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Methylation of multiple genes as a candidate biomarker in non-small cell lung cancer

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ABSTRACT

Aberrant DNA methylation is a common phenomenon in human cancer. The aims of this study were to investigate the methylation profiles of non-small cell lung cancer (NSCLC) in the Chinese population. Twenty tumor suppressor genes (TSGs) were determined of the methylation status using methylation-specific PCR in 78 paired NSCLC specimens and adjacent normal tissues, as well as in 110 Stage I/II NSCLC and 50 cancer-free plasmas. The results showed that, nine genes (APC, CDH13, KLK10, DLEC1, RASSF1A, EFEMP1, SFRP1, RARβ and $p16^{INK4A}$) demonstrated a significantly higher frequency of methylation in NSCLC compared with the normal tissues ($P \leqslant 0.001$), while the others (RUNX3, hMLH1, DAPK, BRCA1, $p14^{ARF}$, MGMT, NORE1A, FHIT, CMTM3, LSAMP and OPCML) showed relatively low sensitivity or specificity. Furthermore, methylation of multiple genes was more frequent in cancerous tissue, CpG island methylator phenotype positive (CIMP+) cases were detected in 65.38% of (51/78) NSCLC while only in 1.28% (1/78) of adjacent normal tissues ($P < 0.001$), and CIMP+ was associated with advanced stage ($P = 0.017$), lymphatic metastasis ($P = 0.001$) and adverse 2-year progression-free survival ($P = 0.027$). The nine genes validated in tissues also showed a significantly higher frequency of tumor-specific hypermethylation in NSCLC plasma, as compared with the cancer-free plasmas, and a 5-gene set (APC, RASSF1A, CDH13, KLK10 and DLEC1) achieved a sensitivity of 83.64% and a specificity of 74.0% for cancer diagnosis. Thus, the results indicated that methylated alteration of multiple genes plays an important role in NSCLC pathogenesis and a panel of candidate epigenetic biomarkers for NSCLC detection in the Chinese population was identified.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths in the world [1]. Non-small cell lung cancer (NSCLC) comprises the majority of lung cancer and has an increasing incidence and mortality in the last two decades in China. In addition to genetic factors such as mutations and susceptibility differences in polymorphism, the role of epigenetic abnormity that occur without changes in nucleotide sequence, has been implicated in lung cancer etiology [2]. DNA methylation is one of the most important epigenetic modifications of the genome and methylation-associated silencing affects many tumor suppressor genes (TSGs) in all existing cellular pathways [3], including apoptosis, cell adherence, DNA repair and cell-cycle control. Since the epigenetic alterations are early and frequent events occurred in carcinogenesis, the aberrant methylation could provide clues to the molecular mechanisms of NSCLC and serve as a valuable biomarker [4,5]. Moreover, it has been demonstrated that methylated DNA can be isolated from "remote media", such as blood, sputum, or bronchoalveolar lavage (BAL), making it well suited for non-invasive detection [6].

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Although multiple studies have attempted to assess the methylated gene profiles in NSCLC [6–10], there is not a large overlap between the top loci identified [6]. In addition, the majority of the previous reports were performed on Western populations. Genetic and environmental distinctions are known to exert a great impact on carcinogenesis and are believed to affect the epigenetic characteristics of tumors. Thus, we would like to investigate the methylation profiles of NSCLC in the Chinese population. In the present study, 20 TSGs potentially important in lung cancer were determined of the methylation status in 78 pairs of NSCLC and adjacent normal tissues, including adenomatous polyposis coli (APC), breast cancer 1 (BRCA1), cadherin 13 (CDH13), CKLF-like MARVEL transmembrane domain containing 3 (CMTM3), death-associated protein kinase 1 (DAPK), deleted in lung and esophageal cancer 1 (DLEC1), EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), fragile histidine triad gene (FHIT), mutL homolog 1 (hMLH1), KLK10 kallikrein-related peptidase 10 (KLK10), limbic system-associated membrane protein (LSAMP), O-6-methylguanine-DNA methyltransferase (MGMT), Ras association domain family member 5 (NORE1A), opioid binding protein/cell adhesion molecule-like (OPCML), $p14^{ARF}$, $p16^{INK4A}$, retinoic acid receptor β (RAR β), Ras association domain family member 1 (RASSF1A), runt-related transcription factor 3 (RUNX3), and secreted frizzled-related protein 1 (SFRP1). Most of these genes were selected based on the literature [6–23], for their highly sensitive and specific methylation in NSCLC, also, three new candidate TSGs (CMTM3, KLK10, LSAMP) were recruited to explore their potential role in NSCLC. Furthermore, an informative set of multiple loci validated in the tissues was analyzed in larger plasma samples, for identification of a non-invasive detection method using DNA methylation markers.

2. Materials and methods

2.1. Study cohort

A total of 78 paired NSCLC specimens and corresponding adjacent normal tissues (distance >5 cm or surgically resected margin) were obtained in Departments of Cardiothoracic Surgery, Jinling Hospital from November 2007 to June 2008. All specimens were immediately snapped frozen in liquid nitrogen and stored at −80 °C until use. Meanwhile, plasma samples were collected from 110 NSCLC patients with Stage I/II (partly matched to tissue specimens) in preoperative examination and 50 cancer-free controls of benign pulmonary diseases or healthy donors. Controls had no previous history of cancer and were frequency matched to cases on the basis of age (within 5 years), gender, and smoking status. The subjects were considered as "smoker" if they had smoked >100 cigarettes in their lifetime and "never smoker" if they responded negatively to the question. All diagnoses were based on pathological evidence. None received preoperative chemotherapy or radiation therapy. Histological classification was conducted according to the 1999 "Histological typing of lung and pleural tumors: third edition" of the WHO, and tumor stage was determined according to the 7th edition guideline suggested by the American Joint Committee on Cancer (AJCC) and the Union Internationale Contre le Cancer (UICC). A part of the patients were under a close follow-up observation for disease progression (recurrence, regional lymph or distant metastasis, death) at 1-month intervals during the first 1 postoperative year, and every 3 months thereafter. Ethical approval was obtained from the hospital and informed consent from all patients prior to sample collection.

2.2. Bisulphite treatment of DNA, methylation-specific polymerase chain reaction (MSP)

Genomic DNA from tissue and plasma was extracted using QIAamp DNA Mini Kit and QIAamp Blood Mini Kit (Qiagen, Hilden, Germany), respectively. After spectrophotometric quantitation, 1 μg of genomic DNA was bisulphite-treated with EZ-DNA methylation Gold Kit (Zymo Research, Orange, CA, USA), according to the manufacturer's instruction, and finally resuspended in 10 μl of TE buffer. For the plasma DNA, all of the 50 μl eluted DNA from the extraction step was bisulphite-treated. Polymerase chain reaction was run in a 25 μl volume containing 2 μl of modified DNA as a template, 10× Buffer, 0.15 mM dNTP, 0.1 mM each primer and 0.5U of Ex Taq Hot Start Version (Takara, Shiga, Japan). All the primer sequences and PCR amplification conditions have been described elsewhere [14–16,20–30] (Supplemental Table 1). Lymphocyte DNA, original or methylated *in vitro* by excessive CpG (SssI) methylase (New England Biolabs, Beverly, MA, USA), was used as unmethylation and methylation positive control (PC). Reagent control (RC) was reagent mixed with water instead of DNA. MSP products were verified by agarose gel electrophoresis. Attentions should be paid to some especial results, if the methylated and unmethylated bands were both detected in a sample, it was incomplete methylation (caused by the DNA impurity, calculated as methylation); if the methylated and unmethylated bands were both not detected in a sample (all of these cases occurred in plasma, calculated as unmethylation), it might be caused by the extremely low DNA content.

2.3. Statistics

The methylation frequency with a 95% confidence interval (95% CI; $\alpha = 0.05$) was calculated using SPSS software (version 13.0, SPSS Inc., Chicago, IL, USA). Difference in frequencies was assessed by Chi-square test or Fisher's exact test. The average number of methylated genes between tumor and normal specimens was compared by student's *t*-test. Clinicopathological correlations were analyzed by multivariate logistic regression. Progression-free survival (PFS) curves were calculated using the Kaplan–Meier method and compared by log-rank testing. Kappa agreement statistics was performed to assess the concordance of methylation between tumor tissue and plasma. The receiver operating characteristics (ROC) curve of both specificity and sensitivity of the sets was constructed as previously described [31].

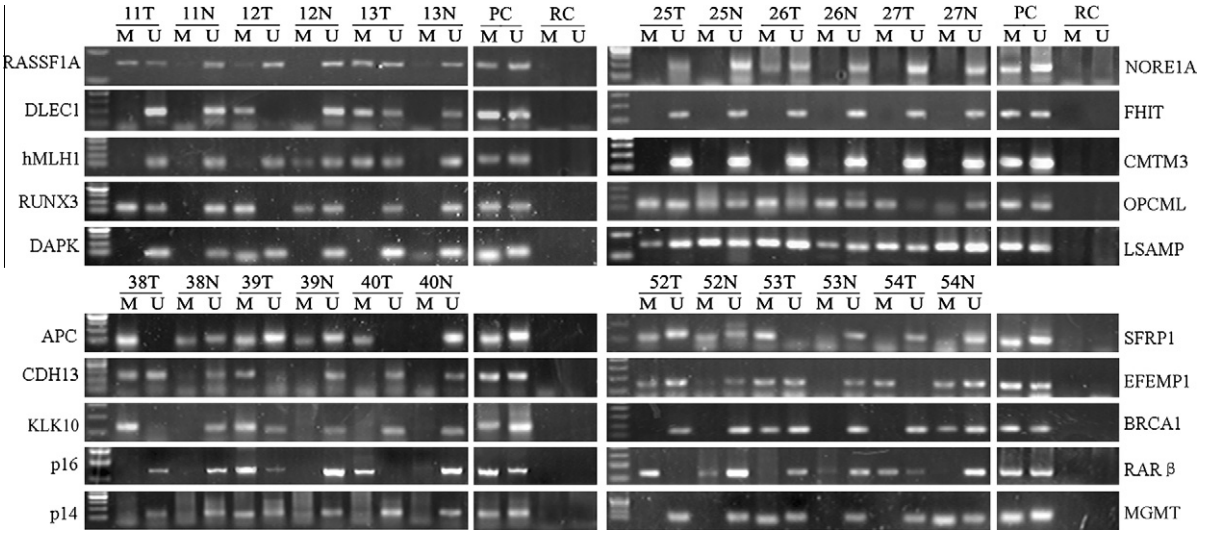

Fig. 1. Representative MSP profiles in matched NSCLC and adjacent normal lung tissues. M, methylation; U, unmethylation; T, tumor; N, normal lung tissues. Lymphocyte DNA, original or methylated *in vitro* by excessive CpG (SssI) methylase, was used as unmethylation and methylation positive control (PC). Reagent control (RC) was reagent mix with water instead DNA.

3. Results

3.1. Methylation profiles in primary tumor samples and corresponding normal lung tissues

We determined the methylation status of 20 candidate genes in 78 pairs of resected NSCLC and adjacent normal lung tissues by MSP (Fig. 1; Table 1). The age of the patients ranged from 35 to 80, with a median of 59, and the numbers of patients in stages I, II, III, IV were 25, 33, 19, 1 (brain metastases), respectively. Comparative analysis classified the 20 genes examined into three groups. The first group was the genes demonstrating frequent methylation in both cancerous and normal tissues (LSAMP, OPCML). The second group was that showing low frequencies of methylation in cancerous and normal tissues. Of which, four genes (BRCA1, $p14^{ARF}$, MGMT, NORE1A) were rarely methylated and two genes (FHIT, CMTM3) were never methylated in the evaluation set. The others belonged to the third group that showed significantly higher frequencies of methylation in tumor compared with normal tissues. Especially, the nine genes (APC, CDH13, KLK10, DLEC1, RASSF1A, EFEMP1, SFRP1, RAR β, $p16^{INK4A}$) were more statistically significant ($P \leqslant 0.001$).

Table 1
Methylation profiles in NSCLC tissue and matched normal tissue ($n = 78$).

Genes	Methylation frequencies		95% CI (P-value[A])
	Cancerous tissue n (%)	Normal tissue n (%)	
APC	44 (56.41)	10 (12.82)	3.952–15.596 (<0.001)
CDH13	38 (48.72)	8 (10.25)	3.533–19.559 (<0.001)
KLK10	36 (46.15)	5 (6.41)	4.560–34.342 (<0.001)
DLEC1	32 (41.03)	3 (3.85)	5.038–60.041 (<0.001)
RASSF1A	31 (39.74)	6 (7.69)	3.066–20.431 (<0.001)
EFEMP1	28 (35.90)	7 (8.97)	2.300–14.024 (<0.001)
SFRP1	25 (32.05)	6 (7.69)	2.169–14.769 (<0.001)
RAR β	24 (30.77)	7 (8.97)	1.809–11.236 (0.001)
$p16^{INK4A}$	20 (25.64)	5 (6.41)	1.782–14.227 (0.001)
RUNX3	18 (23.08)	8 (10.25)	1.066–6.465 (0.032)
hMLH1	13 (16.67)	4 (5.13)	1.149–11.911 (0.037)
DAPK	11 (14.10)	3 (3.85)	1.098–15.341 (0.047)
BRCA1	8 (10.25)	2 (2.56)	0.892–21.151 (0.098)
$p14^{ARF}$	5 (6.41)	1 (1.28)	0.602–46.227 (0.210)
MGMT	4 (5.13)	2 (2.56)	0.365–11.555 (0.681)
NORE1A[a]	1 (2.5)	0 (0)	0.928–1.025 (1.000)
FHIT[a]	0 (0)	0 (0)	–
CMTM3[a]	0 (0)	0 (0)	–
LSAMP[a]	35 (87.5)	30 (75)	0.718–7.587 (0.152)
OPCML[a]	28 (70)	22 (55)	0.761–4.788 (0.166)

[A] Chi-square test or Fisher's exact test.
[a] $n = 40$.

3.2. CpG island methylator phenotype (CIMP) in NSCLC and clinicopathological correlation

Excluding NORE1A, FHIT, CMTM3, LSAMP and OPCML, which were not tested in all of the specimens for their clearly low sensitivity or non-specificity, the distribution of CpG island methylation of the other 15 genes is shown in Fig. 2A. Methylation of multiple genes was more frequent in cancerous tissue. In addition, the average methylated-gene numbers were significantly lower in normal tissues (0.98 ± 0.90, means ± SD) than in NSCLC (4.32 ± 2.27, means ± SD) (Fig. 2B, $P < 0.001$).

High-level methylation of multiple genes, the so-called CpG island methylator phenotype (CIMP), was defined by the average number of methylated genes per sample according to the recent reports [32,33]. Thus, in this study, we classified it into two categories, including CIMP+ with four or more methylated genes and CIMP− with less than four methylated genes. The results showed that, methylation of at least one of the 15 genes was present in 74 of 78 (94.87%) cancerous tissues and 51 of 78 (65.38%) adjacent normal tissues ($P < 0.001$) (Fig. 2A). However, CIMP+ cases were detected in 65.38% (51/78) of cancerous tissues while only in 1.28% (1/78) of adjacent normal tissues ($P < 0.001$) (Fig. 2C). These results indicated CIMP+ status was an important characteristic of NSCLC tissues compared with normal tissues.

Then the relationship between CIMP status of the 15 genes and clinicopathological features in NSCLC was analyzed by logistic regression. As shown in Table 2, CIMP+ samples were preferentially observed in advanced stage (33 of 58, 56.90% in I/II stage and 18 of 20, 90.0% in III/IV stage; $P = 0.017$) or harboring lymphatic metastasis (24 of 44, 54.55% in N_0 and 27 of 34, 79.41% in $N_1/N_2/N_3$; $P = 0.001$). However, there was no difference among CIMP status in gender, age, histological type, grade, tumor size or smoking habit. Furthermore, in the 64 NSCLC patients with reliable follow-up data, 46 cases progressed within 2 years after surgery (recurrence, 14; regional lymph and/or distant metastasis, 26; death, 6) and 18 cases kept stable. Survival curves are shown in Fig. 3; the 2 year-PFS in CIMP+ patients was inferior to CIMP− patients (mean 13.8 months (95%CI: 11.8–15.9) *vs.* 17.8 months (95%CI: 15.2–20.4), $P = 0.027$).

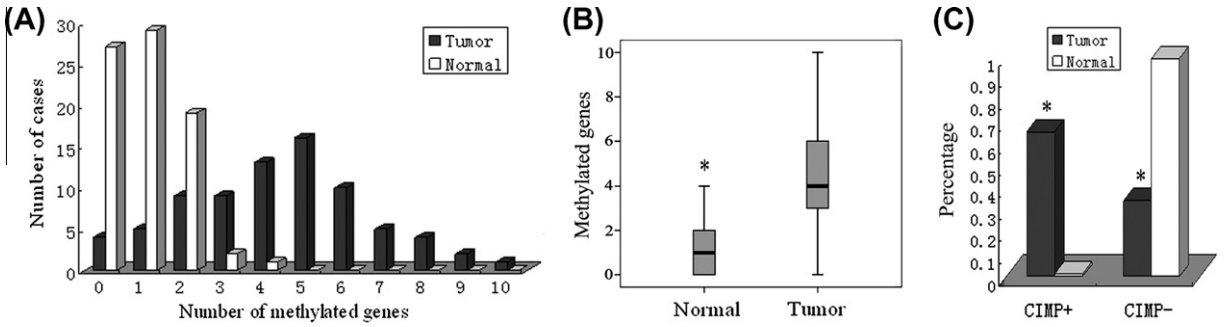

Fig. 2. Histogram of 15 methylated genes distribution. (A) Number of methylated genes per sample in 78 paired NSCLC and normal tissues. (B) Average methylated-gene numbers is significantly different between NSCLC and adjacent normal tissues. *$P < 0.001$. (C) Frequency of the CpG island methylator phenotype (CIMP) in NSCLC and adjacent normal tissues, CIMP+ (more than three methylated genes); CIMP− (3 or 3 less methylated genes). CIMP+ was observed in 65.38% (51/78) of NSCLC, and 1.28% (1/78) of normal lung tissues. CIMP− was observed in 34.62% (27/78) of NSCLC, and 98.72% (77/78) of normal lung tissues. *$P < 0.001$.

Table 2
Logistic regression analysis of risk factors associated with CIMP + NSCLC patients ($n = 78$).

Characteristics	Category	CIMP+	RR (95% CI)	*P*-value
Gender	Male ($n = 58$)	37	1.086 (0.193–6.115)	0.925
	Female ($n = 20$)	14		
Age	<60 ($n = 22$)	12	1.284 (0.296–5.572)	0.739
	⩾60 ($n = 56$)	39		
Histological type	Squamous cancer ($n = 36$)	25	0.449 (0.118–1.715)	0.242
	Adenocarcinoma ($n = 30$)	18	(Squamous *vs.* non-squamous cancer)	
	Others ($n = 12$)	8		
Differentiation	Well ($n = 13$)	6	0.666 (0.185–5.457)	0.702
	Moderate ($n = 46$)	30	(Well/moderate *vs.* poor/undifferentiated)	
	Poor/undifferentiated ($n = 19$)	15		
Tumor size	⩽3 cm ($n = 25$)	14	1.026 (0.255–4.124)	0.971
	>3 cm ($n = 53$)	37		
Stage	I/II ($n = 58$)	33	8.419 (1.472–48.157)	0.017*
	III/IV ($n = 20$)	18		
Lymph metastasis	N_0 ($n = 44$)	24	25.047 (3.966–158.168)	0.001*
	$N_1/N_2/N_3$ ($n = 34$)	27		
Smoking habit	Never ($n = 29$)	17	0.627 (0.170–2.313)	0.483
	Smoker ($n = 49$)	34		

CIMP+: CpG island methylator phenotype positive; RR: Relative risk; 95% CI: 95% confidence interval.
* $P < 0.05$.

3.3. Evaluation of a 9-gene set in plasma for sensitive/specific detection of NSCLC

Based on their conspicuous sensitivity/specificity observed in tissue samples, an informative set of 9-gene (APC, CDH13, KLK10, DLEC1, RASSF1A, EFEMP1, SFRP1, RAR β and $p16^{INK4A}$) was selected for further analysis in plasma samples of 110 early stage NSCLC and 50 cancer-free controls. As shown in Table 3, all of the nine genes demonstrated significantly higher frequencies of methylation in cancerous plasmas compared with the cancer-free controls. The top five genes remained in the highly sensitivity category: APC (47.27%, 52/110), RASSF1A (36.36%, 40/110), CDH13 (33.64%, 37/110), KLK10 (29.09%, 32/110) and DLEC1 (25.45%, 28/110). Furthermore, by using comparative analysis of 58 stage I/II NSCLC patients with matched tumor tissues and plasma samples, we found the concordance of the 9-gene methylation status in plasma samples and tumor tissues was satisfactory (Table 4).

Next, we evaluated the detectability of the nine genes' combinational analysis. In this study, "true positive" (TP) is defined as a NSCLC case with at least one gene methylated, whereas "false negative" (FN) is a NSCLC case with no gene methylated; "false positive" (FP) is defined as a non-cancer case with at least one gene methylated, whereas "true negative" (TN) is a non-cancer case with no genes methylated. Both sensitivity (%), TP/(TP + FN) and specificity (%), TN/(TN + FP) of each gene sets were calculated (Table 5). The sensitivity and specificity were 47.27% (52/110) and 90.0% (45/50) for APC alone; 61.82% (68/110), 82.0% (41/50) for APC and RASSF1A; and 71.82% (79/110), 80.0% (40/50) for APC, RASSF1A, and CDH13 together. An inclusion of KLK10 in the 4-gene set improved the sensitivity to 79.09%, but reduced the specificity to 76.0%. Further recruitment of DLEC1 or SFRP1 into the 5-gene set slightly improved the sensitivity to 83.64%, while compromising the specificity to 74.0% and 72.0%, respectively. Further addition to a six or more gene set would not efficiently improve the detectability for the relatively poor specificities (Table 5).

4. Discussion

DNA methylation at CpG islands within or near the promoter regions has been verified as an important epigenetic regulatory mechanism of gene expression. Hypermethylation will lead to the change of chromatin framework,

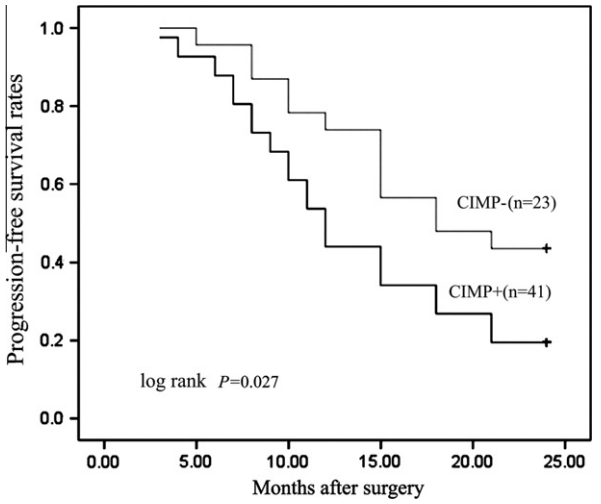

Fig. 3. Kaplan–Meier analysis of correlation of CIMP status and progression-free survival (PFS) among 64 NSCLC patients with follow-up data. The 2-year PFS in patients with CIMP+ was inferior to that with CIMP– (mean 13.8 months *vs.* 17.8 months, $P = 0.027$).

Table 3
Methylation profiles in plasma of Stage I/II NSCLC and cancer-free controls.

Genes	Cancerous plasma cases (%), $n = 110$	Non-cancer plasma cases (%), $n = 50$	P-value[a]
APC	52 (47.27)	5 (10.0)	<0.001
CDH13	37 (33.64)	2 (4.0)	<0.001
KLK10	32 (29.09)	2 (4.0)	<0.001
DLEC1	28 (25.45)	1 (2.0)	<0.001
RASSF1A	40 (36.36)	4 (8.0)	<0.001
EFEMP1	24 (21.82)	3 (6.0)	0.012
SFRP1	26 (23.64)	2 (4.0)	0.002
RAR β	22 (20.0)	3 (6.0)	0.033
p16INK4A	25 (22.73)	4 (8.0)	0.027

[a] Chi-square test or Fisher's exact test.

Table 4
Concordance of DNA methylation pattern in plasma and tumor DNA.

Plasma sample		NSCLC tissue		Kappa value
		M	U	
APC	M	24	3	0.758
	U	4	27	
CDH13	M	20	2	0.784
	U	4	32	
KLK10	M	17	1	0.772
	U	5	35	
DLEC1	M	15	0	0.835
	U	4	39	
RASSF1A	M	18	2	0.847
	U	2	36	
EFEMP1	M	15	1	0.834
	U	3	39	
SFRP1	M	13	1	0.820
	U	3	41	
RAR β	M	13	0	0.906
	U	2	43	
$p16^{INK4A}$	M	12	1	0.812
	U	3	42	

M, methylated; U, unmethylated.

which represses transcription directly, by inhibiting the binding of specific transcription factors, and indirectly, by recruiting methyl-CpG-binding proteins, thus leading to the down-regulation or silence of TSGs and then contributing to cancer initiation and progression [34]. Clinical investigating associations between abnormal methylation and cancer diagnosis or prognosis have been conducted in various cancers [5]. For example, Brock et al. [8] reported that methylation of the promoter region of p16, CDH13, RASSF1A, and APC in patients with stage I NSCLC treated with curative intent by means of surgery was associated with early recurrence. We currently investigate the methylation profiles of NSCLC, using a panel of 20 genes with presumed or known roles in various cellular functions, to further confirm their functional importance and identify candidate epigenetic biomarkers for NSCLC in Chinese population.

Many methods have been used for the detection of DNA methylation, but the most common procedure is bisulfite modification followed by methylation-specific PCR. Using this method, we analyzed the methylation frequencies in NSCLC compared with the adjacent normal tissues, and found that a group of 12 genes (APC, CDH13, KLK10, DLEC1, RASSF1A, EFEMP1, SFRP1, RAR β, $p16^{INK4A}$, RUNX3, hMLH1 and DAPK) showed a high sensitivity and cancerous specificity, suggesting these genes play an important role in NSCLC pathogenesis. Except for the KLK10 gene, all the others have been previously reported to be high frequent and functionally important in the methylation state in lung cancer, involved in cell signaling (APC, SFRP1, RUNX3), cell-cycle control ($p16^{INK4A}$, RASSF1A), cell proliferation/differentiation (DLEC1, RARβ), cell adhesion, invasion/metastasis (CDH13, EFEMP1), apoptosis (DAPK) and DNA repair (hMLH1) [8–20]. KLK10 is a member of the human tissue kallikrein family of secreted serine proteases, exon 3 methylation as a basis for tumor-specific loss of KLK10 expression has been found in breast, ovarian, prostate, gastric and hepatocellular cancers [29,35,36]. Our results implied the hypermethylated silence of KLK10 also existed in NSCLC [37], which further clarified its physiological role in carcinogenesis.

The eight remaining genes showed low sensitivity or specificity of methylation in NSCLC. Of which, cell cycle ($p14^{ARF}$, NORE1A), cell adhesion (OPCML) and DNA repair (BRCA1, MGMT, FHIT)-related genes were considered as classic TSGs and played important roles in NSCLC [17–23]. The contradictions between our results and previous researches may be due to sample heterogeneity, including race, area and bias of sample collection. It is of note that, the new candidate TSG CMTM3, located at 16q22.1, has been reported to be silenced by aberrant promoter methylation in many carcinomas (gastric, breast, nasopharyngeal, esophageal and colon) and inhibiting of cancer cell growth through inducing apoptosis [27], but was never methylated in 40 cases of NSCLC tested in our study. In contrast, LSAMP gene, a member of IgLONs immunoglobulin superfamily involved in modification of neurite outgrowth and

Table 5
Representative receiver operating characteristics (ROC) of the informative sets for NSCLC detection.

No.	Gene sets	TP/FN	FP/TN	Sensitivity (%), TP/(TP + FN)	Specificity (%), TN/(TN + FP)
1	APC	52/58	5/45	47.27	90.0
2	APC, RASSF1A	68/42	9/41	61.82	82.0
3	APC, RASSF1A, CDH13	79/31	10/40	71.82	80.0
4	APC, RASSF1A, CDH13, KLK10	87/23	12/38	79.09	76.0
5	APC, RASSF1A, CDH13, KLK10, DLEC1	92/18	13/37	83.64	74.0
(5)	APC, RASSF1A, CDH13, KLK10, SFRP1	92/18	14/36	83.64	72.0
(5)	APC, RASSF1A, CDH13, KLK10, $p16^{INK4A}$	91/19	15/35	82.73	70.0
6	APC, RASSF1A, CDH13, KLK10, DLEC1, SFRP1	95/15	15/35	86.36	70.0
(6)	APC, RASSF1A, CDH13, KLK10, DLEC1, RAR β	93/17	15/35	84.55	70.0
(6)	APC, RASSF1A, CDH13, KLK10, DLEC1, EFEMP1	94/16	15/35	85.45	70.0
(6)	APC, RASSF1A, CDH13, KLK10, DLEC1, $p16^{INK4A}$	95/15	16/34	86.36	68.0
7	APC, RASSF1A, CDH13, KLK10, DLEC1, SFRP1, $p16^{INK4A}$	97/13	17/33	88.18	66.0
(7)	APC, RASSF1A, CDH13, KLK10, DLEC1, SFRP1, EFEMP1	97/13	17/33	88.18	66.0
(7)	APC, RASSF1A, CDH13, KLK10, DLEC1, $p16^{INK4A}$, EFEMP1	97/13	18/32	88.18	64.0
8	APC, RASSF1A, CDH13, KLK10, DLEC1, SFRP1, $p16^{INK4A}$, EFEMP1	99/11	19/31	90.0	62.0
9	APC, RASSF1A, CDH13, KLK10, DLEC1, SFRP1, $p16^{INK4A}$, EFEMP1, RAR β	99/11	21/29	90.0	58.0

TP, true positive; FN, false negative; FP, false positive; TN, true negative.
No. was number of the genes in each set.

cell–cell recognition, which was silenced due to promoter methylation in a considerable number of clear cell renal cell carcinoma [38] and osteosarcoma [28], showed a high frequency of methylation in both NSCLC and adjacent normal lung tissues. It is the first report of methylation of these two genes in NSCLC, although no statistical significance has been proven, maybe further researches are needed.

Originally, CpG island methylator phenotype (CIMP) is a subset of colorectal carcinoma (CRC) characterized by synchronous hypermethylation of multiple promoter CpG island loci and peculiar clinicopathologic findings, such as close association with microsatellite instability (MSI), proximal tumor location, female preponderance, low frequency of mutation of the oncogene KRAS and poor clinical outcomes [39,40]. Following researches confirmed that a form of CIMP exists in other solid tumors including NSCLC [32,33,41]. Despite the slight differences in definition, CIMP is always characterized by high degrees of methylation. This suggests that CIMP is not restricted to specific tumor types, but rather a general phenomenon that results in concordant aberrant DNA methylation and nvolves different genes depending on tumor type [40]. In this study, excluding NORE1A, FHIT, CMTM3, LSAMP and OPCML, which were not examined in all of the 78 paired specimens, we analyzed the CIMP status of NSCLC using a panel of the other 15 genes. The results showed that multiple genes' methylation was more frequent in cancerous tissue. Methylation of at least one of the 15 genes was present in 94.87% of cancerous tissues and 65.38% of adjacent normal tissue. However, CIMP+ cases (more than three of the 15 genes) were detected in 65.38% of cancerous tissues while only 1.28% of adjacent normal tissues. CIMP+ status was an important characteristic of NSCLC tissues compared with normal tissues, and might be a crucial step in carcinogenesis. Furthermore, CIMP+ cases was closely associated with advanced stage, lymph metastasis, and adverse 2-year PFS, implying that epigenetic alterations are also involved in the progression of NSCLC and CIMP+ status might be a promising tool for classification or prognosis of NSCLC.

It is well known that double-strand DNA fragments often appear in considerable quantities in the serum or plasma of cancer patients, which maybe arise from lysis of tumor cells [42]. Enriched DNA makes it possible to detect tumor-specific DNA alteration in the peripheral blood of patients. As to identify valuable markers for early diagnosis, an informative 9-gene set (APC, CDH13, KLK10, DLEC1, RASSF1A, EFEMP1, SFRP1, RAR β and $p16^{INK4A}$) validated in tissues was selected for further analysis in plasma samples of Stage I/II NSCLC. All of the nine genes were found methylation at a frequency no less than 20% of NSCLC plasmas and no more than 10% of non-cancerous controls, exhibiting a high frequency of tumor-specific manner. Furthermore, comparative analysis of 58 stage I/II NSCLC patients with matched tumor tissues and plasma samples demonstrated that the concordance of the 9-gene methylation status in plasma samples and tumor tissues was satisfactory, which further implies their possible application value. Assaying of a single gene screened no more than 47.27% (APC) of the early stage cancer, multiple loci combinations with more sensitivity were necessary. The tripartite combination of APC, RASSF1A and CDH13 methylation showed a sensitivity of 71.82% and a specificity of 80%; an inclusion of KLK10 and DLEC1 into the 4 or 5-gene set improved the sensitivity to 79.09% and 83.64%, respectively, while the specificity was reduced to 76% and 74%. We noted that further recruitments would not improve the detectability but account for relatively poor specificities.

In conclusion, we confirm a multiple genes' methylated alteration plays an important role in NSCLC pathogenesis, and identify a panel of candidate epigenetic biomarkers for NSCLC detection in Chinese population. The clinical utility of the suggested set(s) from this study remains to be validated in large cohorts in the near future and may be further improved by addition of other biomarkers.

Conflict of interest

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2010.12.011.

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