Promoter hypermethylation of cysteine dioxygenase type 1 in patients with non-small cell lung cancer

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Abstract. In the present study, promoter hypermethylation of cysteine dioxygenase type 1 (CDO1) was evaluated in non-small cell lung cancer (NSCLC) tissues to assess the value of CDO1 as a novel biomarker to improve the diagnosis of NSCLC. Tumor tissue samples and corresponding normal lung tissue samples from 42 patients with NSCLC were obtained at the Department of Thoracic Surgery, The Second Xiangya Hospital (Changsha, China). Conventional methylation-specific PCR (cMSP) and methylation-on-beads followed by quantitative methylation-specific PCR (MOB-qMSP) were used to analyze the tumor and normal lung tissue samples. Using these two methods, promoter DNA hypermethylation of the CDO1 gene was detected in 59.4 and 71.0% of tumor tissues of patients with NSCLC and in 9.4 and 0% of normal lung tissue, respectively. Compared with the rate of methylation in the well-differentiated NSCLC tissues (15.4 and 55.6%, respectively), the rate of CDO1 gene promoter methylation was higher in the poorly differentiated tissues (89.5 and 92.3%, respectively). Overall, it was demonstrated that the MOB-qMSP method had a higher positive detection rate for CDO1 hypermethylation compared with the cMSP method. In conclusion, CDO1 gene promoter hypermethylation was more frequently observed in NSCLC tissues compared with in normal lung tissues, and a high methylation frequency of the CDO1 gene in biopsy specimens of NSCLC was associated with the degree of differentiation.

Introduction

According to global cancer incidence and mortality statistics in 2012, lung cancer has remained the most common malignant tumor type worldwide and has accounted for the highest number of cancer-associated mortalities (1). In 2015, >4 million new cases of cancer and ~3 million cancer-associated mortalities occurred in China, including ~733,000 new lung cancer cases and 610,000 lung cancer-associated deaths (2). The average 5-year survival rate for all cases of lung cancer combined is just 16.8% (3), while it is 55% for localized lung cancers, 27% for regional metastasis and 4% for distant metastasis (4). However, the majority of patients with lung cancer are diagnosed at an intermediate or late stage and, thus, have missed the best opportunity to be cured (4). Therefore, to improve the overall survival rate of patients with lung cancer, it is important to focus on early diagnosis and treatment of lung cancer.

Recent advancements in the promising field of epigenetics have identified a strong association between cancer and epigenetics. DNA methylation is one of the earliest and most important types of epigenetic modification and has an important role in regulating growth, gene expression and genomic stability (5). In previous years, numerous studies have demonstrated that hypermethylation of CpG islands is associated with gene silencing and is an important molecular change during the development of cancer (6,7). In the early stages of cancer, even prior to imaging scans, abnormal DNA methylation may be detected. Therefore, analysis of DNA methylation may be a powerful tool for early diagnosis of lung cancer (8).

Herman *et al* (9) described conventional methylation-specific PCR (cMSP) that is able to rapidly assess the methylation status of the majority of CpG sites within a CpG island. This simple and sensitive method is currently the most widely worldwide. However, during cMSP, bisulfite modification-induced DNA damage and degradation can markedly reduce the sensitivity of methylation detection. As technology advances, quantitative PCR (qPCR) may replace conventional PCR and qPCR is more sensitive and specific compared with cMSP and has a reduced likelihood for operational contamination (10). Hulbert *et al* (11) modified the magnetic bead method to extract DNA, reduced the degradation of DNA during the bisulfite process and combined it with qPCR for detection with higher sensitivity and specificity. This process was named methylation-on-beads followed by quantitative methylation-specific PCR (MOB-qMSP).

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Cysteine dioxygenase type 1 (CDO1) is a mammalian non-heme iron enzyme, the major functions of which are regulation of cysteine levels and participation in metabolic pathways of compounds, including pyruvate and taurine (12). CDO1 is also a tumor suppressor enzyme and previous studies have demonstrated that CDO1 gene promoter methylation leads to silencing of this gene in the development of various types of cancer, including breast cancer (13), hepatocellular cancer (14), gallbladder cancer (15), colorectal cancer (16), gastric cancer (16), prostate cancer (17) and esophageal squamous cell carcinoma (18). In studies investigating lung cancer, methylation of the CDO1 promoter has been observed; Feng *et al* (19) confirmed the association of this with non-small cell lung cancer (NSCLC) and Hulbert *et al* (11) indicated that methylation of the CDO1 promoter has good sensitivity and specificity for detecting lung cancer. However, the vast majority of research available is based on European or American populations and only few studies have been performed among Asian patients. To the best of our knowledge, the present study was the first to use MOB-qMSP to detect CDO1 methylation in China, providing an important reference for the identification of CDO1 gene methylation in Asian patients with NSCLC.

Materials and methods

Patients and samples. In the present study, 42 patients diagnosed with NSCLC were included. The cohort included 30 males and 12 females with a mean age of 60 years (range, 33-83 years). All patients were enrolled from the Department of Thoracic Surgery, The Second Xiangya Hospital of Central South University (Changsha, China) between August 2017 and January 2018. Prior to surgery, all patients received assessments including CT scan of the chest, MRI scan of the brain, color Doppler ultrasound of the abdomen and a radionuclide bone scan. Surgical resection and pathological analyses were performed in all patients and staging was performed according to the most recent Tumor-Node-Metastasis (TNM) guidelines (20). Of the 42 tumor tissue samples, 25 were adenocarcinoma and 17 were squamous cell carcinoma. The matched adjacent normal lung tissues were confirmed by pathologists. The present study was approved by The Ethics Committee of the Second Xiangya Hospital (Changsha, China) and written informed consent was provided from the patients. Tumor and normal tissues were collected immediately after excision and stored at -80˚C.

DNA extraction and methylation analysis. Two methods were used for DNA extraction from tissue and bisulfite conversion (kits were used according to the manufacturer's protocol: i) cMSP including the use of a traditional DNA Purification kit (DP304 TIANamp Genomic DNAkit, Qiagen China Co., Ltd.) and an EZ DNA Methylation kit (D5005 EZ DNA Methylation-Gold kit, Zymo Research Corp.) and ii) MOB-qMSP, including the use of silica super magnetic beads (cat. no. MD1471 MagneSil KF; Paramagnetic Particles; Promega Corporation) in the process of DNA isolation and bisulfite conversion (11). The methylation and unmethylation CDO1 gene primers were designed using Methyl Primer Express 1.0 (Thermo Fisher Scientific, Inc.) and a TaqMan probe and β-actin primers were used (Table I).

DNA extraction from samples was performed using 30 mg of tissue and 40 μl Proteinase K (Qiagen China Co., Ltd.). After digestion and multiple elution, CT Conversion reagent was used for DNA bisulfite conversion to obtain the methylated DNA. cMSP was performed in a 50-μl PCR mixture consisting of 9.25 μl Premix Ex Taq (Takara Bio, Inc.), 1 μl forward primer, 1 μl reverse primer, 2 μl DNA and 36.75 μl water. The thermocycling conditions included 35 cycles of 98˚C for 10 sec, 60˚C for 30 sec and 72˚C for 60 sec. After amplification, each PCR product mixture was separated on a 2% agarose gel, added nucleic acid gel stain (cat. no. GG1301; 500μl GenGreen nucleic acid gel stain; Beijing Dingguo Changsheng Biotechnology Co., Ltd.) and visualized under Blue Light Gel Imager (wavelength, 440 nm; cat. no. G500312-0001; Sangon Biotech Co., Ltd.) in room temperature.

Modification technology-based magnetic beads were used for DNA extraction and bisulfite conversion (11). For DNA extraction, 30 mg of tissue was added to 300 μl Buffer AL and 40 μl Proteinase K (Qiagen China Co., Ltd.), followed by incubation (50˚C, overnight). After DNA bisulfite conversion on magnetic beads, the methylated DNA was obtained. MOB-qMSP was performed in 20-μl PCR mixture consisting of 10 μl Premix Ex Taq, 0.4 μl forward primer, 0.4 μl reverse primer, 0.8 μl probe, 0.4 μl ROX Reference Dye, 2 μl DNA and 6 μl water. An ABI StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for the reaction with the following conditions: 95˚C for 20 sec, 45 cycles at 95˚C for 1 sec and 60˚C for 20 sec. After amplification, the results were directly observed on the ABI StepOnePlus Real-Time PCR system.

Statistical analysis. All data were analyzed with SPSS 21.0 statistical software (IBM Corp.). The rate of DNA methylation was assessed using the χ^2 or Fisher's exact test. The two detection methods for CDO1 gene methylation were compared using McNemar's test. κ statistics were used to evaluate the concordance between the two methods. $P<0.05$ was considered to indicate statistically significant difference.

Results

Patient characteristics. The general data and pathological features of the 42 patients are summarized in Table II. Subjects with smoking indexes of ≥400 and <400 were equally represented. Among the pathological features, adenocarcinoma accounted for 62% of cases, while squamous cell carcinoma accounted for 38% of cases. According to the TNM staging system, 32 cases were in the early stages (I/II) and 10 cases were in the late stages (III/IV). Regarding the degree of tumor differentiation, there were 20 cases of high/moderate differentiation and 22 of poor differentiation.

In the present study, the promoter DNA methylation levels of the CDO1 gene in tissues from 42 patients with NSCLC were compared; in 32 cases, cMSP was used and MOB-qMSP was used in 31 cases. The results obtained with the two methods indicated that CDO1 methylation was significantly higher in tumor tissues compared with normal tissues. Of the 32 cases assessed using cMSP, CDO1 methylation was detected in 19 tumor tissues and in 3 normal lung tissues. Of the 31 cases assessed using MOB-qMSP, CDO1 methylation

Table I. PCR primers and TaqMan probes for conventional methylation-specific PCR and methylation-on-beads followed by quantitative methylation-specific PCR.

Gene	Primer sequence (5'-3')	Size, bp
CDO1, methylation		96
Forward	TTTTTGGGACGTCGGAGATAAC	
Reverse	CGAAAAAACCCTACGAACACG	
CDO1, unmethylation		102
Forward	GATTTTTGGGATGTTGGAGATAAT	
Reverse	AAAACAAAAAAACCCTACAAACACA	
CDO1		97
Forward	AGGCGGGGAGATTTTGCG	
Reverse	CCTAAAACGCCGAAAACAACG	
Probe	6FAM-CGGTTTACGCGTATATTTTCGGTTTT-TAMRA	
β-actin		103
Forward	TAGGGAGTATATAGGTTGGGGAAGTT	
Reverse	AACACACAATAACAAACACAAATTCAC	
Probe	6FAM-GTGGGGTGGTGATGGAGGAGGTTTAG-TAMRA	

CDO1, cysteine dioxygenase type 1.

Table II. Demographic characteristics of 42 patients with NSCLC.

Characteristic	n (%)
Age, years	
Mean ± standard deviation	60±9.51
Range	33-83
Sex	
Male	30 (71)
Female	12 (29)
Smoking index	
≥400	21 (50)
<400	21 (50)
Histology	
Adenocarcinoma	26 (62)
Squamous carcinoma	16 (38)
TNM stage	
I/II	32 (76)
III/IV	10 (24)
Differentiation	
High/moderate	20 (48)
Poor	22 (52)

Smoking index, average root number of smoking per day multiplied smoking years; NSCLC, non-small cell lung cancer; TNM, tumor-node-metastasis.

was detected in 22 tumor tissues and in 0 normal lung tissues (Fig. 1; Table III). Furthermore, 21 pairs of samples were assessed with cMSP and MOB-qMSP and the κ statistical result of 0.47 indicated a low concordance between the two methods for assessing promoter DNA methylation. In addition, MOB-qMSP had a higher positive detection rate for CDO1 hypermethylation compared with cMSP (Table IV).

Further analysis revealed that CDO1 gene hypermethylation was significantly different between highly/moderately and poorly differentiated tissues ($P<0.05$), with the CDO1 methylation rate being higher in poorly differentiated NSCLC tissue. However, no significant association was observed regarding any of the other characteristics assessed (Table V).

Discussion

In somatic cells, abnormal hypermethylation of the promoter region may lead to silencing and inactivation of tumor suppressor genes, which is one of the important molecular changes in tumor development. Increased levels of gene promoter methylation may contribute to the initiation and progression of NSCLC, indicating a close association between DNA methylation and NSCLC (7). CDO1 gene hypermethylation has been reported in various types of cancer and numerous studies have assessed CDO1 gene promoter methylation levels in lung cancer (21-23). CDO1 has been confirmed to be valuable for the diagnosis of NSCLC (24). Another study indicated that CDO1 may potentially serve as a molecular biomarker for multiple human cancers (25).

cMSP is one of the most widely used techniques to detect DNA methylation of a locus of interest; it is able to detect the methylation status rapidly and with high sensitivity (26). In the process of DNA methylation, high bisulfite concentrations lead to DNA degradation and inappropriate conversion (27), reducing the sensitivity of methylation detection. In the present study, MOB-qMSP was also used to detect the promoter methylation of the target gene whilst reducing the

Table III. Promoter hypermethylation detection using cMSP and MOB-qMSP in patients with NSCLC.

Method	Tumor tissues, n (%)	Normal tissues, n (%)	P-value
cMSP			
Methylation	19 (59.4)	3 (9.4)	<0.001
Unmethylation	13 (40.6)	29 (90.6)	
MOB-qMSP			
Methylation	22 (71.0)	0 (0.0)	<0.001
Unmethylation	9 (29.0)	31 (100.0)	

NSCLC, non-small cell lung cancer; cMSP, conventional methylation-specific PCR; MOB-qMSP, methylation-on-beads followed by quantitative methylation-specific PCR.

Table IV. Comparison of two methods to detect CDO1 gene hypermethylation.

	MOB-qPCR			
cMSP	Methylation	Unmethylation	P-value	κ
Methylation	8	0	0.041	0.471
Unmethylation	6	7		

CDO1, cysteine dioxygenase type 1; cMSP, conventional methylation-specific PCR; MOB-qMSP, methylation-on-beads followed by quantitative methylation-specific PCR.

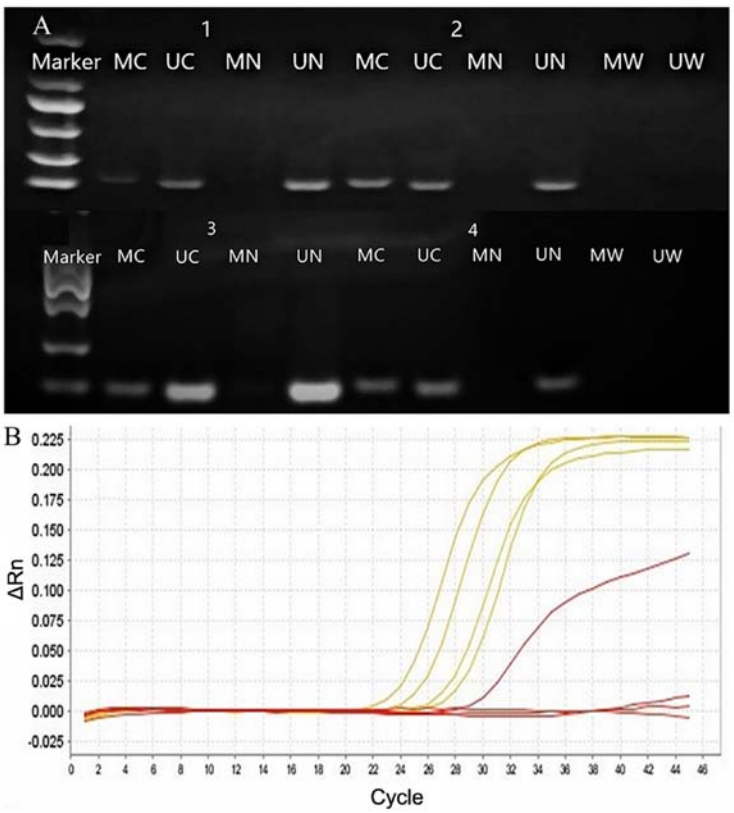

Figure 1. CDO1 gene promoter methylation in NSCLC was detected using gel electrophoresis and quantitative PCR. Numbers 1-4 represent four groups of corresponding tissue samples (tumor vs. normal lung tissue). The DL2000 DNA Marker contains six discrete DNA fragments ranging in size from 100 bp to 2 kb (100, 250, 500, 750, 1,000 and 2,000 bp). (A) Amplification of bisulfite-treated CDO1 from cancer and normal tissues. (B) β-actin, yellow curve; CDO1 gene methylation in tumor tissue, red curve above; CDO1 gene methylation in normal lung tissue, red curve at the bottom. NSCLC, non-small cell lung cancer; CDO1, cysteine dioxygenase type 1; MC or UC, methylation or unmethylation in tumor tissue; MN or UN, methylation or unmethylation in normal lung tissue; MW, methylation in water; UW, unmethylation in water. ΔRn, normalized reporter value, fluorescence emission of the product at each time point-fluorescence emission of the baseline.

Table V. Association between promoter hypermethylation of cysteine dioxygenase type 1 and clinical characteristics.

	cMSP				MOB-qMSP			
Characteristic	M	U	M%	P-value	M	U	M%	P- value
Age, years				0.149				0.054
≥60	13	5	72.2		14	2	87.5	
<60	6	8	42.9		8	7	53.3	
Sex				0.684				>0.999
Male	15	9	62.5		16	6	72.7	
Female	4	4	50.0		6	3	66.7	
Smoking index				0.149				0.113
≥400	12	4	75.0		13	2	86.7	
<400	7	9	43.8		9	7	56.3	
Histology				0.713				>0.999
LUAD	11	9	55.0		14	6	70.0	
LUSC	8	4	66.7		8	3	72.7	
TNM stage				0.703				>0.999
I/II	13	10	56.5		17	7	70.8	
III/IV	6	3	66.7		5	2	71.4	
Differentiation				<0.001				0.045
High/moderate	2	11	15.4		10	8	55.6	
Poor	17	2	89.5		12	1	92.3	

Smoking index, average root number of smoking per day multiplied smoking years; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; cMSP, conventional methylation-specific PCR; MOB-qMSP, methylation-on-beads followed by quantitative methylation-specific PCR; M, methylated; U, unmethylated; TNM, tumor-node-metastasis.

degradation of DNA during the bisulfite conversion process. This method has higher sensitivity and specificity compared with cMSP (11). In the present study, the results obtained with the two methods were consistent, as both methods demonstrated that the methylation rates of CDO1 in tumor tissues from patients with NSCLC were significantly higher compared with those in normal lung tissues (P<0.05). In addition, the two methods were applied to assess 21 samples in parallel and the results showed that MOB-qMSP had a higher positive detection rate compared with MSP (71.0 vs. 59.4%). This may be due to the method of combining DNA with magnetic beads prior to bisulfite conversion, which markedly reduces the degradation of DNA and increases the sensitivity of detection (11).

Lung cancer is an age-associated disease (28-30) and alterations in DNA methylation may be due to the harmful effects exerted by tobacco smoking (31). Levine *et al* (32) indicated that the association between DNA methylation and lung cancer is stronger among older patients and those who are current smokers. Breitling *et al* (33) suggested that DNA methylation serves a role in a variety of smoking-associated outcomes. Although no statistically significant differences were observed in the present study regarding the association between age or smoking on CD01 methylation levels, there still may be an association between these factors. The lack of significance in the present study may be due to the study limitations, such as a small sample size. High-quality studies with a rational design, including large-scale controlled trials, are required in the future.

In addition, in the present study, no significant difference in CDO1 gene methylation was identified between adenocarcinoma and squamous cell carcinoma, or between the early and advanced stage, which was consistent with the results of Ooki *et al* (23). However, it is noteworthy that there were statistically significant differences in CDO1 gene methylation between lung cancer tissues with high/moderate and poor differentiation. This suggested that methylation of the CDO1 gene may be an adjuvant marker for evaluating the degree of malignancy of NSCLC; however, further analysis is required to validate these results. For example, it would be valuable to further verify the correlation between CDO1 hypermethylation and NSCLC using *in vitro* experiments.

In the present study, CDO1 gene promoter methylation was more frequently observed in lung tumor tissues compared with normal lung tissues and this high methylation frequency of the CDO1 gene was associated with the degree of differentiation of NSCLC. As the degree of differentiation may be linked to the degree of malignancy, CDO1 promoter hypomethylation may have value in evaluating the prognosis of patients with NSCLC. However, further high-quality studies including a larger sample size and regular follow-ups are required to confirm these results.

In conclusion, the present study indicated that CDO1 promoter methylation has a role in NSCLC in the Chinese

population and that methylation levels are associated with the degree of differentiation of NSCLC. MOB-qMSP has higher sensitivity and specificity compared with cMSP and is more accurate method as it reduces DNA degradation. If confirmed in further studies, detection of CDO1 gene promoter methylation may be used as an adjunct to NSCLC diagnosis, as well as identification of pathological differentiation and guidance for the prognosis of patients with NSCLC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CC and FY designed the study concept. WY, XW and MS collected the data of patients. WY, YL, CC and BW analyzed the data. WY drafted the manuscript. CC and AH provided technical and material support. AH also participated in the acquisition of data. CC, FY and AH reviewed or revised of manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of The Second Xiangya Hospital (Changsha, China; approval no. 2014S006), and written informed consent was provided by all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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