

The Methylation Analysis of KLF11 and PCDH9 Genes in Patients with Non-Small Cell Lung Cancer

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Abstract

Background: Lung cancer is the leading cause of cancer-related deaths worldwide and the 5-year survival rate is still very poor due to the lack of effective tools for early detection. Epigenetics and especially studies on DNA methylation have given important information towards a better achievement of lung cancer pathogenesis in the recent decades. The inactivation of tumor suppressor genes via promoter hypermethylation is an obvious mechanism and is straightly related to carcinogenesis. In this study, we compared the methylation status of KLF11 and PCDH9 genes in non-small cell lung cancer and adjacent normal tissues.

Methods: Genomic DNA was extracted from 30 tumor tissues, bisulfite treated and were analyzed in terms of promoter methylation status of KLF11 and PCDH9 genes by high resolution melting method. Statistical analysis was carried out by chi-square test.

Results: No significant difference in methylation level at the PCDH9 promoter region in NSCLC tumors compared with non-tumor tissues was observed ($P = 0.3132$, chi-square test). In contrast, the difference in methylation levels between normal and tumor tissue samples for the promoter of the KLF11 gene was quite significant ($P = 0.0001$).

Conclusions: Promoter methylation of KLF11 gene is an important mechanism in the development of NSCLC, therefore, it could be used as one of the potential therapeutic goals for molecular targeted therapy and epigenetic treatment. The role of the PCDH9 gene in the development of lung cancer is complex and requires more research and a larger statistical population.

Keyword: NSCLC, methylation, KLF11, PCDH9, MS-HRM

1. Introduction

Lung cancer is the leading cause of cancer-related death worldwide (Parkin, Bray, Ferlay, & Pisani, 2005). About 1.8 million new cases of lung cancer were diagnosed in 2012, which accounted for 12.9% of the world's total cancer incidence (Ferlay et al., 2013). Despite recent advances in the treatment of lung cancer (e.g., targeted therapy), the overall prognosis for the disease remains dismal with an estimated 5-year survival rate in the United States of 18% (Siegel, Miller, & Jemal, 2015). The two major forms of lung cancer are non-small cell lung cancer (NSCLC; about 85% of all lung cancers) and small-cell lung cancer (about 15% of lung cancer). NSCLC is divided into three major histologic subtypes: adenocarcinoma, squamous cell carcinoma and large cell carcinoma (Burstein & Schwartz, 2008).

Like many other cancers, the development of lung cancer is a multistep process involving epigenetic, genetic and environmental factors that cause the dysregulation of oncogenes and tumor suppressor genes (Ansari, Shackelford, & El-Osta, 2016). In the recent decade, epigenetic alterations have been proposed to play an important role in the initiation, progression or invasiveness of cancer (Issa, 2004). These alterations can be classified into histone modifications, DNA methylation, microRNAs (miRNAs) and nucleosome remodeling

(Portela & Esteller, 2010). The DNA hypermethylation of 5'-CpG islands has been shown to be a major reason of silencing of tumor suppressor genes in human NSCLC tumors (Baylin & Herman, 2000; Jones & Baylin, 2002).

Kruppel-like transcription factor 11 (KLF11), a member of the Sp1/Kruppel-like factor zinc finger transcription factor (Sp1/KLF) family, was first found in osteoblast cells and later shown to be widely expressed in many tissues such as the lung, stomach, mammary gland, kidney and colon, with the highest expression in normal pancreatic tissue (Kuroda et al., 2009; Spittau & Krieglstein, 2012). KLF11 shares a highly conserved C-terminal DNA-binding domain that contains three C2H2 zinc finger motifs. The zinc finger motif facilitates binding to GC-rich promoter elements which, in turn, regulate multiple cellular events, including suppressing cell cycle/proliferation and promoting apoptosis (Bureau et al., 2009; Fernandez-Zapico et al., 2011). As an inhibitory transcription factor, KLF11 participates in TGF- β signaling pathway mainly through regulating the TGF- β induced expression of SMAD7 gene (Spittau & Krieglstein, 2012).

PCDH9 gene (protocadherin 9) is a member of cadherin superfamily which is a calcium-dependent cell-cell adhesion molecule (Redies, Vanhalst, & Van Roy, 2005). This gene has been mapped to 13q21.32 and it encodes a protein that is expressed in a broad tissue type (Frank & Kemler, 2002). PCDH9 is thought to function in cell adhesion, mainly in the central nervous system, but is also expressed in a variety of tissues including lung tissue (Strehl, Glatt, Liu, Glatt, & Lalonde, 1998). In this study, we compared the methylation status of KLF11 and PCDH9 gene in NSCLC and normal lung tissues.

2. Material and Methods

2.1 Patients and Samples

Thirty patients with the diagnosis of NSCLC were selected to be included in the study from surgery department of Masih Daneshvari hospital. Tehran, Iran. Written informed consents were obtained from the patients. Tumor and adjacent normal tissues were obtained at the time of surgery before any treatment. The tissues were snap-frozen and stored at -70°C until use. Patients' clinico-pathological characteristics were reviewed from medical records, including age, gender, histological type, pathological (TNM) stage and smoking history.

2.2 DNA Extraction

DNA was extracted from normal and tumor tissues by using the Qiagen DNA Extraction Mini Kit according to the manufacturer's recommendation (Qiagen, United States). DNA was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Integrity and size of DNA were assessed by 1.0% agarose gel electrophoresis.

2.3 Bisulfite Treatment

Bisulfite conversion was performed with 1000ng of genomic DNA using EZ DNA Methylation Kit (Zymo Research, Orange, CA), according to the manufacturer's protocol. All of the converted DNA was stored at -70°C until use.

2.4 Preparation of Controls

DNA extracted from the blood was used as a unmethylated control. Fully methylated control DNA was prepared by treatment of blood DNA, with *M.sssI* enzyme according to the manufacturer's recommendation (Thermo Scientific, United States). A series of standard dilutions of methylated DNA was prepared by diluting fully methylated DNA with unmethylated bisulfate-treated DNA.

2.5 MS- High Resolution Melting (MS-HRM)

Methylation-sensitive high resolution melting (MS-HRM) method was performed using the bisulfite-modified DNA as the template. The amplification was conducted in a 20 μL reaction volume containing 1 μL DNA template, 4 μL of 5x HOT FIREPol[®]EvaGreen[®] HRM Mix (ROX), 14 μL nuclease-free water and 6 pmol/ μL of forward and reverse primers (1 μL). Designed primers and their sequences were being shown as follows: KLF11 forward: 5'-GGTGGGGTTGTAGGAGGT-3' and reverse: 5'-ATGCCAACRACRCCAAAAACAAA-3' (198bp product length); PCDH9 forward: 5'-GAGTATGTTTGTATTTTATGGAAT-3' and reverse: 5'-CGAACTCTAATTAACCTACATCTT-3' (189bp product length). DNA melting with high resolution is a three-stage process. First stage: initial denaturation at 95°C for 15 min; The second stage consists of three steps in 40 cycles: 94°C for 15 seconds, 60°C (for KLF11) and 58°C (for PCDH9) for 20 seconds (annealing time), 72°C for 30 seconds (extension); and a final extension at 72°C for 5 min. Third stage (The melting curve continuous stage) was performed as follows: denaturation at 95°C for 15 seconds, 60°C (for KLF11) and 58°C (for PCDH9) for 1 min, followed by HRM step ramping from 58°C - 60°C to 95°C , rising 0.1 $^{\circ}\text{C}$.

2.6 Statistical Analysis

The chi-square test was used to compare the methylation levels in the NSCLC tumor and normal lung tissue DNA by GraphPad Prism v7.03 software. A $p < 0.05$ was considered statistically significant.

3. Results

3.1 Patients' Characteristics

From the 30 patients included in this study, 57% had adenocarcinoma and 43% had squamous cell carcinoma; 27% were female and 73% were male, and from age point of view, 3% were under 40, 3% between 40 and 50, 47% between 50 and 60 and 47% between 60 and 70 years old. 73% were non-smokers and 27% were smokers. The proportion of patients with pathological stages I, II, III, and IV was 30%, 36.66%, 33.34% and 0%, respectively. Patients' characteristics are shown in Table 1.

Table 1. Demographic and clinic-pathological Characteristics of the 30 surgically resected lung cancer patients.

Variable	Value (n = 30)
Gender	
Male	22 (73%)
Female	8 (27%)
Age (years)	
Under 40 (37)	1 (3%)
Between 40-50 (47)	1 (3%)
Between 50-60 (55.5 ± 0.07)	14 (47%)
Between 60-70 (63.5 ± 0.05)	14 (47%)
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%)

3.2 Promoter Methylation Status of PCDH9

Genomic DNA from lung tissues was converted by sodium bisulfite, and the methylation level at the PCDH9 promoter region was compared with tumor tissues (n = 30) and non-tumor tissues (n = 30) using methylation-sensitive HRM. A 10% cut-off methylation level was used for the analysis. Normal samples were methylated under 10 percent. 29 out of 30 tumor samples were methylated under 10% like normal samples. One of the tumor samples showed a methylation level of 14% and it was classified as hypermethylated. MS-high resolution melting analysis didn't show any significant difference in methylation level at the PCDH9 promoter region in NSCLC tumors compared with non-tumor tissues ($P = 0.3132$, chi-square test). Graphs related to MS-high resolution melting analysis of PCDH9 gene promoter methylation was shown in Figure 1.

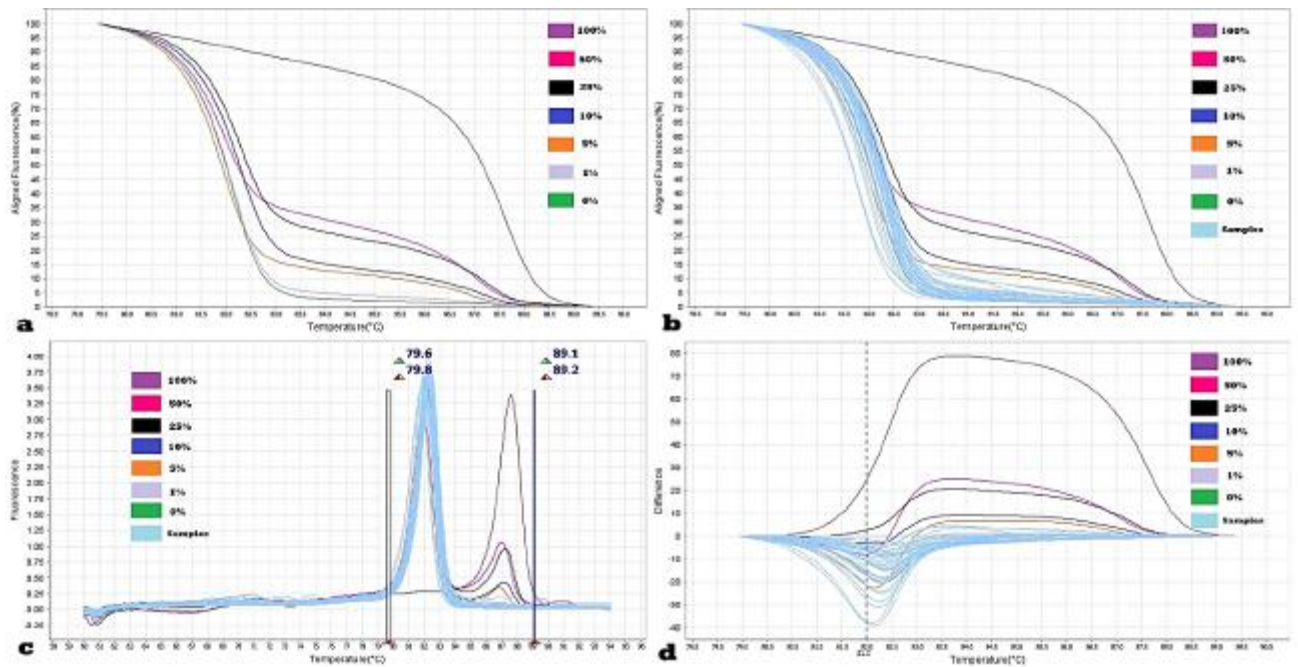


Figure 1. Graphs related to MS-high resolution melting analysis of PCDH9 gene promoter methylation. (a) the normalized graph for PCDH9 controls (b) normalized graph for PCDH9 controls with samples (c) melting curve graph of controls and samples related to PCDH9 gene promoter (d) difference plot graph of controls and samples related to the PCDH9 gene promoter

3.3 Promoter Methylation Status of KLF11

As shown in Table 2, All normal samples were methylated under 10 percent. In contrast, the results for the tumor samples were completely different. MS-high resolution melting analysis showed that 96.67% (29 out of 30) of tumor samples were hypermethylated in comparison with normal paired samples for KLF11 promoter gene (Figure 2). The difference in methylation levels between normal and tumor samples for the promoter of the KLF11 gene is quite significant ($P = 0.0001$, chi-square test). Diagrams related to promoter methylation analysis of KLF11 and PCDH9 genes was shown in Figure 3.

Table 2. The methylation status of the sample for the KLF11 gene.

Methylation (%)	Number of tumor Samples (n=30) (%)	Number of normal samples (n=30) (%)
<10	1 (3.3%)	30 (100%)
10-25	3 (10%)	0
25-50	15 (50%)	0
50-75	7 (23.3%)	0
75-100	4 (13.4%)	0

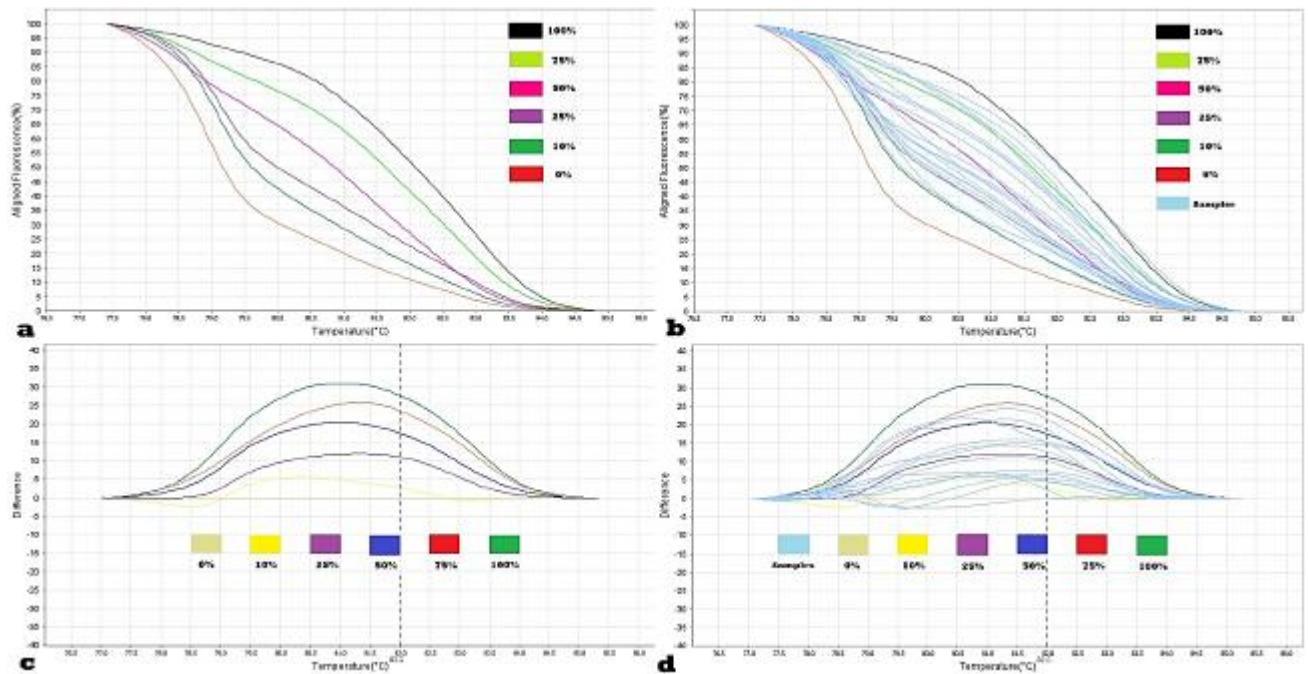


Figure 2. Graphs related to MS-high resolution melting analysis of KLF11 gene promoter methylation. (a) the normalized graph for KLF11 controls (b) normalized graph for KLF11 controls with tumor samples (c) difference plot graph for KLF11 controls (d) difference plot graph for KLF11 controls with some tumor and normal samples

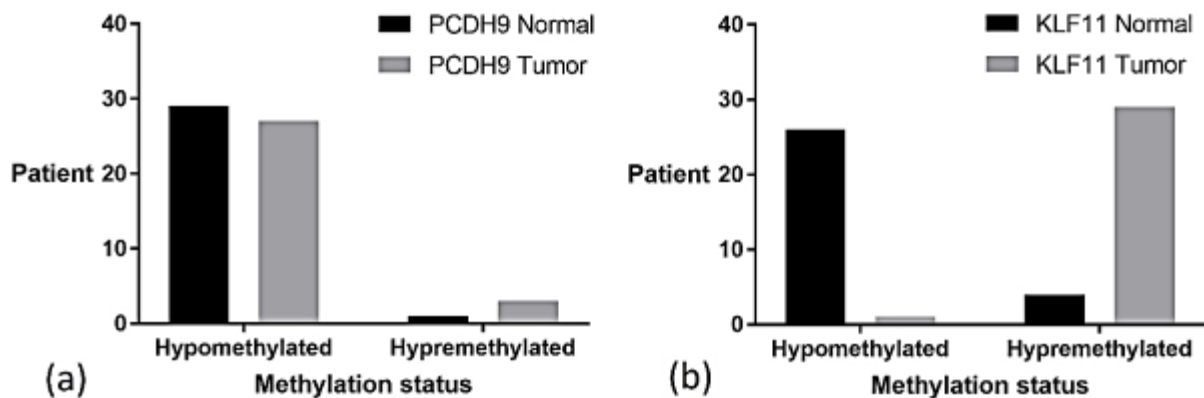


Figure 3. Diagram related to (a) PCDH9 promoter methylation status; no significant difference in methylation level at the PCDH9 promoter region in NSCLC tumors compared with non-tumor tissues was observed ($P = 0.3132$, chi-square test). (b) KLF11 promoter methylation status; The difference in methylation levels between normal and tumor samples for the promoter of the KLF11 gene is quite significant ($P = 0.0001$, chi-square test)

4. Discussion

The hypermethylation of DNA in promoters and regulatory regions of tumor suppressor genes is a momentous epigenetic occurrence in development of NSCLC. On the basis of this occurrence, the tumor suppressor genes are going to become silence and it is now recognized as a main and causal epigenetic incident in various kinds of cancer (Belinsky, 2005). Different methods have been employed for detection of the methylation status of genes promoter regions. Among them, MS-HRM has been used in many studies for sensitive, high throughput assessment of DNA methylation (Dahl & Guldborg, 2007). In this study, we have analyzed the methylation status of KLF11 and PCDH9 genes promoter in 30 NSCLC patients by MS-HRM.

MS-high resolution melting analysis showed that 96.67% (29 out of 30) of NSCLC tumor samples were hypermethylated compared to non-tumor lung samples for KLF11 promoter gene ($p = 0.0001$).

Aberrant promoter methylation of the KLF11 gene has been reported in several human cancers. According to the research of Wang et al. the expression of KLF11 was significantly reduced in ovarian cancer tissues. They also investigated the methylation level between normal and tumor ovarian tissues in 31 Chinese patients. Compared with the normal samples, the overall methylation level was dramatically increased in the tumor tissues ($P = 0.0001$), and this significant difference was observed through all stages of the disease (G. Wang et al., 2015). In another study, Potapova et al. demonstrated that epigenetic inactivation of KLF11 occurs in myelodysplastic syndrome. Their studies showed that this gene is hypermethylated in 25.2% of cases affected by myelodysplastic syndrome (Potapova et al., 2010).

KLF11 plays an important role in regulating TGF- β signaling pathway. This pathway controls a wide spectrum of cellular activities including differentiation, proliferation, and apoptosis (Ellenrieder, 2008). KLF11 can regulate the TGF- β pathway directly through the SMAD7-mediated negative loop. In one side, KLF11 is able to bind to GC-rich elements within the SMAD7 promoter sequence to suppress TGF- β -induced SMAD7 expression and hence releases TGF- β signaling from the SMAD7-mediated inhibitory effect (Gohla, Krieglstein, & Spittau, 2008). According to the result obtained in this study, with the hypermethylation of this gene, it is expected that dysregulation of this pathway could be involved in developing lung cancer. On the other hand, the samples in this study are often in the early stages, the 96.67% hypermethylation of the samples indicates that the KLF11 gene often has a hypermethylated and reduced expression at the early stages of lung cancer.

The PCDH9 promoter didn't show any significant difference in methylation level in NSCLC tumors compared to non-tumor tissues ($P = 0.3132$). Formerly, the loss of protocadherin family proteins has been shown to contribute to the development of various human cancers, such as prostate cancer (M.-W. Chen et al., 2002), liver cancer (Okazaki, Takahashi, Kojima, Masuho, & Koga, 2002), colon cancer (Okazaki et al., 2002), renal cancer (Stassar et al., 2001), breast cancer (Yu et al., 2008), astrocytoma (Waha et al., 2005), nasopharyngeal and lung cancers. The cause of protocadherin loss is due to gene promoter hypermethylation, mutation or gene deletion (Imoto et al., 2006; Ying et al., 2006).

The expression of PCDH9 decreases in Hepatocellular carcinoma (HCC), which is due to DNA methylation and histone deacetylation (Lv et al., 2017). According to Chen et al. loss of PCDH9 expression is associated with the differentiation of tumor cells and metastasis and predicts poor survival in gastric cancer (Y. Chen, Xiang, Zhang, Wang, & Yu, 2015). Wang et al. showed that the expression of PCDH9 was decreased in primary gliomas (C. Wang et al., 2014).

This gene is also involved in the regulation of the epithelial-mesenchymal transition (EMT) (Zhu et al., 2014). In this research, we didn't find any significant difference in methylation level at the PCDH9 promoter region. 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 because of the PCDH9 gene's function, we expect the hypermethylation of this gene occurs at the late stages of lung cancer. Obviously, the reduced expression of this gene causes metastasis and this process occurs in the advanced stages of the disease. According to the description provided, the role of this gene in the development of lung cancer is complex and requires more research and a larger population.

5. Conclusion

Promoter hypermethylation of KLF11 gene is an important mechanism in the development of NSCLC and because of its important role; it can be used as one of the potential therapeutic goals for molecular targeted therapy and epigenetic treatment. The role of the PCDH9 gene in the development of lung cancer is complex and requires more research and a larger population.

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