RESEARCH ARTICLE

Methylation of RASSF1A and CDH13 Genes in Individualized Chemotherapy for Patients with Non-small Cell Lung Cancer

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Abstract

Background: This study aimed to evaluate the methylation of RASSF1A and CDH13 gene promoter regions as a marker for monitoring chemotherapeutic efficacy with personalized medicine for patients with NSCLC, in the hope of providing a new direction for NSCLC individualized chemotherapy. Materials and Methods: 42 NSCLC patients and 40 healthy controls were included. Patient blood samples were collected in the whole process of chemotherapy. Methylation of RASSF1A and CDH13 gene promoter regions was detected by the methylation specific polymerase chain reaction (MSP). Results: The rate of RASSF1A and CDH13 gene methylation in 42 cases of NSCLC patients was significantly higher than in 40 healthy controls (52.4% to 0.0%, 54.8% to 0.0%, $p<0.05$). After the chemotherapy, the hyper-methylation of RASSF1A and CDH13 genes in PR group and SD group decreased significantly ($p<0.05$), and was significantly different from that in PD group ($p<0.05$), but not as compared with healthy controls ($P>0.05$). With chemotherapy, RASSF1A and CDH13 promoter region methylation rate in 42 cases of patients showed a declining trend. Conclusions: The methylation level of RASSF1A and CDH13 gene promoter region can reflect drug sensitivity of tumors to individualized treatment.

Keywords: RASSF1A gene - CDH13 gene - DNA methylation - non-small cell lung cancer - individualized chemotherapy

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Introduction

Lung cancer is one of the malignant tumors which has the highest morbidity and mortality in the world (Shin et al., 2014). The majority of patients with non-small cell lung cancer (NSCLC) wouldn't even find out the situation until the cancer has reached its late stage, lost the chance of operation. So the chemotherapy is still the important method for the treatment of advanced non small cell lung cancer patients (Song et al., 2013). However, due to individual differences in sensitivity to chemotherapy drugs, the effect of chemotherapy is not ideal, even lead to the progression of disease. Therefore, in order to prolong the survival of patients with NSCLC, to improve the quality of life of these patients, the application of individualized chemotherapy is particularly important (Xinbing et al., 2013). Because of the methylation of promoter region is a reversible process, detection of gene methylation levels may provide guidance for the individualized chemotherapy (Blattler et al., 2013). It has been suggested that methylation of RASSF1A and CDH13 genes promoter region is closely related to the occurrence of NSCLC.As tumor suppressor gene, RASSF1A and CDH13 genes methylation occur frequently in NSCLC (Jung et al., 2012; Milica et al., 2012).

Materials and Methods

The object of study

We analyzed 42 patients who diagnosed with NSCLC from 2013 June to 2014 February at The First Affiliated Hospital of Dalian Medical University. Age 38-78 years old, (62.39±9.38) years old. 32 cases of male, 10 cases female. Histopathological classification was assessed according to the World Health Organization (WHO) criteria. There were 10 cases of Squamous cell carcinoma, 32 cases of adenocarcinoma. Tumor-node-metastasis (TNM) staging followed the American joint Committee on Cancer (AJCC) staging system as revised in 2010 (7th edition). TNM stage was stage I in 4 (9.5%), stage II in 2 (4.8%), stage III in 14 (33.3%) and stage IV in 22 (52.4%). Select 40 cases of healthy persons which were not suffering from any disease at the same period as healthy control group.which were correlation in age, gender with the lung cancer group.

Methods

Specimen collection: Collected venous blood samples before the first time of chemotherapy, after the secend time of chemotherapy, after the fourth time of chemotherapy and after the sixth time of chemotherapy,

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EDTA anticoagulant.

DNA extraction: Using Blood genomic DNA Extraction Kit (centrifugal column type) from Beijing Tiangen Biotech, extracting DNA from the blood according to the operating instructions.

Sodium Bisulfite modification: Sodium Bisulfite modificating the genomic DNA, the un-methylated cytosine (C) in DNA sequence turn to Urine pyrimidine (U) (Zinn et al., 2007).

DNA purification and desulfonation reaction: Purificated the DNA by Wizard DNA Clean Up System (America Promega company).Desulphurizated the DNA by 0.3 mol/L NaOH at room temperature for 5 min.And then, precipitated the DNA with cold ethanol, solubled the DNA in 20 μl distilled water.

Methylation specific polymerase chain reaction (MSP): The primers were designed according to the literature, identify methylation specific sequence (M) and un-methylation specific sequence (U) respectively. RASSF1A-M sense primer:5'GTGTTAACGCG TTGCGTATC3', RASSF1A-M antisense primer: 5'AACCCCGCGA ACTAAAAACGA3', The amplification product was 119bp; RASSF1A-U sense primer: 5'TTTGGTTGGAGT GTGTTAATGTG3', RASSF1A-U antisense primer:5'CAAACCCCAC AAACTAAAAACAA3', The amplification product was 125bp.CDH13-M sense:5'TCGCGGGGTT CGTTTTTCGC3', CDH13-M antisense: 5'GACGTTTTC ATTCATACACGCG3', The amplification product was 243bp; CDH13-U sense:5'TTGTGGGG TTGTTTTTTGT3', CDH13-U antisense: 5'AACTTTTCAT TCATACACACA3', The amplification product was 243bp (Qiang et al., 2009; Vo et al., 2013; Milica et al., 2012).

The reaction system was 20μl, where 2×Tag PCR Master Mix 10μl, purified DNA 2μl, ddH_2O 6μl. Amplification the the solution after mixing sufficiently.

The reaction conditions of RASSF1A gene: 94°C: 5min, 94°C: 30sec, 55°C (M)/60°C (U): 30sec, 35 Cycles, 72°C: 30sec, 72°C: 10min

The reaction conditions of CDH13 gene: 94°C: 5min, 94°C: 30sec, 55°C: 30sec, 35 Cycles, 72°C: 30sec, 72°C: 10min

After the amplification, electrophoresis 10μl sample at agarose gel of 2% concentration.

Statistical processing

Statistical analysis was performed using SPSS17.0 statistical software, the measurement data using mean±standard deviation (±s); the count data using multifrequency x^2 test.There were statistically significant differences when $p<0.05$.

Results

Among the 42 NSCLC patients, 22 cases were hyper-methylation of RASSF1A gene, the methylation rate was 52.4%. There was no 1 case in 40 healthy persons occured RASSF1A gene promoter region hyper-methylation. The rate of RASSF1A gene methylation in 42 cases of NSCLC patients is significant higher than in 40 cases of healthy controls ($p<0.05$) (Figure 1).

Among the 42 NSCLC patients, 23 cases were hyper-methylation of CDH13 gene, the methylation rate was 54.8%.There was no 1 case in 40 healthy persons occured CDH13 gene promoter region hyper-methylation. The rate of CDH13 gene methylation in 42 cases of NSCLC patients is significant higher than in 40 cases of healthy controls ($p<0.05$) (Figure 2).

Hyper-methylation of RASSF1A and CDH13 genes was not associated with gender, age, smoking, pathological type and TNM staging system (Table 1).

After the chemotherapy, the hyper-methylation of RASSF1A and CDH13 genes in partial remission (PR) group and stable disease (SD) group decreased significantly ($p<0.05$), and was significantly different from that in progressive disease (PD) group ($p<0.05$), but had no statistical difference compared with that in healthy controls ($p>0.05$) (Table 2).

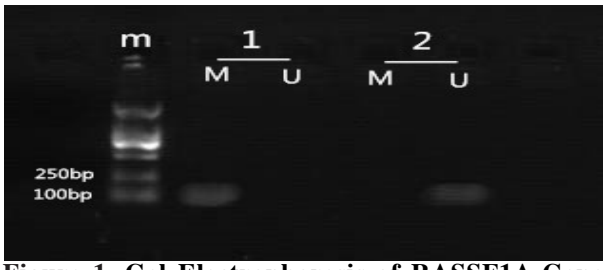

Figure 1. Gel Electrophoresis of RASSF1A Gene Methylation in Blood of NSCLC Patients and Healthy Controls. M:Methylated; U:Un-methylated; 1:NSCLC patients; 2:Healthy controls

Figure 2. Gel Electrophoresis of CDH13 Gene Methylation in Blood of NSCLC Patients and Healthy controls. M:Methylated; U:Un-methylated; 1:NSCLC patients; 2:Healthy controls

Table 1. Relationship between Promoter Methylation Status of Genes and Clinicopathologic Features

Clinicopathologi-c features	NO. of patients (n)	RASSF1A gene Methylation frequency(%)	*P*	CDH13 gene Methylation frequency(%)	*P*
Gender			0.207		0.288
Male	32	46.9(15/32)		59.4(19/32)	
Female	10	70.0(7/10)		40.0(4/10)	
Age/year			0.9		0.213
≤60	13	53.8(7/13)		69.2(9/13)	
>60	29	51.7(15/29)		48.3(14/29)	
Smoking status			0.352		0.559
Former	22	45.4(10/22)		59.1(13/22)	
Never	20	60(12/20)		50.0(10/20)	
Pathologic type			0.865		0.706
Squanmous carcinoma	10	50.0(5/10)		60.0(6/10)	
Adenocarcinoma	32	53.1(17/32)		53.1(17/32)	
TNM stage			0.319		0.803
I-II	6	33.3(2/6)		50.0(3/6)	
III-IV	36	55.6(20/36)		55.6(20/36)	

Table 2. The Relationship between the Methylation of RASSF1A and CDH13 Genes Promoter Region and the Effect of Chemotherapy

Group	Number(n)	RASSF1A			CDH13		
		Before chemotherapy(%)	After chemotherapy(%)	*P*	Before chemotherapy(%)	After chemotherapy(%)	*P*
PR	4	100.0(4/4)	0.0(0/4) *	0.008	100.0(4/4)	0.0(0/4)*	0.008
PSD	23	26.1(6/23)	4.3(1/23) *	0.042	34.8(8/23)	8.7(2/23) *	0.034
PD	15	80.0(12/15)	66.7(10/15)	0.417	80.0(12/15)	60.0(9.15)	0.24
Healthy controls	40	0.0(0/40)			0.0(0/40)		

*Ps: compared with PD group, P<0.05; compared with healthy controls, P>0.05

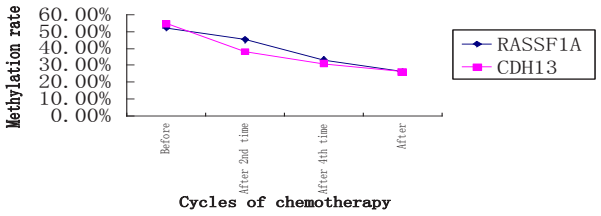

Figure 3. Changes of RASSF1A and CDH13 Genes Promoter Region Methylation in the Course of Chemotherapy

In 42 cases of NSCLC patients, the methylation rate of RASSF1A gene before the first time of chemotherapy, after the second time of chemotherapy, after the fourth time, after the sixth time of chemotherapy was 52.4%, 45.2%, 33.3%, 26.2%.It showed a gradual downward trend.Meanwhile, the methylation rate of CDH13 gene was 54.8%, 38.1%, 31.0%, 26.2%, showed a gradual downward trend too (Figure 3).

Discussion

With the influence of various environment, the morbidity and mortality of lung cancer have been growing fast in recent years.Lung cancer threatens people's health and the quality of human life (Nunomiya et al., 2014). Hyper-methylation of promoter region of tumor suppressor genes occurs, so that the gene silencing, and increase the risk of cancer (Shicheng et al., 2014). Chemotherapy is still the important method for the treatment of advanced non small cell lung cancer patients.However, due to individual differences in sensitivity to chemotherapy drugs, the effect of chemotherapy is not ideal, even lead to the progression of disease (Linda et al., 2009). Therefore, in order to prolong the survival of patients with NSCLC, to improve the quality of life of these patients, the application of individualized chemotherapy is particularly important (Pitroda et al., 2014). The methylation of RASSF1A and CDH13 genes occur frequently in NSCLC.

As an important tumor suppressor gene, RASSF1A involved in regulation of intracellular biological events, such as cell growth, differentiation and apoptosis, regulation of cell cycle, promote microtubule stability, to inhibit the occurrence and development of tumor (Vo et al., 2013). The hyper-methylation of RASSF1A gene promoter region, will inactivation this gene, RASSF1A lost the tumor suppressor function, induced lung cancer.

CDH13 is a special cadherin cell adhesion molecules. As the media of normal cells adhering to each other, cadherin cell adhesion molecule plays an important role in the establishment of cell polarity, by inducing cell cycle arrest, inhibiting tumor invasion and tumor amplification. Therefore, CDH13 gene plays an important role in inhibition of tumor development (Qiang et al., 2009). When the CDH13 gene promoter region occurs hyper-methylation, CDH13 gene silencing, increases the risk of cancer.

In this study, we evaluated the hyper-methylation status of tumor suppression genes associated with NSCLC, such as RASSF1A and CDH13. We showed that the methylation level of RASSF1A and CDH13 genes in NSCLC patients was much higher than that in health persons.The difference was statistically significant (p<0.05). This experiment selects the blood specimens from patients with lung cancer instead of tissue samples, which can easy to take samples. Not only reduce the suffering of patients but also provided the possibility for the detection of patients with advanced lung cancer which can not taken operation.

According to Response Evaluation Criteria in Solid Tumors (RECIST), there were 4 cases of partial remission (PR), 23 cases of stable disease (SD) and 15 cases of progressive disease (PD) .The hyper-methylation of RASSF1A and CDH13 genes in PR group and SD group decreased significantly (p<0.05), and was significantly different from that in PD group (p<0.05), but had no statistical difference compared with that in healthy controls (p>0.05). With chemotherapy, RASSF1A and CDH13 promoter region methylation rate in 42 cases of patients showed a declining trend. It showed the RASSF1A and CDH13 gene promoter region of patients who sensitive to the chemotherapy tent to demethylation. These tumor suppressor gene restored the activity, inhibited of tumor development, stabilized the disease (Yong et al., 2013). In contrast, the methylation level of RASSF1A and CDH13 gene promoter region of patients in PD group had no significant change. These patients with low sensitivity to chemotherapeutics drugs.Gene promoter region methylation belongs to epigenetic, is a reversible process. This study showed that after chemotherapy, RASSF1A, CDH13 gene promoter methylation level has prompted to the effect with chemotherapy drugs, can be the adjustment of the guide clinical individualized treatment.

In conclusion, we analyzed gene promoter methylation status by using MSP. We found that RASSF1A was hyper-methylated in 52.4%, CDH13 in 54.8% of the NSCLC samples. Showed the methylation of RASSF1A and CDH13 gene promoter has a higher incidence in NSCLC.The methylation level of RASSF1A and CDH13

gene promoter region can reflect on the drug sensitivity of tumor, adjust individualized treatment.

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