

Gene's Expression and Methylation Status of ZBTB2 and TRAF6 In Non-Small Cell Lung Cancer Patients

Shima Hojabri Mahani ¹, Hassan Tasezar ², Morteza Karimi pour ³, Shohre Zare Karizi ⁴

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

² MSc molecular genetics, Islamic Azad University, Pishva, Varamin, IR Iran

³ Department of Molecular Medicine, Biotechnology Research Center, Pasture Institute of Iran, Tehran,

⁴ Department of Biology, Varamin Pishva Branch, Islamic Azad University, Pishva, Varamin, IR Iran



Abstract

Background: The uncontrolled proliferation of cells in the lung tissue leads to lung cancer. There are several forms of this type of cancer that each has its own symptoms. One of the major types of lung cancer is NSCLC that accounting for 85% of this cancer. Various factors including epigenetic alteration play a role in the incidence of lung cancer that DNA methylation is one of the most important of them. Studies have shown that methylation changes in the promoter region of genes may occur in the early stages of lung cancer. Therefore in this paper, the expression levels and status of methylation of ZBTB2 and TRAF6 genes were checked in the NSCLC tissue.

Methods: The expression levels and methylation status of ZBTB2 and TRAF6 genes were investigated in 30 patients with NSCLC tissue and 30 adjacent normal tissues, so that the expression level was performed by use of Real Time-PCR method and methylation status was conducted using methylation specific-High resolution melting (MS-HRM) method.

Results: The results of both genes showed that there was no significant difference between the expression levels of tumor samples compared to adjacent normal samples ($P>0.05$). Also, the results of the methylation status showed that the ZBTB2 gene was methylated in 2 samples of 30 tumor samples and the TRAF6 gene was methylated in one sample of 30 tumor samples. So wasn't found significant difference in methylation status of two genes in the samples ($P>0.05$). However, further investigations are required to confirm our findings because this study was designed as a pilot study.

Keywords - Non-Small Cell Lung Cancer, Epigenetic, DNA Methylation, ZBTB2, TRAF6, Real Time-PCR, MS-HRM.

I. INTRODUCTION

One of the most important cancers that each year has the highest rates of cancer deaths, is lung cancer [1]. Currently, The survival of lung cancer is 5 years after the diagnosis of the disease. Although in recent decades, chemotherapy and radiotherapy have been effective in improving the survival and quality of life of patients with lung cancer and also reduced mortality [2]. Lung cancer is categorized into two types of SCLC (small cell lung cancer) and NSCLC (non-small cell lung cancer), so that the type of NSCLC accounts for about 85% of lung cancers. The type of NSCLC also is divided into three categories: SqCc (squamous cell carcinoma), AdC (adenocarcinoma) and LcC (large cell carcinoma) [3–5]. Patients with lung cancer show symptoms

like chest pain, chronic cough, blood in the sputum or saliva and appetite loss [6]. Each year, lung cancer leads to the death of 1.6 million people worldwide. In Japan, about 25 percent of deaths from cancer in men in 2015 were due to lung cancer. In 2017, about 222500 cases of lung cancer were identified in the United States, of which 155870 of them died [7, 8]. Recent studies have shown that environmental, genetic and epigenetic factors are involved in the development of lung cancer [9, 10]. Epidemiological studies show that environmental factors such as smoking, exposure to tobacco smoke, asbestos and radon are associated with the incidence of lung cancer [11]. Genetic factors include mutations and variations in gene expression levels, but it should be noted that epigenetic alterations are

involved in lung cancer more than mutations. Epigenetic agents that involved in the development of lung cancer are DNA methylation, histone modifications and non-coding RNA expression [12]. DNA methylation is one of the most important epigenetic mechanisms in the development of cancer [5]. Aberrant DNA methylation by DNMTs enzyme (DNA methyltransferases) in the CpG islands of the gene promoter regions causes silencing downstream genes and is known to be the main mechanism for disabling tumor suppressor genes in lung cancer [3, 13]. In the last decade, epigenetic changes have played a significant role in the onset, progression and invasion of cancer. So far, many studies have been done on changes in the level of expression and methylation of various genes in lung cancer so that these studies confirm the presence of changes multiple gene expression and methylation in cancerous lung specimens [14]. In this study the expression level and status of methylation of TRAF6 and ZBTB2 genes were investigated in NSCLC tissue. The ZBTB2 gene is a member of the POK transcription factor family and is a strong repressor of the important pathway of ARF-HDM2-p53-p21 in cell cycle regulation, this gene suppresses the transcription of ARF, P51, and P21 genes, but activates the HDM2 gene. ZBTB2 via its POZ domain and zinc fingers directly interacts with SP1 then it is involved in the repression of transcription activation, also ZBTB2 via its zinc fingers directly interacts with p53 and prevents P53 binding and then suppresses transcription by P53. In particular, the ZBTB2 gene is a potent transcriptional suppressor that inhibits p53 and sp1, leading to the arrest of the p21 cell cycle [15–17]. Recent studies have shown that TRAF6 is involved in some cancers. TRAF6 is a component of intracellular signal transduction proteins and is part of the TNFR family members [18] and this gene plays an important role in inherent immune responses [19]. Differences in the expression of TRAF protein have been reported in human cancers and TRAF2 and TRAF6 have the highest levels of expression in human cancer cells [20, 21]. The expression patterns of TRAF protein vary widely so that highest levels of TRAF1 and TRAF5 expression exist in the tonsils, spleen, lungs, testicles, and thymus [22–24]. This gene also plays important role in the growth, inflammation, metastasis, apoptosis and tumor progression [25].

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. 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.

2.2 RNA extraction and cDNA preparation

Total RNA was isolated from each tumor tissue and adjacent non-tumor tissue by using RiboEx (GeneAll, Korea) according to the manufacturer's specifications. The concentration of total RNA in the final eluate was determined by spectrophotometry. The synthesis of cDNA (240 ng of total RNA per 20 µL reaction mixture) was performed using the Prime Script RT reagent kit (Perfect Real Time) RR037A (Takara, Japan) according to the manufacturer's specifications. The obtained cDNAs were stored in -80°C until use.

2.3 Real-time quantitative PCR

Real-time PCR was performed using an StepOnePlus™ Real-Time PCR Systems (ABI Applied Bio-systems, Thermo Fisher Scientific, USA) in a 15-µl reaction containing 7.5-µl of RealQ Plus 2x Master Mix Green High ROX™ (Ampliqon, Denmark), 1-µl of cDNA, 5.5-µl of H₂O and 1-µl of mixed forward and reverse primers (3 Pmol/µl concentration). Real-time PCR amplifications were done as follows: for these selected genes, PCR amplification was set to an initial 95°C for 15 min and then for TRAF6 and ZBTB2 genes, a total of 40 cycles, 95°C for 15 seconds and 60°C for 1 min (step and hold). All samples were analyzed in duplicate. For internal control, GAPDH was used. The primers used for real-time PCR are listed in Table 2.

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%)

Table 2. Primer sequences of two selected genes and GAPDH.

Target genes		Sequences (5' → 3')	Product length
TRAF6	Forward	TTGCCATGAAAAGATGCASAG	85bp
	Reverse	AGCCTGGGCCAACATTCTC	
ZBTB2	Forward	CCCGTAGTAACCTAAGGCAACA	114bp
	Reverse	CATTAAGGGGATGCACTTCACC	
GAPDH	Forward	CATCAAGAAGGTGGTGAAGCA	120bp
	Reverse	GCGTCAAAGGTGGAGGAGTG	

2.4 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. 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. All of the converted DNAs were stored at -80 °C until use.

2.5 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.

2.6 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 and 60°C for 20 seconds (annealing time for TRAF6 and ZBTB2, respectively), 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/60°C to 95°C, rising 0.3%. The primers used for amplification are listed in Table 3.

Table 3. Designed primer sequences for two selected genes.

Target genes		Sequences (5' → 3')	Product length
TRAF6	Forward	GGGGGTAGGAGGGGAAATTATT	108bp
	Reverse	CCGCCTTTAATCTCGTTAATAAACTA	
ZBTB2	Forward	GGGGGTAGGAGGGGAAATTATT	108bp
	Reverse	CCGCCTTTAATCTCGTTAATAAACTA	

2.7 Statistical analysis

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

III. RESULTS

3.1 The results obtained of expression changes of ZBTB2 and TRAF6 genes by Real Time-PCR method

The expression status of ZBTB2 gene showed that out of 30 tumor samples, 14 samples (46.6%) increased of expression and 16 samples (53.4%) decreased of expression (Fig1.C). Also, the expression status of TRAF6 gene demonstrated that 13 (43.3%) out of 30 tumor samples showed increased of expression and the rest of the samples (56.7%) showed a decrease in expression (Fig1.D). So, there were no significant difference between the expression level of these genes in tumor samples compared with normal samples ($P>0.05$) (Fig1.A and B).

3.2 The results obtained of methylation status of ZBTB2 and TRAF6 genes by MS-HRM method

The MS-HRM method was used to methylation status changes of ZBTB2 and TRAF6 genes in 30 samples

NSCLC tissues and 30 adjacent non-tumor tissues of the same subjects. For investigating the methylation status were considered 0%, 5%, 10%, 25%, 50% and 100% methylated controls. Also cut off for this study was determined 5% methylation, so that the samples of higher than 5% were considered as methylated and the samples of lower than 5% were considered as un-methylated. The investigation on ZBTB2 gene reported that 2 samples (6.6%) of 30 tumor samples were methylated with compared to normal samples and 28 other tumor samples (93.4%) were showing no methylation changes with compared to normal samples (Fig2.A and B). Also, the results of the TRAF6 gene showed that 29 samples (96.7%) of the tumor samples did not show any methylation changes and only 1 sample out of 30 tumor samples (3.3%) was methylated compared to normal samples (Fig2.C and D). Finally, it was concluded that there was no significant difference between the methylation of tumor samples compared to normal samples ($P>0.05$).

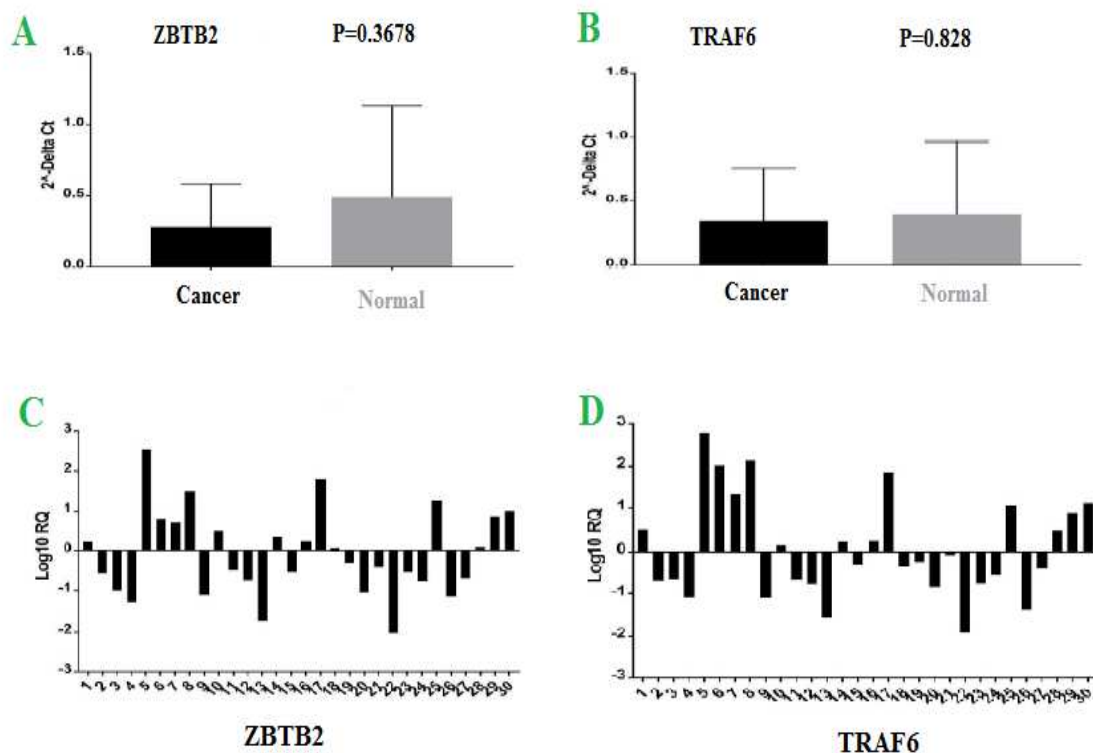


Fig1. The graph (A) related to expression level of ZBTB2 gene in NSCLC samples compared to non-tumor adjacent samples and (B) related to expression level of TRAF6 gene in NSCLC samples compared to non-tumor adjacent samples. The graph (C and D) related to the number of samples that show an increase or reduction of expression.

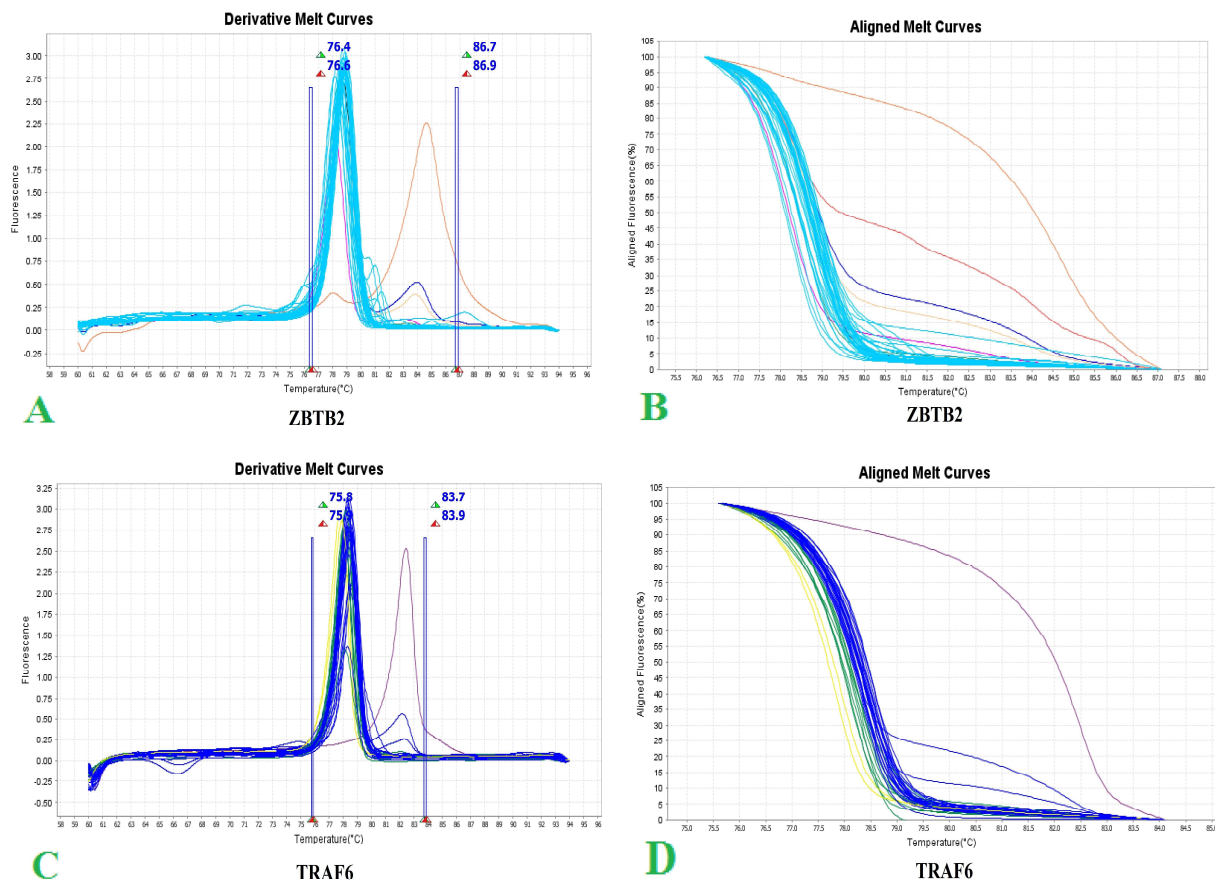


Fig2. The graphs A and B related to ZBTB2 gene methylation status with controls and samples. The graphs C and D related to TRAF6 gene methylation status with controls and samples. So that controls are from down to up consists of 0%, 5%, 10%, 25%, 50% and 100% methylated.

IV. DISCUSSION

Over the last few years, a great number of studies have reported aberrant patterns of gene expressions and methylation in various cancers including NSCLC. Despite the many advances in treating of lung cancer, the survival rate of NSCLC patients is still poor. Therefore, understanding of the different mechanisms involved in the onset and progression of lung cancer can provide the basis for better treatment. In this study, we have analyzed the expression and methylation level of ZBTB2 and TRAF6 genes in thirty NSCLC patients by real-time quantitative PCR and MS-HRM method.

Earlier researches on these genes determine different results in various cancers. Aberrant gene expressions of TRAF6 has been reported in several human cancers. Meng et al. demonstrated that the expression rate of TRAF6

mRNA was significantly increased in osteosarcoma tissues rather than normal bone tissues [26]. TRAF6 was also overexpressed in pancreatic cancer tissues. This gene was significantly upregulated in muscles of gastric cancer compared to normal controls [22]. Considering the mentioned studies and the roles of this gene in normal lung tissues, we expect the overexpression of TRAF6 gene occurs in NSCLC patients, but in this case, overexpression of TRAF6 gene was observed only in 13 NSCLC patients (43.3%). Also, MS-high resolution melting analysis didn't show any significant difference in methylation level for this gene.

As previously mentioned, The ZBTB2 gene is a member of the POK transcription factor family and is a strong repressor of the important pathway of ARF-HDM2-p53-p21 in cell cycle regulation [15, 16]. The role of this gene in gastric cancer has already been studied and the results

showed that the ZBTB2 gene expression was significantly reduced by miR-149 [27]. In this study, we didn't see any significant difference in expression level between tumor and normal NSCLC tissues for these genes but the ZBTB2 expression was reduced in 16 out of 30 (53.3%) NSCLC tissues compared to adjacent normal tissues.

We expect the gene expression and methylation difference between normal and tumor samples were occur in this patients but our expectation was not fulfilled. One of the reasons that could be proposed for this contradiction is that the samples in this study are often at early stages of non-small cell lung cancer. However, the roles of the ZBTB2 and TRAF6 genes in the evolution of lung cancer are complicated and require more research and a larger population.

In summary, we didn't see any significant difference in expression and methylation level in this patients for these selected genes. However, further investigations are required to confirm our findings because this study was designed as a pilot study.

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