adnan Khosravi8, Sadegh Shirian9, Armita Narimani10, Maral Emami11, Mahsa Rekabi12, Abdolreza Mohamadnia11,13

Abstract

Background: Lung cancer has recently shown the highest incidence among all cancers. microRNAs (miRNAs) are the molecules playing a role in regulating gene expression and contributing to many pathogenic mechanisms. Therefore, these molecules could be used as biomarkers for the detection, anticipation, and treatment of cancer. With this in mind, we decided to investigate and compare the expression of miR-1, miR-133, miR-191, and miR-24 and also the expression differences in these four RNA molecules between lung cancer patients and the controls. Methods: A total of 50 patients with lung cancer participated in this study. In addition, 50 healthy blood samples were selected as the control group. Real-time PCR determined the expression levels of miRNA. The RNAs extracted from the patients’ white blood cells were initially synthesized, and then cDNA was extracted. Finally, the synthesized cDNA was amplified using real-time PCR, and its expression was compared with the control group. Results: The result indicated a low expression level of miR-1 and miR-133, and a high expression level of miR-191 and miR-24 in the blood of patients with lung cancer compared to the healthy subjects. Conclusion: Our findings revealed that miR-1, miR-133, miR-191, and miR-24 are oncogenes, and their expression could result in cancer. It appears that a therapy to overexpress miR-1 and miR-133 and downexpress miR-191 and miR-24 could contribute to the treatment of lung cancer.

Keywords: Lung cancer- apoptosis- miRNA1- miRNA133- miRNA191- miRNA24- real-time PCR

Asian Pac J Cancer Prev, 23 (5), 1565-1570

Introduction

Lung cancer is a type of cancer beginning in the lungs and is considered among the most common malignancies and the first prevalent cancer in the world. However, the function of cell apoptosis failure in cancer has not fully been proven yet (Lodish et al., 2000; Ghadimi et al., 2017; Karimi et al., 2017).

MicroRNAs (miRNAs) are a large subgroup of non-coding RNAs of 18-25 nucleotides that are evolutionally protected (Jin et al., 2013; Kim and Reitmair, 2013). These molecules control the gene expression after transcription by inhibiting mRNA translation or inducing dissociation (Negri et al., 2009). The interaction of miRNAs with...
target genes determines their role in growth, planned death, cell differentiation, and proliferation and verifies the direct function of miRNA in cancer (Schaefer et al., 2010).

MiRNA-133a is downregulated in many human malignancies and correlated with tumor progression. However, its roles and its related molecular mechanisms in lung cancer are still unknown. MiR-133a was identified to be a tumor suppressor as its transfection mimics in PANC-1 cells was able to reduce cell proliferation, invasion, and migration, promote cell apoptosis in vitro and suppress tumorigenicity in vivo. These findings suggest a significant role of miR-133a in the molecular etiology of cancer and implicate its potential application in gene therapy of cancers (Qin et al., 2014). MiR-191 has recently been reported to be abnormally expressed in several cancers (>20) and various other disorders, including type 2 diabetes, Crohn’s and Alzheimer’s diseases, and pulmonary hypertension. This RNA type regulates important cellular processes, such as cell proliferation, differentiation, apoptosis, and migration, by targeting prominent transcription factors, chromatin remodelers, and cell cycle associated genes. Studies have demonstrated miR-191 to be an excellent biomarker for cancer diagnosis and prognosis, leading to two patent already in its kitty (Nagpal and Kulshreshtha, 2014). miR-1 was shown to be predominantly downregulated in almost all examined human cancers. As a tumor suppressor miRNA, miR-1 is involved in post-transcriptional regulation of crucial tumor associated gene expression and represents a promising target for anticancer therapy. Re-expression of miR-1 can suppress cancer cell proliferation, promote apoptosis, and reverse drug resistance in cancers both in vitro and in vivo. Lately, the regulatory mechanisms of miR-1 expression have been studied in various cancers in different model systems. Recent investigations from our group and others have revealed that miR-1 is frequently downregulated in various types of cancer. Through targeting multiple oncogenes and oncogenic pathways, miR-1 represses cancer cell proliferation and metastasis and promotes apoptosis by ectopic expression (Nakajima et al., 2006; Gagan et al., 2012; Han et al., 2014). MiR-24 is one of the most abundant miRNAs in cervical cancer (Wang et al., 2008), and its upregulation has been observed in oral carcinoma (Lin et al., 2010). Evidence has evinced that miR-24 can inhibit the apoptosis of both cancer cells (Qin et al., 2010) and cardiomyocytes (Qian et al., 2011) and can accelerate cell proliferation (Zaidi et al., 2009). It appears that miR-24 can repress the expression of the tumor suppressor p16INK4a (Lal et al., 2008).

With respect to the increasing prevalence of lung cancer, researchers have extensively focused on studying this kind of cancer. However, few studies have been conducted in molecular apoptosis. Therefore, this study was undertaken to compare the expression levels of miR-133, miR-191, miR-1, and miR-24 in lung cancer patients with the control group. Moreover, we compared the expression differences between these four molecules in the under study groups.

Materials and Methods

Sampling

This study was conducted in 2018 at Masih Daneshvari Hospital of Shahid Beheshti University of Medical Sciences, Tehran, Iran after receiving the ethical code (IR. SBMU.RETECH.REC.1397.580). A total of 50 patients with lung cancer who did not undergo any treatment and 50 healthy subjects were enrolled in the study at the Clinical Laboratory of Masih Daneshvari Hospital. The clinical diagnosis of cancer was confirmed using histopathologic tests on tumor tissue samples. From each subject, 1 mL of peripheral blood mononuclear cell was taken and combined with anticoagulant to prevent clotting. All human protocols for this work were reviewed and approved by the Committee of Christian Hospital, USA, in conformity to the Helsinki Statement. A written informed consent was obtained from each participant (Table 1).

Total RNA extraction

Total RNA was extracted from whole blood samples using Trizol reagent (Invitrogen, Life Technologies, USA) (Rio et al., 2010). Quantitative and qualitative analyses of the extracted RNA were performed by optical absorption spectroscopy using a spectrophotometer (Sigma, USA) and by agarose gel electrophoresis, respectively. The size fractionation of DNA fragments was evaluated by gel electrophoresis on a 1% agarose gel.

cDNA generation

To generate cDNA from miRNA, we used hairpin primer and an RNA-dependent reverse transcriptase using Master Mix Kit (Takara, Japan) on a Rotor-Gene (Qiagen, Germany). All RNA samples were treated with DNaseI enzyme to avoid DNA contamination.

Real-time PCR reaction

The PCR reaction was carried out (Jamaati et al., 2016; Heydar et al., 2018) in a 20-µl total volume, 12.5 µl of Master Mix (Ampliqon, Denmark), 2 µl of primers, 1.5 µl of cDNA template, and 4 µl of deionized distilled water. To perform the real-time PCR reaction, we used the primers included in Table 1 using the following cycling conditions: 95°C for 10 min, and 40 cycles at 95°C for 15 sec, and 60°C for 1 min. Each complete amplification stage was followed by a dissociation stage at 95°C for 15 sec and 60°C for 30 sec. The temperature was then raised from 60°C to 95°C (0.03°C/s), and fluorescence intensity data were collected continuously over the ramping stage for 20 min. Melting curve analysis was conducted according to the dissociation stage data, and reactions with a single peak at expected temperature melting (Tm) were considered for further analysis.

Statistical analysis

All data were analyzed using GraphPad Prism version 2. Comparison of the expression of miRNAs between the lung cancer patients and healthy controls was performed using SPSS 21.1 software and Mann-Whitney test. p ≤ 0.05 was considered to be statistically significant.
Expression of miRNA1, miRNA133, miRNA191, and miRNA24, as Good Biomarkers, in Non-Small Cell Lung Cancer

ΔCT of each individual was studied. The mean ratios of the healthy (control) group to patients were 1 ± 0.145 and 0.51 ± 0.021 for miR-1 and 1.011 ± 0.123 and 0.292 ± 0.040 for miR-133 (Figures 1 and 2).

Comparing the expression levels of miR-1 and miR-133 between two under study groups showed a significant reduction in the patient group compared to the control one, with P = 0.025 and P = 0.039, respectively. Besides, the mean ΔCT of miR-1 and miR-133 indicated a

Results

Comparison of miRNA levels between lung cancer patients and healthy controls

The mean CT and expression levels of miR-1, miR-133, miR-191, and miR-24 were evaluated in all the healthy subjects and patients. Following the expressions of the four miRNAs, the U6 gene expression was analyzed and utilized as an internal control. For this purpose, the

Figure 1. The Expression Levels of miR-1 between the Two under Study Groups, which were Significantly Different

Figure 2. The Expression Levels of miR-133 between the Two under Study Groups, which were Significantly Different

Table 1. Association of miR-1, miR-133, miR-191, and miR-24 Expressions with Clinicopathological Features

| Variable                  | N  | miR-1 expression | P   | miR-133 expression | p   | miR-191 expression | P   | miR-24 expression | P   |
|---------------------------|----|------------------|-----|--------------------|-----|--------------------|-----|--------------------|-----|
| Age                       |    |                  |     |                    |     |                    |     |                    |     |
| ≤35                       | 8  | 0.512            | 0.236| 2.312              | 3.211|
| 35-50                     | 10 | 0.578            | 0.222| 0.751              | 2.431| 0.423              | 3.413| 0.532              |
| 51-65                     | 18 | 0.556            | 0.231| 2.253              | 3.651|
| >66                       | 14 | 0.525            | 0.212| 2.367              | 3.214|
| Gender                    |    |                  |     |                    |     |                    |     |                    |     |
| Male                      | 24 | 0.535            | 0.844| 0.24               | 0.548| 2.317              | 0.546| 3.216              | 0.346|
| Female                    | 26 | 0.523            | 0.23 | 2.375              | 3.412|
| Grade                     |    |                  |     |                    |     |                    |     |                    |     |
| I                         | 9  | 0.513            | 0.261| 2.287              | 3.241|
| II                        | 11 | 0.523            | 0.034| 0.238              | 0.013| 2.356              | 3.181| 0.541              |
| III                       | 10 | 0.565            | 0.247| 2.411              | 0.413| 3.621              |      |                    |
| IV                        | 20 | 0.548            | 0.271| 2.246              | 3.234|
| Lymphatic metastasis      |    |                  |     |                    |     |                    |     |                    |     |
| Negative                  | 27 | 0.556            | 0.004| 0.271              | 0.643| 2.316              | 3.401| 0.001              |
| Positive                  | 23 | 0.235            | 0.244| 2.357              | 0.214| 3.112              |      |                    |
| Differentiation degree    |    |                  |     |                    |     |                    |     |                    |     |
| Low                       | 12 | 0.533            | 0.921| 0.251              | 2.431| 3.121              |      |                    |
| Middle                    | 17 | 0.537            | 0.242| 0.892              | 2.287| 0.021              | 3.412| 0.021              |
| High                      | 21 | 0.581            | 0.222| 2.319              | 3.523|
| Surgery                   |    |                  |     |                    |     |                    |     |                    |     |
| Yes                       | 24 | 0.555            | 0.551| 0.271              | 0.521| 2.351              | 3.452| 0.361              |
| No                        | 26 | 5.12             | 0.238| 2.411              | 0.821| 4.236              |      |                    |
two and five time reduction of the expression levels in the healthy individuals and patients, respectively. The mean ratios of the healthy (control) group to the patients were 1 ± 0.072 and 3.211 ± 0.323 for miR-24 and 1± 0.085 and 2.312 ± 0.130 for miR-191, with P = 0.016 and P = 0.002, respectively. Fold change for miR-24 was 3.2, while that of miR-191 was 2.3 (Figures 3 and 4).

Comparison of miRNA levels with lung cancer clinicopathological features

The present study analyzed the relationship of miR-1, miR-133, miR-191, and miR-24 with lung cancer clinicopathological features, including age, gender, clinical stage, lymphatic metastasis, differentiation degree, and surgery history (Table1). The expression levels of miRNAs in lung cancer patients had no link to age, gender, and tumor size (p > 0.05). However, miR-1 showed an association with grade and lymph node metastasis (P = 0.034 and P = 0.004, respectively). Statistical analysis for a relationship between the mRNA expression levels of miR-133 and clinicopathological parameters exhibited a significant difference in grade (P = 0.013). Statistical analysis also represented that the miRNA expression levels of miR-24 in lung cancer tissue were significantly associated with differentiation degree (P = 0.021). In addition, a strong connection was observed between miR-191 and lymph node metastasis (P < 0.001).

Discussion

Lung cancer is one of the major cancers with a highly impact on society and economics in terms of prevalence. Based on the latest global estimates, 13% of people with cancer suffer from lung cancer (Boffetta, 2018). According to Iran’s Statistics Center, this cancer type is ranked eight among the most common cancers in the country (Mohabbi et al., 2018). Lung cancer is a disease characterized by an uncontrolled cell growth in tissues of lung, and if untreated, this growth can spread beyond the lung, as well as to adjacent and other parts of the body, through a process named metastasis. Small cell lung cancers, known as oat cell cancer and non-small cell lung cancer are two main types of lung cancer (Ho et al., 2007). miRNAs are non-coding ribonucleic acids able to control the gene expression after transcription by decomposing miRNA or inhibiting their translation. These molecular structures are involved in the control of physiological and pathological cell processes, and the majority can act as an oncogene or tumor suppressor. The identification of miRNAs and their target molecules contribute to understand the factors leading to cancer. Therefore, these compounds can be used as biological markers for the diagnosis, prediction, and treatment of cancer (Wouters et al., 2011).

The results of our study indicated that miR-1 expression decreased significantly in tumor tissues as compared to the normal tissue. Besides, the miR-1 expression level showed a direct correlation with cancer progression. miR-1 is likely to be one of the targets of C/EBPα, through which it inhibits cell proliferation and induces apoptosis. It would be of interest to determine whether the binding of C/EBPα to miR-1-1 and miR-1-2 gene promoters is essential for their transactivation (He et al., 2007). The study of Mohd and colleagues (…) disclosed that miR-1 probably affects some mediators downstream of p53 as DOXR-induced expressions of p53 and its target PUMA were comparable in control and miR-1-expressing cells. Furthermore, ectopic miR-1 induced apoptosis in A549 cells in response to the potent anticancer drug doxorubicin. Enhanced activation of caspases 3 and 7, cleavage of their substrate PARP-1, and depletion of anti-apoptotic Mcl-1 contributed to the sensitivity of miR-1-expressing cells to doxorubicin. Thus, miR-1 has potential therapeutic application against lung cancers (Nasser et al., 2008).

The present study of detected the downregulation miR-133 expression level in patients with lung cancer. Researchers have also found that miR-133a expression is significantly downregulated in pancreatic cancer tissue samples and cell lines, and there was a significantly
correlation of decreased miR-133a expression with aggressive clinicopathological features and poor survival (Szafranska et al., 2007). Our results explored increased miR-191 and miR-24 in patients, but not in the control group. In another study, miR-191 was found to be abnormally expressed in more than 20 different cancers and suggested to be a key player in some of these cancers. Moreover, the altered expression of miR-191 has been associated with various other diseases such as type 2 diabetes, neurodegenerative diseases, and more recently with innate immunity (Saha et al., 2008). Croce and associates have emphasized that both miR-191 and miR-425 promote cell proliferation in breast cancer, exhibiting overlapping functions. In contrast, another survey highlighted that miR-191, but not miR-425, promotes erythroid enucleation (Farzanegan et al., 2021). Therefore, with very limited data, we can speculate that miR-191 may display specific or overlapping functions considering the tissue and conditions. Likewise, the aberrant expression of miR-24 has been identified in varied cancer types, and miR-24 has both tumor-suppressive and oncogenic features depending on the cellular context. In many cancers, miR-24 has been exhibited to have great capability to discriminate and monitor between cancer patients and controls (Nguyen et al., 2013; Liu et al., 2015; Zhao et al., 2015).

In summary, our findings revealed that some miRNAs have ability to decrease and increase apoptosis in patients with lung cancer. It seems that a therapy to reduce miRNA expression could be effective for the treatment of this type of cancer. However, more future research is needed to confirm the results of this study and to identify the mechanism of action of these molecules.

Author Contribution Statement

B.F., M.F., and A.M. conceived the idea. N.B. and S.P. analyzed the data. M.E. and S.S.H. drafted the article. A.K.H., H.D.Z.KH, M.R. contributed to research procedures. M.K.D. collected the data and performed the article. A.K.H., H.D.Z.KH, M.R. contributed to research and diagnostic utility in oncology. Int J Mol Sci, 14, 4934-68.

Lal A, Kim HH, Abdelmohsen K, et al (2008). p16INK4a translation suppressed by miR-24. PLoS One, 3, e1864.

Lin SC, Liu CJ, Lin JA, et al (2010). miR-24 up-regulation in oral carcinoma: positive association from clinical and in vitro analysis. Oral Oncol, 46, 204-8.

Liu R, Zhang H, Wang X, et al (2015). The miR-24-Bim pathway promotes tumor growth and angiogenesis in pancreatic carcinoma. Oncotarget, 6, 43831.

Lodish H, Berk A, Zipursky SL, et al (2000). Molecular cell biology 4th edition. National Center for Biotechnology Information, Bookshelf.

Mohrbi E, Nahvijou A, Hadji M, et al (2018). Iran Cancer Statistics in 2012 and projection of cancer incidence by 2035. Basic Clin Cancer Res, 9.

Nagpal N, Kulshreshtha R (2014). miR-191: an emerging player in disease biology. Front Genet, 5, 99.

Nakajima N, Takahashi T, Kitamura R, et al (2006). MicroRNA-1 facilitates skeletal myogenic differentiation without affecting osteoblastic and adipogenic differentiation. Biochem Biophys Res Commun, 350, 1006-12.

Nasser MW, Datta J, Nuovo G, et al (2008). Down-regulation of micro-RNA-1 (miR-1) in lung cancer suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin-induced apoptosis by miR-1. J Biol Chem, 283, 33934-405.

References

Boffetta P (2018). Classic Epidemiology of Lung Cancer. In ‘IASLC Thoracic Oncology (Second Edition)’, Eds Elsevier, pp 1-8. e3.

Farzanegan B, Bahrami N, Birjandi B, et al (2021). Down-expression of mirna-98 and over-expression of mirna-9 can result in inadequate immune system response against lung cancer. Biointerface Res Appl Chem, 11, 13893-902.

Gagan J, Dey BK, Layer R, et al (2012). Notch3 and MeC2e are mutually antagonistic via Mkp1 and miR-1/206 in differentiating myoblasts. J Biol Chem, 2012, M112.378414.

Ghadimi K, Bahrami N, Fathi M, et al. (2017). Diagnostic value of LunX mRNA and CEA mRNA expression in pleural fluid of patients with non-small cell lung cancer. Minerva Pneumologica, 56, 90-5.

Han C, Yu Z, Duan Z, et al (2014). Role of microRNA-1 in human cancer and its therapeutic potentials. BioMed Res Int, 2014.

He X, He L, Hannon GJ (2007). The guardian’s little helper: microRNAs in the p53 tumor suppressor network. Cancer Res, 67, 11099-101.

Heydar H, Mansouri K, Noroooznezhad M, et al. (2018). Bevacizumab inhibits angiogenic cytokines in head and neck squamous cell carcinoma: from gene to the protein. Int J Hematol oncol Stem Cell Res, 12, 136.

Ho MM, Ng AV, Lam S, et al (2007). Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. Cancer Res, 67, 4927-33.

Jamaati H, Bahrami N, Ahatinki M, et al (2016). Real-time RT-PCR Detection of HCN4 and ADAM8 genes in ventilator-associated pneumonia patients Hospitalized in intensive care unit. J Cell Mol Anesthesia, 1, 163-7.

Jin XL, Sun QS, Liu F, et al (2013). microRNA 21-mediated suppression of sprouty1 by Pokemon affects liver cancer cell growth and proliferation. J Cell Biochem, 114, 1625-33.

Karimi S, Bahrami N, Sharifi K, et al (2017). Investigating gene expression level of MUC1 and CEA in pleural fluid of NSCLC lung cancer patients with real-time RT-PCR method. Minerva Pneumol, 56, 18-24.

Kim T, Reitmair A (2013). Non-coding RNAs: functional aspects and diagnostic utility in oncology. Int J Mol Sci, 14, 4934-68.

Lal A, Kim HH, Abdelmohsen K, et al (2008). p16INK4a translation suppressed by miR-24. PLoS One, 3, e1864.

Lin SC, Liu CJ, Lin JA, et al (2010). miR-24 up-regulation in oral carcinoma: positive association from clinical and in vitro analysis. Oral Oncol, 46, 204-8.

Liu R, Zhang H, Wang X, et al (2015). The miR-24-Bim pathway promotes tumor growth and angiogenesis in pancreatic carcinoma. Oncotarget, 6, 43831.

Author Contribution Statement

This study was conducted in Masih Daneshvari Hospital, Shahid Beheshti University of Medical Sciences (Theran, Iran) in collaboration with Anesthesia and Critical Care Department, Shahid Beheshti University of Medical Sciences (Tehran, Iran). Authors would like to express their gratitude to the staff and professors.

Funding statement

This work was supported by Shahid Beheshti University of Medical Sciences. (Tehran, Iran)

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

Asian Pacific Journal of Cancer Prevention, Vol 23
Negrini M, Nicoloso MS, Calin GA (2009). MicroRNAs and cancer—new paradigms in molecular oncology. *Curr Opinion Cell Biol*, **21**, 470-9.

Nguyen T, Rich A, Dahl R (2013). MiR-24 promotes the survival of hematopoietic cells. *PLoS One*, **8**, e55406.

Qian L, Van Laake LW, Huang Y, et al (2011). miR-24 inhibits apoptosis and represses Bim in mouse cardiomyocytes. *J Exp Med*, **208**, 549-60.

Qin W, Shi Y, Zhao B, et al (2010). miR-24 regulates apoptosis by targeting the open reading frame (ORF) region of FAF1 in cancer cells. *PLoS One*, **5**, e9429.

Qin Y, Dang X, Li W, et al (2014). miR-133a functions as a tumor suppressor and directly targets FSCN1 in pancreatic cancer. *Oncol Res*, **21**, 353-63.

Rio DC, Ares M, Hannon GJ, et al (2010). Purification of RNA using TRizol (TRI reagent). Cold Spring Harbor Protocols, 2010, pdb. prot5439.

Saba R, Goodman CD, Huzarewich RL, et al (2008). A miRNA signature of prion induced neurodegeneration. *PLoS One*, **3**, e3652.

Schaefer A, Jung M, Kristiansen G, et al (2010). MicroRNAs and cancer: current state and future perspectives in urologic oncology. *Urologic Oncology: Seminars and Original Investigations*, Elsevier, pp 4-13.

Szafranska A, Davison T, John J, et al (2007). MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene*, **26**, 4442.

Wang X, Tang S, Le S-Y, et al (2008). Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. *PLoS One*, **3**, e2557.

Wouters MD, van Gent DC, Hoeijmakers JH, et al (2011). MicroRNAs, the DNA damage response and cancer. *Mutat Res Fundam Mol Mech Mutagen*, **717**, 54-66.

Zaidi SK, Dowdy CR, Van Wijnen AJ, et al (2009). Altered Runx1 subnuclear targeting enhances myeloid cell proliferation and blocks differentiation by activating a miR-24/MKP-7/MAPK network. *Cancer Res*, **69**, 8249-55.

Zhao G, Liu L, Zhao T, et al (2015). Upregulation of miR-24 promotes cell proliferation by targeting NAIF1 in non-small cell lung cancer. *Tumor Biol*, **36**, 3693-701.

This work is licensed under a Creative Commons Attribution-Non Commercial 4.0 International License.