

Assessing Methylation of Mir-146b in Patients with Non-Small Cell Lung Cancer

Masoumeh Masrouri¹, Elham sadat Hosseini manesh², Mahsa Sadeghiazad³, Morteza Karimipoor⁴
Shohreh Zare Karizi⁵

¹MA molecular genetics, Islamic Azad University, Pishva, Varamin, IR Iran

²MA molecular genetics, Islamic Azad University Central Tehran Branch, IR Iran

³MA Microbial biotechnology, Payame Noor University of Tehran East, IR Iran

⁴Department of Molecular Medicine, Biotechnology Research Center, Pasture Institute of Iran, Tehran, Iran

⁵Department of Biology, Varamin Pishva Branch, Islamic Azad University, Pishva, Varamin, IR Iran



Abstract

Background: One of the most important epigenetic factors in lung cancer is aberrant DNA methylation. So far, many studies have been performed on the methylation of various genes and microRNAs in various cancers, especially lung cancer. So because of the high importance of microRNAs in cancer. In this study, mir-146b methylation was checked in NSCLC tissues.

Method: Analysis methylation of mir146b was investigated in 30 samples NSCLC tissues and 30 adjacent normal tissues using by MS-HRM method.

Results: Study on mir146b methylation showed that there was no significant difference between the methylation levels of this microRNA in tumor samples compared with healthy samples ($P>0.05$). However, this study was designed as a pilot study, and further investigations are required to confirm our findings.

Keywords - Lung Cancer; NSCLC; Mir-146b; MS-HRM Method.

I. INTRODUCTION

One of the most common cancers in world is lung cancer and malignant changes in the epithelial cells of the lung can lead to this cancer (1, 2). The performance of current treatments for lung cancer depends on the time of diagnosis and if the tumor is detected at an early stage, the chance of survival is higher. Only 13% of patients with lung cancer survive more than 5 years (3). Two major types of lung cancer are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (4). The type of NSCLC is the most common type that about 85% of lung cancer belong to its, also this type of lung cancer includes adenocarcinoma (AdC), squamous cell carcinoma (SqCC), and large cell carcinoma (5, 6). In the development of lung cancer are involved genetic factors, epigenetics and environmental factors which of environmental factors can be indicated to

smoking, exposure to tobacco smoke, asbestos, arsenic, radon gas and air pollution (7, 8). In 2016, around 224390 cases of lung cancer were diagnosed in the United States, of which 158080 died and in 2014, about 8251 people died of lung cancer in Australia (9, 10). DNA aberrant methylation that one of the most important epigenetic changes is deeply involved in the development of human cancers. (11, 12). In particular, the aberrant methylation of the CPG Islands in the promoter region causes the gene to be muted (13). So far, many studies have examined the role of microRNAs in various cancers, especially lung cancer (14, 15). The study of microRNAs in cancer patients can be used as new diagnostic or pre-diagnostic methods (16). Many studies have been conducted on mir146b in lung cancer (17, 18). Mir146 was first identified as an immune regulator in response to microbial infections. Further studies showed that

mir-146a/b was in the NF-KB dependent pathway and was induced by TLR. This micro-RNA inhibits the NF-KB pathway by targeting two essential proteins in the message transmission path called TRAF6 and IRAK, also studies have shown that mir146b acts as a tumor suppressor (19-21). So far the expression level of mir146b has been checked in lung cancer (22), therefore in this study, we investigated the methylation changes of mir-146b by using the MS-HRM method.

II. MATERIAL AND METHODS

2.1 Patients and Samples

Human NSCLC tissues (n = 30) and adjacent non-tumor tissues were obtained from patients at Masih daneshvari Hospital, Tehran, Iran, with informed consent from each patient. The type of the disease was diagnosed by the pathologists and the patients did not receive any type of treatment. Clinicopathologic characteristics of thirty NSCLC patients are presented in table 1.

Table 1. Clinicopathologic characteristics of thirty NSCLC patients.

Variable	Value (n = 30)
Gender	
Male	22 (73%)
Female	8 (27%)
Smoking history	
Never-smoker	22 (73%)
Current smoker	8 (27%)
Cell type	
Adenocarcinoma	17 (57%)
Squamous cell carcinoma	13 (43%)
Stage	
I	9 (30%)
II	11 (36.66%)
III	10 (33.34%)

2.2 DNA Extraction and Bisulfite treatment

DNA was extracted by using the Qiagen DNA Extraction Mini Kit (Qiagen, United States) from each tumor and normal tissues according to the manufacturer's specifications. The concentration of DNA was determined by spectrophotometry and Integrity of DNAs were assessed by 1.0% agarose gel electrophoresis. Then, 1000ng Genomic DNA of each tumor and normal tissues were treated with Sodium bisulfite by using EZ DNA Methylation Kit (Zymo Research, Orange, CA), according to the manufacturer's recommendation. The entire converted DNA was stored at -70 °C until use.

2.3 Preparation of controls

For unmethylated control, we used white blood cell DNA and then, fully methylated DNA was made by treatment a same white blood DNA extracted with *M. SssI* according to the manufacturer's recommendation (Thermo Scientific, United States). Finally, by diluting fully methylated DNA with unmethylated bisulfate-treated DNA, all controls with different ratios (10%, 25%, 50% and 75% methylated controls) were constructed.

2.4 MS- High resolution melting (MS-HRM)

MS-High Resolution Melting was performed in a 20-μl reaction containing 4-μl of 5x HOT FIREPol®EvaGreen® HRM Mix (ROX), 1-μl of bisulphite converted DNA, 14-μl of H₂O and 1μl of mixed forward and reverse primers (6 Pmol/μl concentration) by using an StepOnePlus™ Real-Time PCR Systems (ABI Applied Bio-systems, Thermo Fisher Scientific, USA). The thermocycler conditions were as follows: initial denaturation at 95°C for 15 min; followed by 40 cycles of 94°C for 15 seconds, 58°C for 20 seconds (annealing time), 72°C for 30 seconds (extension time); and a final extension at 72°C for 5 min. The melting curve stage was performed as follows: denaturation at 95°C for 15s, 60°C and 58°C for 1 min, followed by HRM step ramping from 58°C to 95°C, rising 0.3%. The primers used for amplification are listed in Table 2.

Table 2. Designed primer sequences for Mir-146b.

Target genes		Sequences (5'→ 3')	Product length
Mir-146b	Forward	GGGGGTAGGAGGGGAAATTATT	108bp
	Reverse	CCGCCTTAATCTCGTTAATAAACTA	

2.5 Statistical analysis

Statistical analysis was performed using the GraphPad Prism v7.03 (GraphPad Software Inc., USA) and Chi-square test. For all tests, a P value <0.05 was considered statistically significant.

III. RESULTS

In this paper, the methylation status of mir-146b was examined in 30 samples NSCLC tissues and 30 adjacent normal tissues by MS-High resolution melting method. As mentioned in the materials and methods section, in this study controls were considered 0%, 10%, 25%, 50%, 75% and 100% methylated that figure 1 illustrates results obtained of controls. Also, for more certainty two 50% methylated controls were provided. Cut off for this study was considered 10%. This study reported that were hypermethylated 1 (3.33%) out of the 30 NSCLC samples. In contrast, none of the normal samples were in the promoter region of methylated, so that there was no significant difference of methylation status between tumor samples with adjacent normal samples ($P=0.31$). (Fig. 2 and 3 show the results of the mir-146b methylation).

IV. DISCUSSION

DNA hypermethylation of tumor suppressor genes was an important epigenetic event in the progression of early changes in the progression of NSCLC, the silencing of genes through promoter hypermethylation is now recognized as a major and causal epigenetic event. It is also closely associated with transcriptional silencing of genes (23). In this paper, we have analyzed Mir146-b gene promoter methylation status in 30 NSCLC patients by the MS-HRM method.

As mentioned, mir146b acts as a tumor suppressor and earlier research on this miRNA determines different results in various cancers. Li et al demonstrated that expression of mir146b significantly reduced in NSCLC patients and they also shown low levels of miR-146b expression are associated with poor prognosis (24). In another study, it was found that the expression of mir-146b was downregulated in human prostate cancer tissues (25). According to the results obtained in these studies, we expected to see the hypermethylation of this gene in this patients, but MS-high resolution melting analysis didn't show any significant difference in methylation level between tumor and non-tumor adjacent tissues. One of the reasons that could be proposed for this contradiction, is that the samples in this study are often at the early stages of lung cancer and according to the research by He et al, mir-146b inhibits epithelial-mesenchymal transition by regulating FOXM1 in lung cancer cells and this process occurs in the advanced stages of the disease (26). Moreover, the decrease of mir-146b expression levels in cancerous lung tissues can be related to the mutation in this gene as well as to different epigenetic factors or regulatory factors at transcriptional level or to post-transcriptional factors.

In summary, we didn't see any significant difference in methylation level between tumor and adjacent normal lung tissues for mir-146b. However, this study was designed as a pilot study, and further investigations are required to confirm our findings.

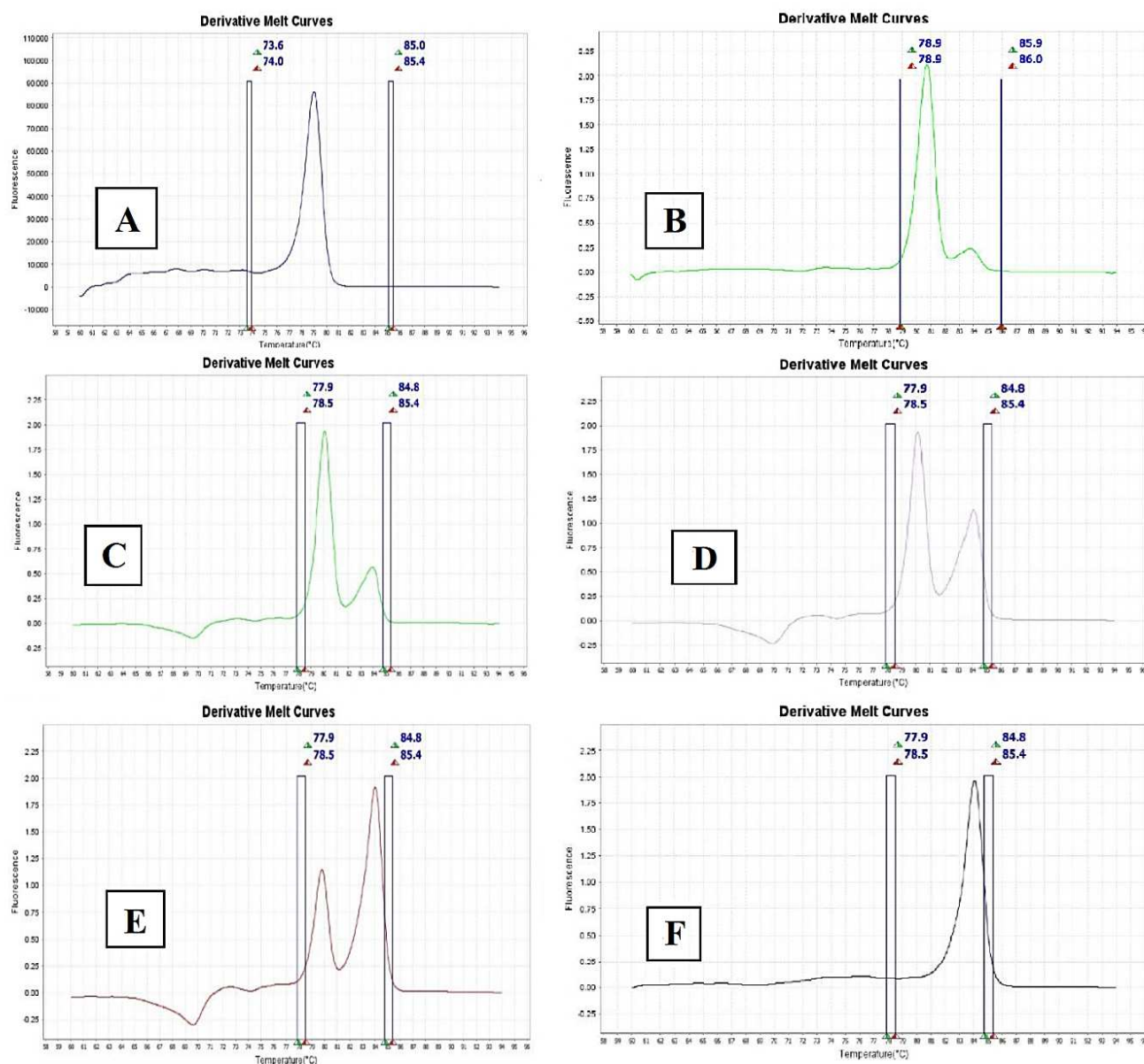


Figure 1. The graph (A) related to 0% methylation control, (B) related to 10% methylation control, (C) related to 25% methylation control, (D) related to 50% methylation control, (E) related to 75% methylation control and (F) related to 100% methylation control.

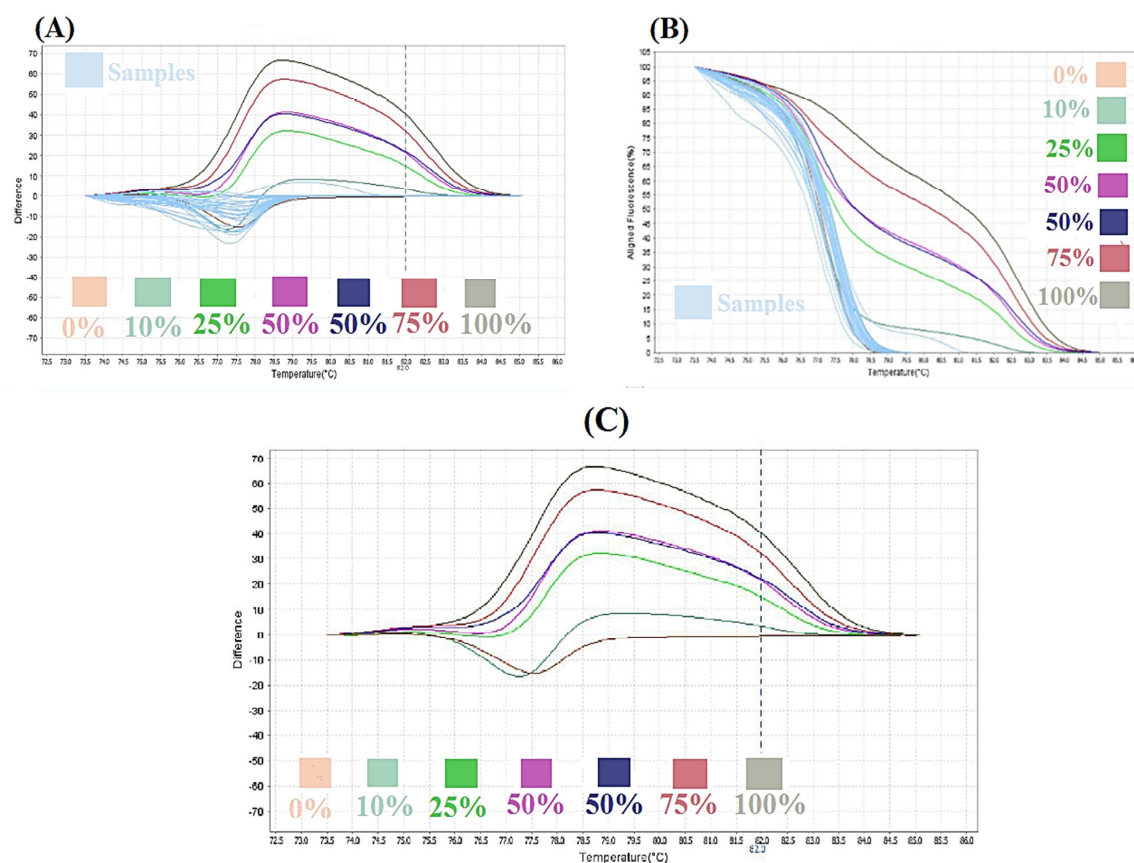


Figure 2. The graphs related to the mir-146b with controls and samples. (A) related to difference plot of mir-146b methylation, (B) related to aligned melt curves of mir-146b methylation and (C) related to difference plot of mir-146b methylation control so that controls are from down to up consists of 0%, 10%, 25%, 50%, 50%, 75% and 100% methylation.

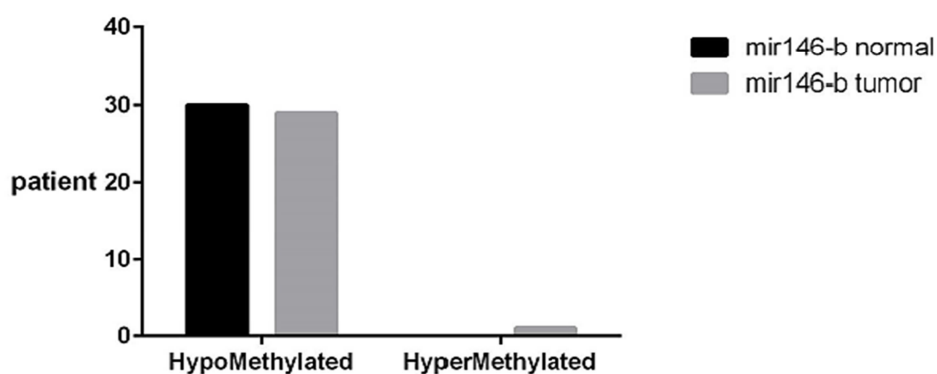


Figure 3. The graph related to methylation of mir-146b in NSCLC tumor samples compared to non-tumor adjacent samples.

REFERENCES

- [1] Tang N, Guo J, Zhang Q, Wang Y, Wang Z. Greater efficacy of chemotherapy plus bevacizumab compared to chemo-and targeted therapy alone on non-small cell lung cancer patients with brain metastasis. *Oncotarget*. 2016;7(3):3635.
- [2] Jemal A, Center MM, DeSantis C, Ward EM. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiology and Prevention Biomarkers*. 2010;19(8):1893-907.
- [3] Risch A, Plass C. Lung cancer epigenetics and genetics. *International journal of cancer*. 2008;123(1):1-7.
- [4] Tanaka K, Kumano K, Ueno H. Intracellular signals of lung cancer cells as possible therapeutic targets. *Cancer science*. 2015;106(5):489-96.
- [5] Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304(5676):1497-500.
- [6] Cruz CSD, Tanoue LT, Matthey RA. Lung cancer: epidemiology, etiology, and prevention. *Clinics in chest medicine*. 2011;32(4):605-44.
- [7] Council NR. Health effects of exposure to radon: BEIR VI: National Academies Press; 1999.
- [8] Turner MC, Krewski D, Pope III CA, Chen Y, Gapstur SM, Thun MJ. Long-term ambient fine particulate matter air pollution and lung cancer in a large cohort of never-smokers. *American journal of respiratory and critical care medicine*. 2011;184(12):1374-81.
- [9] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA: a cancer journal for clinicians*. 2016;66(1):7-30.
- [10] Yu XQ, Luo Q, Kahn C, Grogan P, O'Connell DL, Jemal A. Contrasting temporal trends in lung cancer incidence by socioeconomic status among women in New South Wales, Australia, 1985–2009. *Lung Cancer*. 2017;108:55-61.
- [11] Langevin SM, Kratzke RA, Kelsey KT. Epigenetics of lung cancer. *Translational Research*. 2015;165(1):74-90.
- [12] Lee Y-C, Wang H-P, Wang C-P, Ko J-Y, Lee J-M, Chiu H-M, et al. Revisit of field cancerization in squamous cell carcinoma of upper aerodigestive tract: better risk assessment with epigenetic markers. *Cancer prevention research*. 2011:canprevres. 0096.2011.
- [13] Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene*. 2002;21(35):5427.
- [14] Esteller M. Non-coding RNAs in human disease. *Nature Reviews Genetics*. 2011;12(12):861.
- [15] Eilertsen M, Andersen S, Al-Saad S, Richardsen E, Stenvold H, Hald SM, et al. Positive prognostic impact of miR-210 in non-small cell lung cancer. *Lung Cancer*. 2014;83(2):272-8.
- [16] Kota SK, Balasubramanian S. Cancer therapy via modulation of micro RNA levels: a promising future. *Drug discovery today*. 2010;15(17-18):733-40.
- [17] Rabinowits G, Gerçel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. *Clinical lung cancer*. 2009;10(1):42-6.
- [18] Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer cell*. 2006;9(3):189-98.
- [19] Omrane I, Kourda N, Stambouli N, Privat M, Medimegh I, Arfaoui A, et al. MicroRNAs 146a and 147b biomarkers for colorectal tumor's localization. *BioMed research international*. 2014;2014.
- [20] Suzuki Y, Kim HW, Ashraf M, Haider HK. Diazoxide potentiates mesenchymal stem cell survival via NF- κ B-dependent miR-146a expression by targeting Fas. *American Journal of Physiology-Heart and Circulatory Physiology*. 2010;299(4):H1077-H82.
- [21] Chou C-K, Chen R-F, Chou F-F, Chang H-W, Chen Y-J, Lee Y-F, et al. miR-146b is highly expressed in adult papillary thyroid carcinomas with high risk features including extrathyroidal invasion and the BRAFV600E mutation. *Thyroid*. 2010;20(5):489-94.
- [22] Hsu S-D, Chu C-H, Tsou A-P, Chen S-J, Chen H-C, Hsu PW-C, et al. miRNAmap 2.0: genomic maps of microRNAs in metazoan genomes. *Nucleic acids research*. 2007;36(suppl_1):D165-D9.
- [23] Belinsky SA. Silencing of genes by promoter hypermethylation: key event in rodent and human lung cancer. *Carcinogenesis*. 2005;26(9):1481-7.
- [24] Li Y, Zhang H, Dong Y, Fan Y, Li Y, Zhao C. MiR-146b-5p functions as a suppressor miRNA and prognosis predictor in non-small cell lung cancer. *Journal of Cancer*. 2017;8(9):1704.
- [25] Ding H-Y, Qian W-Q, Xu J. MicroRNA-146b acts as a potential tumor suppressor in human prostate cancer. *Journal of BU ON: official journal of the Balkan Union of Oncology*. 2016;21(2):434-43.
- [26] He Y, Yu Y-C, Zhu H-C, Jing X-J, Wei N, Wang P-D, et al. MicroRNA-146 inhibits epithelial-mesenchymal transition by regulating FOXM1 in lung cancer cells. *Int J Clin Exp Pathol*. 2017;10(3):3085-92.