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Assessing Gene Expression and Methylation of KMT2D and IGF2 Genes in Patients with Non-Small Cell Lung Cancer

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Abstract

Background: Aberrant promoter methylation of CpG islands is an important mechanism for regulation of gene expression. Recent data suggest that epigenetic abnormalities may occur very early in lung carcinogenesis. Systemic methylation changes may be a diagnostic marker for tumor development or prognosis. In this study, the expression and methylation of KMT2D and IGF2 genes were investigated in the lung cancer tissue compared to the adjacent normal tissue.

Methods: The status of methylation of KMT2D and IGF2 genes were investigated in 30 patients with NSCLC after genomic DNA extraction using bisulfite treatment and MS-HRM method and the expression of these genes were checked by Real-Time PCR method in same samples.

Results: For KMT2D gene, the expression and methylation level increased in 46.6% and 6.67% (respectively) for tumor samples comparison with normal samples ($P>0.05$). Also, for IGF2 gene 50% tumor samples overexpressed and 50% tumor samples showed that reduced expression comparison with the normal samples ($P>0.05$). In addition, 96.66% of tumor tissues did not show any change in methylation level for IGF2 gene promoter ($P>0.05$).

Conclusion: This study showed that expression and methylation level of KMT2D and IGF2 genes did not change in NSCLC tumor samples compared to normal samples. However, this study was designed as a pilot study, and further investigations are required to confirm our findings.

Keywords: Lung cancer, DNA methylation, IGF2, KMT2D, MS-HRM, Real-time PCR, NSCLC

1. Introduction

Lung cancer is the leading cause of cancer-related death in males and females worldwide (Ginsberg, 1997; Marby, Nelkin, & Baylin, 1998). Estimates of the American Cancer Society for Lung Cancer in the United States for 2017 were about 222500 new cases of lung cancer (116,990 cases for men and 105,551 for women) and 155870 deaths from lung cancer (84590 cases for men and 71280 cases for women)[From: https://www.cancer.org/cancer/lung-cancer.html]. Clinically, lung cancer is divided into 2 groups: small cell lung cancer (SCLC) and non- small cell lung cancer (NSCLC). Approximately 85% of lung tumors are NSCLC, which includes squamous cell carcinoma, adenocarcinoma and large cell carcinoma (Risch & Plass, 2008). Symptoms of lung cancer include chronic cough, blood in sputum or saliva, chest pain, fatigue, and weight loss and appetite loss (From: https://www.cancer.org/cancer/lung-cancer.html). Previous studies have demonstrated that lung cancer development involves environmental, genetic and epigenetic factors (Dumitrescu, 2012; P. Lee, 2001). Epidemiological studies indicate that cigarette smoking, as well as exposure to asbestos and radon, have a strong causal association with lung cancer (Alberg, Ford, & Samet, 2007). Somatic genetic aberrations, such as mutations and copy number alterations, play a well-known role in oncogenesis, epigenetic alterations are in fact more frequent than somatic mutations in lung cancer (Brzeziańska, Dutkowska, & Antczak, 2013). Epigenetic

alterations, including DNA methylation, histone modifications, and non-coding RNA expression, have widely been reported in the literature to play a major role in the genesis of lung cancer (Langevin, Kratzke, & Kelsey, 2015). DNA methylation is the most studied epigenetic regulatory mechanism (Ansari, Shackelford, & El-Osta, 2016), Epigenetic alterations, such as aberrant DNA methylation, are deeply involved in human cancer development (Kalari & Pfeifer, 2010; Y.-C. Lee et al., 2011), Especially, aberrant DNA methylation of a CpG island (CGI) in a promoter region causes silencing of its downstream gene and is known as a major mechanism for inactivation of tumor suppressor genes in human lung cancers (Esteller, 2002; Risch & Plass, 2008). CpG island methylation is completed by different DNA methyltransferases (DNMTs) that can lead to gene silencing (Forde, Brahmer, & Kelly, 2014; Patel et al., 2010). In the last decade, epigenetic alterations have been proposed to play an important role in the initiation, progression or invasiveness of cancer (Issa, 2004). So far, many studies have been conducted on the relationship between methylation and lung cancer, so that studies confirm the presence of multiple gene hyper-methylation in lung cancer samples (Wu et al., 2012). KMT2D (lysine (K)-specific methyltransferase 2D), formerly named MLL2 (myeloid/lymphoid or mixed-lineage leukemia 2, also known as ALR/MLL4), is a histone methyltransferase that plays an important role in regulating gene transcription. In particular, it targets histone H3 lysine 4 (H3K4), whose methylations serve as a gene activation mark (Bunn Jr et al., 2016; Guo et al., 2013). Additional cancers that have recently been found to be driven by an aberrant KMT2D/KMT2C pathway, with frequencies ranging from 5% to 40%, include renal (Dalgliesh et al., 2010), prostate, bladder (Gui et al., 2011), gastric, hepatic and lung cancer (Guo et al., 2013).

The IGF2 gene acts as an oncogene in cancer cells (Chava et al., 2012). The gene operates in normal body tissues in pathways such as the RAS/MAPK pathway, the PI3K/AKT pathway, and the AKT/mTOR pathway (Fernandez & Torres-Alemán, 2012). Studies have shown that IGF pathway involvement is present in a wide range of malignancies, including non-small cell lung cancer (NSCLC) and small lung cancer (SCLC). Increasing plasma levels of IGF is also associated with an increased risk of lung cancer (Dziadziuszko, Camidge, & Hirsch, 2008).

In this paper were investigated the expression and status of methylation of KMT2D and IGF2 genes in NSCLC cancer by using Real-Time PCR and MS-HRM[1] method.

2. Materials and Methods

2.1 Study Population- RNA Extraction and Synthesis of cDNA

Assessing the expression and methylation pattern of KMT2D and IGF2 genes were investigated in patients with non-small cell lung cancer from 30 samples with tumor tissue and adjacent tumor tissue (as normal sample). Patients with non-small cell lung cancer have been diagnosed by pathologist and from the Messiah Daneshvari Hospital (Tehran. Iran) entered the study. A tumor and adjacent tumor (as normal tissue) were collected from each patient and immediately transferred to the nitrogen tank, then stored in a freezer -70◦C. Clinical information related to the patients was studied and collected on a regular basis (Table 1).

Table 1. Study population characteristics

	male	female	Smoking history	lymph node metastasis	Pathological stage I- III	IA-IIIA	IB-IIIB
ADC n=17	11 Age(35-60) → 7 53.5* Age(60-80) → 4 69.75*	6 Age(50-60) 56.6*	5	10	1	8	8
SqCC n=13	12 Age(40-60) → 4 53* Age(60-70) → 8 63.12*	1 Age(62)	3	3	0	7	6
***Mean ± SD**							

Total RNA was isolated from each tumor tissue and adjacent non-tumor tissue by using RiboEx (GeneAll, Korea) according to the manufacturer's specifications and synthesis of cDNA was performed by using HyperScriptTM

[1] -Methylation Specific-High Resolution Melting

Reverse Transcriptase kit (GeneAll, korea) according to the relevant instructions.

2.2 Real-Time PCR

The total volume of the reaction was considered to be 15µl, So that were used the amount of 7.5µl RealQ Plus 2x Master Mix Green High ROX™ (Ampliqon, Denmark), 0.5µl from the forward primer (6Pmol/µl Concentration), 0.5µl from reverse primer (6Pmol/µl Concentration) and 1µl cDNA (10ng concentration) in each reaction (The sequence of primers is shown in Table 2).

Table 2. Designed Primers for Real-Time PCR

Gene	Forward primer (5'→ 3')	Reverse primer (5' →3')	Size (bp)
KMT2D	GCCTGGCTTTGGTGGTTTCA	CCATCCCCACTCAACACCTC	96
IGF2	CCGACTTCCAGACACCAATG	CGGTCCTGCTGAAGTAGAAG	183
GAPDH	CATCAAGAAGGTGGTGAAGCA	GCGTCAAAGGTGGAGGAGTG	120

These conditions were considered for Real-Time PCR: First stage (Holding stage): 95◦C for 15 min. The second stage (Cycling stage) consists of 40 cycles and three step, first step: 95◦C for 15 seconds, second step: 58◦C-62◦C for 25 seconds (for KMT2D primer 58◦C, for IGF2 primer 62◦C) and third step: 72◦C for 30 seconds. The third stage (Melt curve Stage–Step and Hold) consists of three step, first step: 95◦C for 15 seconds, second step: 60◦C for 1 min and third stage: 95◦C for 15 seconds. All samples were analyzed in duplicate. GAPDH was used as an internal control. Gene expression was calculated using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method.

2.3 DNA Extraction and Bisulfite Treatment

DNA extraction was performed by using QIAamp DNA extraction kit (Qiagen, United States) and bisulfite treatment of the extracted DNAs was performed by using the EZ DNA Methylation Gold Kit Zymo Research, United states) according to the relevant instructions.

2.4 Providing Controls

DNA extracted from the Human blood sample was used to prepare controls and after bisulfite treatment was considered as 0% methylated control (un-methylated), Also preparation of 100% methylated control (fully methylated) by using *M.SssI* enzyme (Thermo Scientific) was performed according to the instructions. Finally, for prepare control with different percentages were used of certain amounts 0% methylated and 100% methylated controls.

2.5 MS-HRM

The total volume of the reaction was considered to be 20µl, So that were used the amount of 4µl from HOT FIREPol EvaGreen HRM Mix5x (Rox), 1µl from the forward primer (6Pmol/µl Concentration), 1µl from reverse primer (6Pmol/µl Concentration) and 1µl of bisulfite converted DNA (1000ng concentration) in each reaction (The sequence of primers is shown in Table 3).

Table 3. Designed Primers for MS-HRM

Gene	Forward primer (5'→ 3')	Reverse primer (5' → 3')	Size (bp)
KMT2D	CGTGTGCGTAGAATTGTAGAGTTAT	CTATTTCCTAACCCAAAAACTAAACAAC	206
IGF2	CGAGTAGGTATTTGGGAGTTT	TTTCTTTACCTTCTCCACAC	150

These conditions were considered for MS-HRM: First stage (Holding stage): 95◦C for 15 min. The second stage (Cycling stage) consists of 40 cycles and three step, first step: 95◦C for 15 seconds, second step: 58◦C-60◦C for 25 seconds (for KMT2D primer 58◦C, for IGF2 primer 60◦C) and third step: 72◦C for 30 seconds. The third stage (Melt curve Stage–Continuous) consists of three step, first step: 95◦C for 15 seconds, second step: 60◦C for 1 min and third stage: 95◦C for 15 seconds.

2.6 Statistical Analysis

The difference in expression and methylation between tumor samples and adjacent normal tissues was performed using the Chi-square test or fisher exact test by GraphPad Prism 7.03. For all test, $p < 0.05$ was considered as a significant difference for this study.

3. Results

3.1 Status of Expression and Methylation of KMT2D Gene in Patients with NSCLC

The Real-Time PCR method was used to detect KMT2D gene expression in 30 samples NSCLC tissues and 30 adjacent non-tumor tissues of the same subjects. The results of the KMT2D gene expression showed that 14 (46.6%) tumor samples increased expression in comparison with the normal sample, but 16 (53.4%) tumor samples reduced expression in comparison with the normal samples. Therefore, there was no significant difference between the expression of this gene in tumor samples compared to normal samples (P = 0.46). (Figure 1.A shows the result of the KMT2D gene expression). Also, the MS-HRM method was used to detect KMT2D gene methylation in the same samples and cut off for this study was considered 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. This study reported that were hyper-methylated 2 of the 30 tumor samples of the lung tissue (6.67%). In contrast, none of the normal samples were in the promoter region of methylated (P = 0.515). (Figure 2 and 4.A show the results of the KMT2D gene methylation).

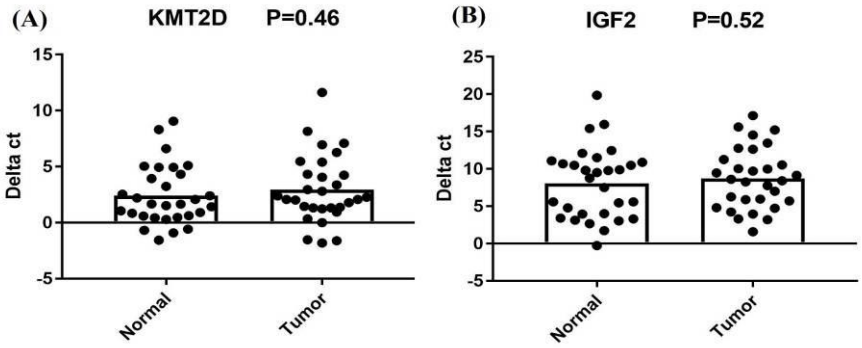

Figure 1. The graph (A) related to expression of KMT2D gene in NSCLC tumor samples compared to non-tumor adjacent samples and the graph (B) related to expression of IGF2 gene in NSCLC tumor samples compared to non-tumor adjacent samples

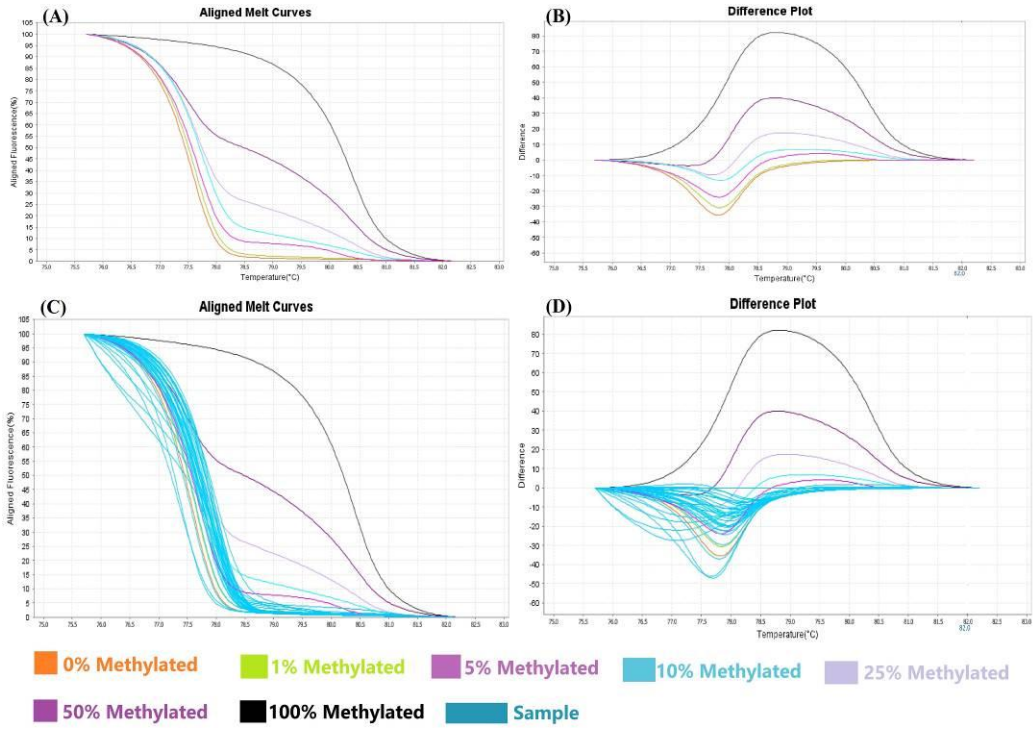

Figure 2. The graphs related to the KMT2D gene with controls and samples. The graphs (A) and (B) represent the controls for the KMT2D gene, So that controls are from down to up consists of 0%, 1%, 5%, 10%, 25%, 50% and 100% methylated. The graphs (C) and (D) represent the samples and controls

3.2 Status of Expression and Methylation of IGF2 Gene in Patients with NSCLC

The gene expression of IGF2 was investigated in 30 samples NSCLC tissues and 30 adjacent non-tumor tissues of the same subjects by Real-Time PCR method. The results obtained of IGF2 gene expression showed that 15 (50%) tumor samples increased expression in comparison with the normal sample so that 15 (50%) tumor samples reduced expression in comparison with the normal sample. Therefore, there was no significant difference between the expression of this gene in tumor samples compared to normal samples (P =0.52). (Fig. 1.B shows the result of the IGF2 gene expression). The methylation of the promoter region of IGF2 gene was also investigated in the same samples by MS-HRM method. Cut off for this study was considered 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. This study reported that was hyper-methylated 1 of the 30 tumor samples of the lung tissue (3.34%), so that were not 30 normal samples adjacent to methylated (P=0.644). (Figure 3 and 4.B show the results of the IGF2 gene).

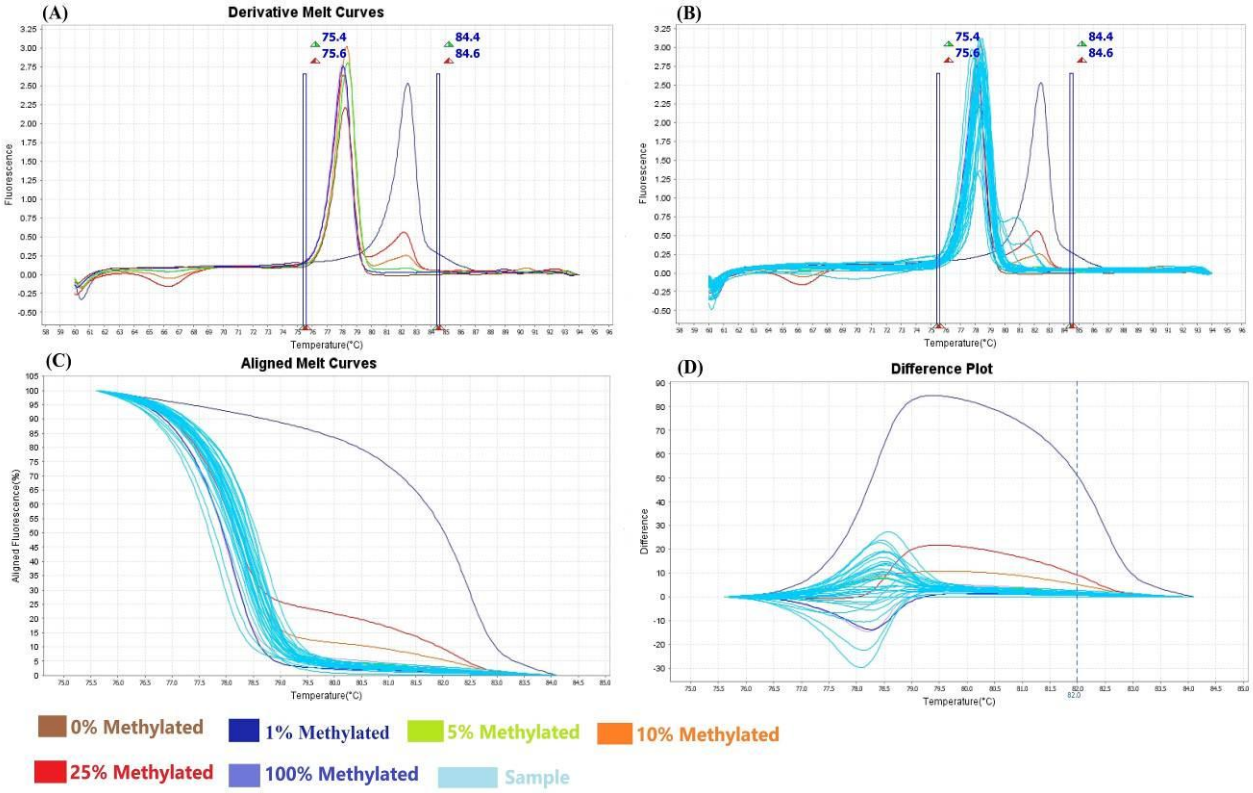

Figure 3. The graphs related to the IGF2 gene with controls and samples. The graphs (A) represent the controls for the IGF2 gene, so that controls are from down to up consists of 0%, 1%, 5%, 10%, 25%, and 100% methylated. The graphs (B), (C) and (D) represent the samples and controls

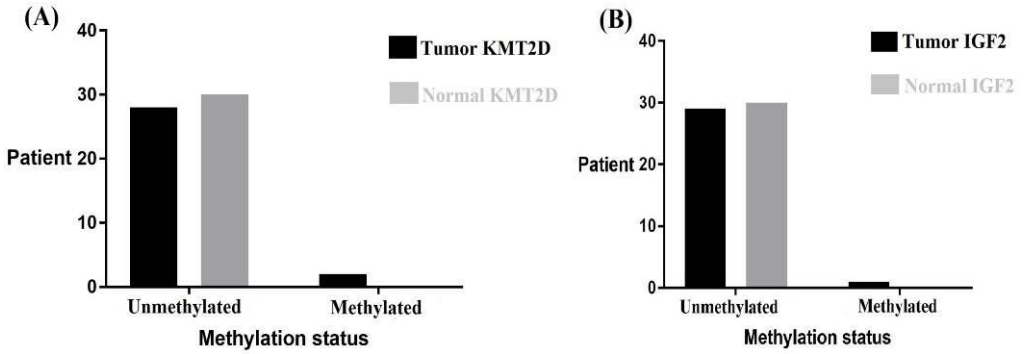

Figure 4. The graph (A) related to methylation of KMT2D gene in NSCLC tumor samples compared to non-tumor adjacent samples and the graph (B) related to methylation of IGF2 gene in NSCLC tumor samples compared to non-tumor adjacent samples

4. Discussion

DNA methylation is a type of chemical modification of DNA that can be inherited without changing the original DNA sequence. As such, it is part of the epigenetic code and is also the best characterized epigenetic mechanism in humans, and changes in methylation patterns play an important role in tumorigenesis (Jones & Baylin, 2002; Lopez, Percharde, Coley, Webb, & Crook, 2009). Particularly, hyper-methylation of normally un-methylated CpG islands in tumor suppressor genes is associated with silencing of gene expression, and may confer growth advantages to these cells that favor cancer development (Baylin, 2002; Baylln, Herman, Graff, Vertino, & Issa, 1997). Growing evidence has shown that hyper-methylation of the tumor suppressor gene promoter represents one of the major molecular alterations in tumor development (Herman & Baylin, 2003). Promoter hyper-methylation can be used as a sensitive marker not only for TSG identification but also for cancer diagnosis and prognosis prediction (Rui, You-Wei, & Long-Bang, 2010). Several methods have been identified for detecting DNA methylation, one of which is bisulfite treatment and using by MS-HRM method.

In this study, this method was used to analyze the methylation status of KMT2D and IGF2 genes promoter in 30 tumor samples of NSCLC and 30 adjacent non-tumor samples from the same individuals also the expression of these genes in the same specimens was also investigated by Real-Time PCR method.

The results of KMT2D gene expression and methylation demonstrate that there are no significant difference between expression and methylation of tumor samples compared to adjacent normal samples (Fig. 1.A and 2). According to recent studies on the KMT2D gene in various cancers, including lung cancer, it has been investigated that the expression of KMT2D gene has been reduced as tumor suppressor in cancerous tissues. In some cases, decrease of KMT2D gene expression is due to mutation occurrence in NSCLC specimens, it has also been reported that the expression of KMT2D gene in tumor samples in advanced stages is reduced compared to tumor samples in the early stages (Figueiredo et al., 2015; Yin et al., 2014). Therefore, in the present study, the expression and methylation changes of KMT2D gene was not observed in NSCLC tumor samples compared to normal samples. The decrease of KMT2D gene expression in cancerous 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. This study also showed that IGF2 gene expression and methylation level in tumor samples was not significantly different compared to normal samples (Fig. 1.B and 3). Recent studies on the expression and methylation of IGF2 gene show increased expression of this gene, followed by hypo-methylation (Chava et al., 2012; Li, Meng, Huang, & Guo, 2009). Considering the mentioned studies and the roles of this gene in normal lung tissues, we expect the overexpression of IGF2 gene occurs in NSCLC patients, but in this case, overexpression of IGF2 gene was observed only in 50 percent of NSCLC patients and as expected the increase of methylation for this gene was not observed.

5. Conclusion

This study showed that expression and methylation level of KMT2D and IGF2 genes did not change in NSCLC tumor samples compared to normal samples. However, this study was designed as a pilot study, and further investigations are required to confirm our findings.

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