

The Methylation Analysis of Mir-210 in NSCLC Patients

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Abstract

Background: Because of the importance of lung cancer worldwide, as well as the deaths from it. So far, various studies have been conducted on this cancer. Epigenetic changes are one of the factors contributing to lung cancer that the most important of these changes are DNA methylation. Various biomarkers such as microRNAs can be used to detect cancer. In addition, many studies have been performed on the relationship between cancer and microRNAs. In this study, the methylation status of mir-210 was investigated in NSCLC tissues.

Method: The methylation status of mir-210 was checked in 30 patients with NSCLC and 30 adjacent normal tissues using the MS-PCR method.

Results: This paper reported that there was no significant difference between methylation level of tumor samples and adjacent normal samples. so that all of the normal samples were in methylated state and only 4 (13.33%) cases of tumor samples were in a unmethylated state ($P>0.05$). The results obtained from our study confirmed that hypo methylation and therefore overexpression of mir-210 didn't occur in early stages of non-small cell lung cancer.

Keywords — Lung Cancer; Non-Small Cell Lung Cancer (NSCLC); Mir-210; MS-PCR Method.

I. INTRODUCTION

Lung carcinoma is the leading cause of cancer deaths in the United States and worldwide for both men and women (1). The lung cancer is categorized in terms of the histology of the tumor into two types of small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) and about 85% of lung cancers are related to the type of NSCLC (2). In 2015, about 133500 cases of lung cancer were diagnosed in Japan, of which approximately 77,200 people died and in 2012, about 29949 people died of lung cancer in France (3, 4). Different factors affect the incidence of lung cancer of which types can be referred to genetic and epigenetic factors and environmental factors. Smoking and occupational exposure to asbestos and radon gas and air pollution contribute to the intensification of lung cancer (5-7). In addition to numerous known genetic variations in lung cancer, several epigenetic changes have been discovered that are involved in the tumorigenic process in lung cancer (8). One of the most important types of epigenetic

mechanisms is DNA methylation (9). Methylation of DNA in lung epithelial cells may be one of the factors contributing to the development of lung cancer (10). Examining different biomarkers like microRNAs can be useful in early detection of cancer (11). So far, many studies have been conducted on microRNAs in different types of cancers and some of these studies have been performed on mir-210 in lung cancer tissues so that have reported miR-210 is overexpressed at late stages of non-small cell lung cancer (12-15). MiR-210 appears as an important regulator of the cellular response to hypoxia and has been called the "micromanagement of the hypoxia pathway" (16). In malignant disease, hypoxia evolves because of insufficient vasculature supporting the growing number of cancer cells. Regulation of miR-210 is reported to be dependent on the transcription factor HIF1 (17). So far the expression level of mir-210 has been investigated in lung cancer. Therefore, due to the importance of mir210 in lung cancer, in this study

methylation of this microRNA was performed using MS-PCR method.

II. MATERIAL AND METHODS

2.1 Human specimens

Human non-small cell lung cancer specimens (n = 30) and adjacent non-tumor tissues were obtained from patients at Masih daneshvari Hospital, Tehran, Iran, with informed consent from each patient. Patient demographic and

clinicopathologic characteristics are shown in Table 1. According to the information presented in Table 1, 8 patients were male and 22 of the patients were female, 17 patients had adenocarcinoma and 13 patients had squamous cell carcinoma. The proportion of patients with pathological stages I, II, III was 9 out of 30, 11 out of 30 and 10 out of 30, respectively.

Table 1. Clinicopathologic characteristics of thirty NSCLC patients.

Variable	Value (n = 30)
Gender	
Male	22 (73%)
Female	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

DNA was extracted from each tumor and non-tumor tissues by using the Qiagen DNA Extraction Mini Kit according to the manufacturer's specifications (Qiagen, United States). The concentration of extracted DNAs in the final eluate was determined by spectrophotometry and the absorbance 260/280 ratio was controlled between 1.8 and 2.0. Integrity and size of DNAs were assessed by 1.0% agarose gel electrophoresis.

2.3 Methylation-specific PCR

Methylation-specific PCR (MSP) was performed using the bisulfite-modified DNA as the template. The

amplification was conducted in a 15µL reaction volume containing 1µL DNA template, 0.5µL dNTPs, 1.5µL PCR buffer, 0.5µL MgCl₂, 1µL of mixed forward and reverse primers (10 Pmol/µl concentration), and 1 U (0.2µL) of HotStart Taq (Qiagen, United States). The thermocycler conditions were as follows: initial denaturation at 95 °C for 15 min; followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s (annealing), 72 °C for 30 s; and a final extension at 72 °C for 5 min. Then, all PCR products were assessed by 1.0% polyacrylamide gel electrophoresis. The primers used for MS-PCR are listed in Table 2.

Table 2. Designed primer sequences for Mir-210.

Target genes		Sequences (5' → 3')	Product length
Mir-210 (methylated)	Forward	AAGTTGGTTTTGCGAGTTTAAAC	101bp
	Reverse	GAAACGAAAAAATCCTATCTATACGC	
Mir-210 (unmethylated)	Forward	AGTTGGTTTTGTGAGTTTAAATG	
	Reverse	CAAAACAAAAAATCCTATCTATACA	

2.4 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. 3. RESULTS

The methylation status of mir-210 was checked using the MS-PCR method in 30 NSCLC tissues and 30 adjacent normal tissues. The results obtained showed that all of the normal samples were in methylation condition so that in all of these specimens only the product of methyl primer was amplified (Fig.1 illustrates the results of all normal samples

with methyl and un-methyl primers). Also, 26 out of 30 tumor samples (86.67%) were in the methylation state and 4 out of 30 tumor samples (13.33%) were in the un-methylation state, so that the product of the un-methyl primer was only observed in 4 tumor samples (Fig.2 shows the results of some tumor samples with methyl primer and un-methyl primer). Finally, the overall result demonstrated that there is no significant difference between the methylation level of tumor samples compared to adjacent normal samples ($P=0.150$). (Fig 3 shows the difference of methylation level in tumor samples with adjacent normal samples).

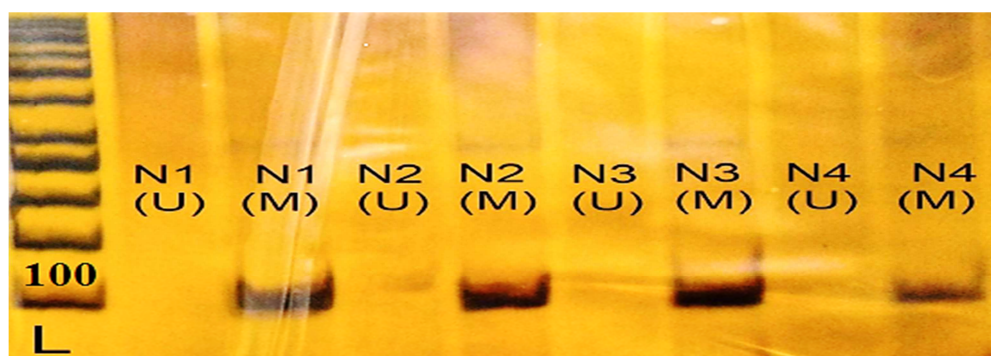


Fig.1. The gel related to normal samples with methyl primer and un-methyl primer. For each samples were tested both primers (methyl primer and un-methyl prime). Abbreviations of (N) related to Normal samples, (M) Methyl primer, (U) Un-methyl primer and (L) Ladder.



Fig.2. The gel related to some tumor samples with methyl and un-methyl primer. For each samples were tested both primers (methyl primer and un-methyl primer). Abbreviations of (T) related to Tumor samples, (M) Methyl primer, (U) Un-methyl primer and (L) Ladder.

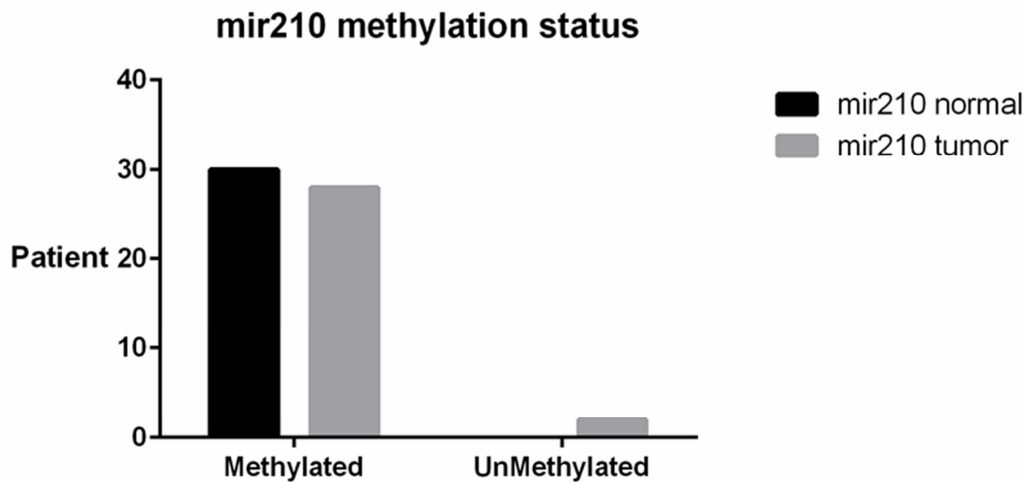


Fig.3. Diagram related to methylation level of mir-210 in tumor samples comparison with adjacent normal samples

IV. DISCUSSION AND CONCLUSION

DNA hyper methylation 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 hyper methylation is now recognized as a major and causal epigenetic event. It is also closely associated with transcriptional silencing of genes (18). Therefore, in this study, we have been analyzed Mir210 gene promoter methylation status in 30 NSCLC patients by the MS-PCR method.

As mentioned, earlier research on this miRNA determines different results in various cancers. Hong et al. demonstrated that the expression rate of mir-210 was significantly increased in breast cancer tissues rather than normal breast tissues and High expression of miR-210 associated with poor survival in patients with breast cancer (19). According to the results obtained from another study, epigenetic silencing of miR-210 increases the proliferation of gastric epithelium during chronic *Helicobacter pylori* infection (14). miR-210 is also overexpressed at late stages of non-small cell lung cancer (15). According to the results obtained from these studies, we expected the hypo methylation of mir210 occurs in these patients, but we didn't see any significant difference in methylation level in NSCLC tumors compared to non-tumor tissues. In another hand, the samples in this study are often at the early stages of lung cancer and we expect that the hypo methylation of mir210 occurs at the advanced stages of lung cancer.

In summary, the results obtained from our study confirmed that hypo methylation and therefore overexpression of mir-210 didn't occur in early stages of non-small cell lung cancer. However, this study was designed as a pilot study, and further investigations are required to confirm our findings.

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