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Promoter Methylation of the *CADM*1 and 4.1*B* Genes Occurs Independently of the *EGFR* or the *KRAS*2 Mutation in Non-Small Cell Lung Cancer

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Abstract

Objective: Targeting mutated EGFR by EGFR-tyrosine kinase inhibitors (EGFR-TKI) is a potent approach to a subset of non-small cell lung cancer (NSCLC). However, the response to EGFR-TKI varies in individual cases even among tumors carrying the same *EGFR* mutation, suggesting the involvement of modifying factors. To characterize possible modifiers, we examined mutation state of the *EGFR* and the *KRAS* genes in Japanese NSCLC and compared them with the methylation state of lung tumor suppressors, the *CADM*1 and 4.1*B*, whose products have potentials to modify the functions of EGFR or KRAS. Materials and methods: A total of 103 Japanese NSCLC and 11 NSCLC cell lines were examined. Genomic DNA of exons 18 - 21 of the *EGFR* and exons 1 and 2 of the *KRAS* were amplified by polymerase chain reaction (PCR), followed by single-strand conformation polymorphism analysis and direct sequencing. Methylation status of gene promoters in NSCLC cells were examined by methylation-specific PCR. Results: Mutations of the *EGFR* and *KRAS* were detected mutually exclusively in 27 and 11 out of 103 NSCLC cases, respectively. *EGFR* mutations were observed exclusively in adenocarcinoma (27 of 69, 41%) and preferentially in tumors from female and non-smokers ($p < 0.00001$). Eight (30%) and 12 (44%) of 27 tumors carrying mutated *EGFR* and 4 (36%) and 8 (73%) of 11 tumors carrying mutated *KRAS* showed methylation of the *CADM*1 and 4.1*B*, respectively. *EGFR*-mutated tumors with methylation of either *CADM*1 or 4.1*B* showed more malignant features than those with unmethylated *CADM*1 and 4.1*B* ($p < 0.05$). Con-

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clusion: Methylation state of the *CADM*1 and 4.1*B* are independent of the mutation status of the *EGFR* or *KRAS* but play roles in the malignant progression of NSCLC. Integration of epigenetic information would be useful for identifying possible modifiers to predict the response or recurrence of lung adenocarcinoma to the EGFR-TKI therapy.

Keywords

Non-Small Cell Lung Cancer, *EGFR* Mutation, *KRAS* Mutation, *CADM*1 Methylation, 4.1*B* Methylation

1. Introduction

Lung cancer is a leading cause of cancer death in developed countries, including USA and Japan, and about 80% - 85% of them are histologically classified as non-small cell lung cancer (NSCLC) [1]. NSCLC develops and progresses with multiple genetic and epigenetic alterations. Mutations of the *EGFR* and the *KRAS* oncogenes and fusion of the *EML*4-*ALK* gene are representative driver mutations occurred in the early stage of NSCLC. On the other hand, mutations of the tumor suppressor *TP*53, *RB*1and *CDKN*2*A* genes, as well as hypermethylation of the tumor suppressor *CADM*1 and 4.1*B* genes, occur in the late stage of tumorigenesis and play important roles in malignant progression, including invasion and metastasis of NSCLC [2]. It is important, therefore, to know whether these genetic and epigenetic alterations accumulate in the same cancer cells and participate synergistically in the malignant progression of tumors or some of the alterations occur mutually exclusively and play roles in independent pathway of lung tumorigenesis.

Recently, several molecular targeting drugs have been developed and clinically used for the treatment of NSCLC, especially for lung adenocarcinoma. EGFR-tyrosine kinase inhibitors (EGFR-TKI), including Gefitinib and Erlotinib, are established for the treatment of lung adenocarcinomas carrying the specific mutations of the *EGFR* gene [3]. ALK-tyrosine kinase inhibitor, Crizotinib, is also effective to a subset of lung adenocarcinoma expressing the fusion gene, *EML*4-*ALK* [4]. On the other hand, Cetuximab, a monoclonal antibody against KRAS protein, is widely used for the treatment of a subset of colorectal cancer with wild-type *KRAS* gene [5], although treatment by Cetuximab or other RAS-targeting drugs has not been established in NSCLC over-expressing KRAS. Targeting to such specific driver oncogenes is getting a major approach to develop new molecular targeting drugs against cancer. However, the responses of tumors to the relevant molecular targeting drugs show variation in individual cases even among tumors expressing the same specific markers, suggesting that additional factors would modulate the responsiveness to the relevant drugs [6].

We have previously identified a tumor suppressor gene, the *CADM1/TSLC*1, on chromosome 11q23, which encodes a transmembrane glycoprotein that is involved in adhesion in various epithelial cells [7]. We have subsequently identified 4.1*B* as a direct binding protein of CADM1 in its cytoplasmic domain [8]. 4.1*B* is an actin-binding protein which transmits the signals of cell adhesion to the cytoskeletal reorganization [9]. We have previously reported that the promoter of the *CADM*1 and 4.1*B* genes are methylated in 45 (44%) and 59 (57%) of 103 NSCLCs, respectively, both of which provide indicators of poor prognosis of lung adenocarcinoma [10] [11]. Promoter methylation as well as loss of expression of CADM1 and 4.1*B* is also observed in various cancers [12]-[14], suggesting that CADM1-4.1*B* is an important tumor suppressor cascade of NSCLC [15]-[17].

Interestingly, it has been reported that CADM1 protein associates with a member of EGFR family proteins, ERBB3 [18], suggesting that CADM1 might possibly modify EGFR pathway. It is also noteworthy that loss of CADM1 expression was specifically reported in an invasive model of murine lung adenocarcinoma in the mutated *KRAS* knock-in mice, suggesting that loss of CADM1 might enhance the malignant features of tumor cells caused by mutated *KRAS* [19]. However, correlation of mutated *EGFR* or *KRAS* and inactivation of *CADM*1 or 4.1*B* in NSCLC have not been reported yet.

Here, we examined the mutation state of the *EGFR* and *KRAS* genes in a series of 103 NSCLC tumors as well as 11 NSCLC cell lines and compared to our previous results of the methylation state of the *CADM*1 and 4.1*B* genes. Then, we analyzed the clinico-pathological features of the tumors carrying these genetic and epigenetic alterations to investigate whether methylation of *CADM*1 or 4.1*B* could serve as modifiers of the malignant phenotype of NSCLC.

2. Materials and Methods

2.1. Surgical Materials and Cell Lines

A total of 103 Japanese subjects (74 men and 29 women; median age: 67 years old) provided informed, written consent in accordance with the ethics board of the University of Tokyo (ID: 20-36-0918) and were included in this study. All subjects were surgically resected and histologically diagnosed as having NSCLC at the National Cancer Research Center Hospital, Japan. After surgical removal, all samples were immediately frozen and stored at −135˚C. NSCLC cell lines, Calu-3, NCI-H441, NCI-H522, SK-LU-1, and NCI-H596 were obtained from the American Type Culture Collection; ABC-1, RERF-LC-MS, RERF-LC-OK and VMRC-LCD were from the Health Science Research Resources Bank, Japan; and A549 and PC-14 cells were from RIKEN Cell Bank, Japan. These cells were cultured according to the supplier's recommendation.

2.2. Single-Strand Conformational Polymorphism (SSCP) Analysis

Genomic DNA was extracted using the AllPrep Mini Kit (Qiagen, Madison, WI, USA). For SSCP analysis, the DNA fragments corresponding to exons 18 - 21 of the *EGFR* and exons 1 and 2 of the *KRAS* as well as their flanking sequences were amplified by PCR using KOD FX polymerase (TOYOBO, Osaka, Japan) and a specific pair of primers [20]. Primers used for PCR were shown in **Table A1**, where one of the primers in each pair was end-labeled with Texas Red. The PCR product was diluted 10 times with a loading buffer (90% deionized formamide, 0.01% New Fuchsin and 10 mM EDTA), heat-denatured for 3 min at 95˚C, cooled on ice for 3 min and then loaded onto the gel (0.5xMDE Gel Solution; BMS, Rockland, ME). Electrophoresis was carried out for 120 min at 20˚C using SF5200 (Hitachi Electronics Engineering, Tokyo, Japan) with cooling systems. The results were analyzed using a DNA Fragment Analyzed (Hitachi Electronics Engineering).

2.3. Sequencing Analysis

PCR products were separated by electrophoresis on agarose gel, and fragments were cut from the gel and purified by Wizard SV Gel and PCR Clean-Up System (Promega. Madison, WI, USA). Sequencing was conducted by reactions with Applied Biosystems' BigDye 3.1 kit on a 3130xl DNA sequencer. Sequence results were analyzed using Lasergene (DNASTAR, Madison, WI, USA), and statistical analysis was done using SPSS software. The significance level was set at $p < 0.05$.

2.4 Methylation-Specific PCR

Genomic DNA was extracted from cells using a Wizard SV Genomic DNA Purification System (Promega) according to the manufacturer's instructions. For methylation analysis, 500 ng of genomic DNA was subjected to bisulfite conversion using a MethylCode Bisulfite Conversion Kit (Invitrogen, Carlsbad, CA, USA). Then, the bisulfate-converted DNA (50 ng) was subjected to PCR to amplify methylated and unmethylated DNA of cancer related genes, including *RASSF*1*A*, *CDKN*2*A*, *APC*, *CDH*1, *GATA*-4 and *GATA*-5, by Platinum Taq DNA Polymerase (Invitrogen). Pairs of primers specific to methylated and unmethylated DNA were as listed in **Table A1** [21]-[25]. Methylation state of the *CADM*1 and 4.1*B* genes in 11 cells were analyzed by bisulfite SSCP as described previously [10] [11].

2.5. Statistical Analysis

Fisher exact test were used for categorical variables. Stat View 5.0 (SAS Institute, Cary, NC) was used for the analysis. Differences with P value of less than 0.05 were considered significant.

3. Results

3.1. Mutation of the EGFR and the KRAS Genes in Japanese NSCLC

SSCP analysis followed by sequencing analysis of exons 18 - 21 and their flanking regions of the *EGFR* gene idenified 27 mutations (26%) out of 103 HNSCC cases (**Figure 1** and **Figure 2**, **Table 1**). These include 14 in-frame deletions of 15 nucleotides corresponding to codon 746 to codon 750, nine T to G transversions at codon 858 (leucine to arginine), and four G to T transversions at codon 719 (glycine to cysteine), all of which are

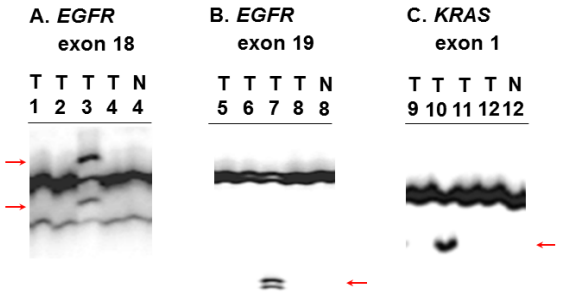

Figure 1. Single-strand conformation polymorphism (SSCP) analysis of exon 18 (A) and exon 19 (B) of the *EGFR* and exon 1 of the *KRAS* genes. T1-T12 are DNAs from NSCLC, while N4, N8, N12 are DNAs from non-cancerous lung tissues from the same patients. T3, T7 and T10 showed shift of the mobility caused by nucleotide substitutions indicated by red arrow. Primer pairs used for amplification of exons 18 - 21 of the *EGFR* gene and exons 1 and 2 of the *KRAS* gene are listed in **Table A1**. Red arrows indicate bands with mobility shift caused by higher structural changes due to nucleotide substitutions or deletions.

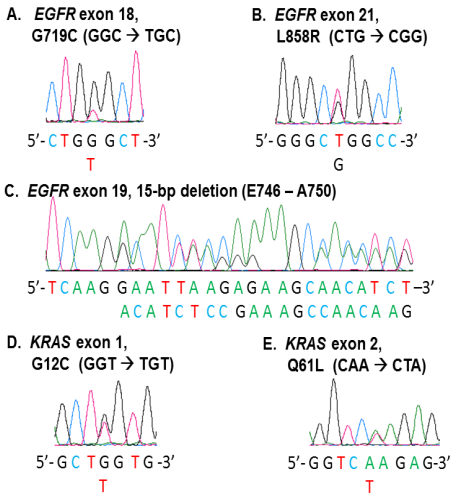

Figure 2. Sequencing analysis of the *EGFR* and the *KRAS* genes. A-C. A G to T transversion of codon 719 (Glycine (GGC) to Cysteine (TGC)) in exon 18 (A), an in-frame deletion of 15-bp from codon 746 to codon 750 in exon 19 (B) and T to G transversion of codon 858 (CTG (Leucine) to CGG (Arginine) (C) of the *EGFR* gene are shown. (D) and (E) A G to T transversion of codon 12 (GGT (Glycine) to TGT (Cysteine)) in exon 1 (D) and a G to T transversion of codon 61 (CAA (Glutamine) to CTA (Leucine)) in exon 2 (E) of the *KRAS* gene are shown.

known mutations of the *EGFR*. On the other hand, SSCP and sequencing analyses of exons 1 and 2 and their flanking regions of the *KRAS* gene identified 11 mutations (11%) out of 103 NSCLC cases, all of which were known activating mutations of the *KRAS* gene (**Figure 1** and **Figure 2**, **Table 2**).

3.2. Clinico-Pathological Features of NSCLC with the *EGFR* or the *KRAS* Mutations

It is widely accepted that the *EGFR* mutation is preferentially observed in female cases of adenocarcinoma without smoking background, whereas the *KRAS* mutation is frequently detected in NSCLC with heavy smoking background. The present study confirmed these characteristics (**Table 1** and **Table 2**). The *EGFR* mutation is

Table 1. *EGFR* mutation and clinicopathological characteristics of primary NSCLCs.

Characteristics	No. of tumors examined	WT	EGFR mutation	(%)	*p*
Total	103	76	27	(26)	
Age (mean)	67.0	3.2	63.8		
Gender					
Male	74	64	10	(14)	
Female	29	12	17	(59)	<0.00001
Smoking status					
Never	26	10	16	(65)	
Ex-smoker	26	20	6	(23)	
Current	51	46	5	(12)	<0.00001
Smoking index					
<799	46	23	23	(54)	
>800	57	53	4	(7)	<0.000001
Histological differentiation					
Adenocarcinoma	69	42	27	(41)	<0.0001
Squamous cell ca.	26	26	0	(0)	
Adenosquamous ca.	2	2	0	(0)	
	6	6	0	(0)	
Adenocarcinoma	69	42	27	(41)	
Histological differentiation					
Well differentiated	22	10	12	(55)	
Moderately diff.	37	22	15	(41)	p < 0.05
Poorly differentiated	10	10	0	(0)	
Patholoical stage					
I	36	21	15	(42)	NS
II	13	8	5	(38)	
III	17	10	7	(41)	
IV	3	3	0	(0)	
KRAS gene					
Mutated	11	11	0	(0)	
Not mutated	92	65	27	(32)	p < 0.05
*CADM*1 gene					
Methylated	45	37	8	(18)	
Unmethylated	58	39	19	(33)	NS
4.1*B* gene					
Methylated	59	47	12	(20)	
Unmethylated	44	29	15	(34)	NS
*CADM*1 or 4.1*B* gene					
Both or either methylated	71	55	16	(23)	
Both unmethylated	32	21	11	(34)	NS

NS: not significant: LCNEC: Large cell neuroendocirne carcinoma.

Table 2. *KRAS* mutation and clinicopathological characteristics of primary NSCLCs.

Characteristics	No. of tumors examined	WT	KRAS mutation	(%)	*p*
Total	103		11	(11)	
Age (mean)	67.0		68.3		
Gender					
Male	74	64	10	(14)	
Female	29	28	1	(3)	NS
Smoking status					
Never	26	25	1	(4)	
Ex-smoker	26	23	3	(12)	
Current	51	44	7	(14)	NS
Smoking index					
<799	46	42	4	(9)	
>800	57	50	7	(12)	NS
Histological differentiation					
Adenocarcinoma	69	63	6	(9)	NS
Squamous cell ca.	26	24	2	(8)	
Adenosquamous ca.	2	1	1	(50)	
LCNEC	6	4	2	(33)	
Patholoical stage					
I	51	47	4	(8)	NS
II	27	22	5	(19)	
III	22	20	2	(8)	
IV	3	3	0	(0)	
*CADM*1 genes					
Methylated	45	41	4	(9)	NS
Unmethylated	58	51	7	(12)	
EGFR					
Mutated	11	11	0	(0)	$p < 0.05$
Not mutated	92	65	27	(32)	
*CADM*1 gene					
Methylated	45	41	4	(9)	NS
Unmethylated	58	51	7	(12)	
4.1*B* gene					
Methylated	59	51	8	(14)	NS
Unmethylated	44	41	3	(7)	
*CADM*1 or 4.1*B* gene					
Both or either methylated	71	62	9	(13)	NS
Both unmethylated	32	30	2	(6)	

NS: not significant; LCNEC: Large cell neuroendocrine carcinoma.

observed exclusively in adenocarcinoma (27/69; 39%) but not in 26 squamous cell carcinoma, 2 adenosquamous carcinoma, or 6 large cell neuroendocrine carcinoma (LCNEC) ($p < 0.0001$). The incidence of the *EGFR* mutation is also significantly higher in female cases (17/29; 59%) than that in male cases (10/74; 14%) ($p < 0.00001$). In addition, the incidence of the *EGFR* mutation is significantly higher in never smokers (16 of 26; 62%) in comparison with that in ex-smokers (6 of 26; 23%) or in current smokers (5 of 51; 10%) ($p < 0.00001$). Moreover, mutation of the *EGFR* and the *KRAS* gene was also detected mutually exclusively as previously reported.

On the other hand, the incidence of the *KRAS* mutation do not show significant difference in male or female cases (10 of 74; 14% and 1 of 29; 3%, respectively) or in current smokers (7 of 51; 14%) in comparison with that in in ex-smokers (3 of 26; 12%) or in never smokers (1 of 26; 4%) (**Table 2**). In contrast to the *EGFR* mutation, the *KRAS* mutation is detected in considerable portions of various histological subtypes of NSCLC, including adenocarcinoma (6 of 69; 9%), squamous cell carcinoma (2 of 26; 8%), adenosquamo cell carcinoma (1 of 2; 50%) and large cell neuroendocrine carcinoma (2 of 6; 33%).

3.3. Mutation of the *EGFR* or the *KRAS* Genes Is Independent of the Promoter Methylation of the *CADM*1 or 4.1*B* Genes in Japanese NSCLC

Next, mutation state of the *EGFR* and *KRAS* gene obtained in this study was compared with the methylation state of the *CADM*1 or 4.1*B* gene promoters that we have previously reported in the same series of NSCLC tumors [10] [11]. As shown in **Table 1**, the *EGFR* mutation was observed in 8 of 45 (18%) NSCLC tumors with hypermethylation of the *CADM*1 and in 19 of 58 (33%) tumors without hypermehylation of the *CADM*1. Similarly, the *EGFR* mutation was observed in 12 of 59 (20%) NSCLC tumors with hypermethylation of the 4.1*B* and in 15 of 44 (34%) tumors without hypermehylation of the 4.1*B*. These incidences do not show any significant difference, suggesting that the *CADM*1 or the 4.1*B* methylation is an independent event of the *EGFR* mutation.

When clinico-pathological features of the *EGFR*-mutated tumors were examined, the *CADM*1 or 4.1*B* methylation appeared to be preferentially observed in tumors with moderately differentiated histology or with stages II or III in comparison with tumors with well differentiated histology or those with stage I, although the difference was not statistically significant. Since CADM1 and 4.1B act in the same cascade of tumor suppression [8] [16], this cascade would be disrupted when either the *CADM*1 or 4.1*B* is methylated, whereas this cascade would be intact only when both the *CADM*1 and 4.1*B* are unmethylated. When we divide 27 *EGFR*-mutated tumors into two groups from this point of view, methylation of either the *CADM*1 or 4.1*B* was more frequently observed in moderately differentiated tumors (12 of 15, 80%) than in well differentiated tumors (4 of 12; 33%) ($p < 0.05$) or in tumors with stages II and III (10 of 12; 87%) than in stage I tumors (6 of 15; 40%) ($p < 0.05$) (**Table 3**).

Similarly, the *KRAS* gene mutation was observed in 4 of 45 (9%) NSCLC tumors with the *CADM*1 methylation, while in 7 of 58 (12%) tumors without the *CADM*1 methylation. The *KRAS* mutation was also observed in 8 of 59 (14%) NSCLC tumors with the 4.1*B* methylation and in 3 of 44 (7%) tumors without the 4.1*B* methylation (**Table 2**). The differences in the incidence are not statistically significant, suggesting that the mutation status of the *KRAS* gene is independent of the methylation status of the *CADM*1 or 4.1*B* genes in Japanese NSCLC. Furthermore, histological differentiation and pathological stages of the *KRAS*-mutated tumors in terms of the methylation status of the *CADM*1 or 4.1*B* did not show any significant difference (**Table A2**).

Finally, we have examined the mutation of the *EGFR* and the *KRAS* genes in 11 NSCLC cell lines and found 1 *EFGR* and 3 *KRAS* mutations, respectively, whereas methylation of the *CADM*1 and 4.1*B* were detected in 4 and 7 cell lines, respectively. The patterns of alteration in individual cells suggest that the *EGFR* and *KRAS* mutations and the *CADM*1 and 4.1*B* methylation are also independent events in NSCLC cell lines (**Table 4**). To know the overall tendency of gene methylation in each cell line, methylation state of 6 additional gene promoters were analyzed by methylation-specific PCR. These include *RASSF*1*A*, *CDKN*2*A*, *APC*, *CDH*1, *GATA*-4 *and GATA*-5 genes. As shown in **Table 4**, NCI-H441 and ABC-1 cells showed total 6 methylated genes, whereas NCI-H522, Calu-3, PC-14, SKLU-1 and VMRC-LCD cells showed only 2 methylated genes.

4. Discussion

Molecular targeting therapies in association with specific genetic markers provide promising approaches to the treatment of NSCLC. However, the effectiveness of or resistance to EGFR-TKI inhibitors to lung adenocarci-

Table 3. Methylation status of the *CADM*1 and the 4.1*B* and clinico-pathological charactersitcs of *EGFR*-mutated lung adenocarcinoma.

Characteristics	*CADM*1 Methylation		4.1*B* Methylation		*CADM*1 *or* 4.1*B* Methylation		Total
	(+)	(−)	(+)	(−)	(+)	(−)	
Histological differentiation							
Well differentiated	2	10	3	9	4	8	12
Moderately differentiated	6	9	9	6	12	3	15
Poorly differentiated	0	0	0	0	0	0	0
		*NS		*NS		*p < 0.05	
Pathological stages							
I	2	13	5	10	6	9	15
II	2	3	2	3	4	1	5
III	4	3	5	2	6	1	7
IV	0	0	0	0	0	0	0
		**NS		**NS		**p < 0.05	
Total	8	19	12	15	16	11	27

NS: not significant; *W/D vs M/D, **I vs II + III.

Table 4. Mutation status of the *EGFR* and the *KRAS* genes and methylation status of the *CADM*1, 4.1*B* and other genes in human NCLCL cell lines.

Cell lines	Mutation		Methylation								No. of methylated genes
	EGFR	*KRAS*	*CADM*1	4.1*B*	*RASSF*1*A*	*CDKN*2*A*	*APC*	*CDH*1	*GATA*-4	*GATA*-5	
PC-14	(+)		(+)	(+)							2
NCI-H596		(+)	(+)	(+)	(+)				(+)	(+)	5
SK-LU-1		(+)	(+)						(+)		2
NCI-H441		(+)		(+)	(+)	(+)	(+)		(+)	(+)	6
Calu-3			(+)	(+)							2
ABC-1				(+)	(+)	(+)	(+)		(+)	(+)	6
A549				(+)	(+)		(+)		(+)		4
RERF-LC-OK				(+)	(+)			(+)	(+)		4
RERF-LC-MS					(+)	(+)	(+)				3
NCI-H522					(+)		(+)				2
VMRC-LCD					(+)					(+)	2

noma shows diversity in individual tumors even if they are carrying the specific *EGFR* gene mutation. One of the possible molecular mechanisms of this variation would be additional factors that modulate the function of EGFR and its signaling in tumor cells. Thus, it would be important to characterize such modifiers for generating more effective personalized therapy. Expression profiling of these tumors would provide valuable information of possible genetic and epigenetics modifiers [26]. Alternatively, a candidate gene approach to examine the expression of specific molecules showing functional cross-talk with EGFR would be also useful. It was reported that inactivation of the *CDKN*2*A* gene could serve as a poor prognostic factor in NSCLC [27].

In the present study, we examined the mutation status of the *EGFR* gene in 103 NSCLC and compared the

results with the methylation status of the *CADM*1 and/or 4.1*B*. CADM1 was chosen because CADM1 was shown to interact with ERBB3, a member of EGFR family proteins, and partly suppresses ERBB3 phosphorylation by recruiting a phosphatase PTPN13 [18]. Since ERBB3 forms a dimer with EGFR, CADM1 could modulate EGFR signaling. As 4.1B is a binding protein of CADM1 and acts in the downstream of CADM1 cascade, we also compared the *EGFR* mutation with methylation status of the 4.1*B*. As widely accepted, we have confirmed that 27 *EGFR*-mutated tumors are exclusively adenocarcinoma ($p < 0.00001$) and developed in female ($p < 0.00001$) and non-smokers ($p < 0.00001$). We have also confirmed that the *EGFR* mutation and the *KRAS* mutation are mutually exclusive (**Table 1**).

The original finding in the present study is independent occurrence of the mutation of a driver oncogene, *EGFR*, and methylation of the *CADM*1 and 4.1*B*, tumor suppressor genes involved in tumor progression in 103 primary tumors and 11 cell lines of NSCLC. This would be reasonable considering that CADM1-4.1*B* cascade transmits the signals of cell adhesion to the development and maintenance of epithelia-like cell structure through reorganizing the cytoskeleton, which is not directly related to growth factor signaling triggered by EGFR [8] [28]. Thus, 27 *EGFR*-mutated tumors can be divided into 16 tumors with methylation of either the *CADM*1 or 4.1*B* genes showing disrupted CADM1-4.1*B* cascade, and 11 tumors with unmethylated *CADM*1 and 4.1*B* genes showing intact CADM1-4.1*B* cascade. Another important finding of this study is that the disruption of CADM1-4.1*B* cascade by methylation of either *CADM*1 or 4.1*B* genes are associated with more malignant phenotype in histological differentiation ($p < 0.05$) and pathological stages ($p < 0.05$) even in *EGFR*-mutated tumors (**Table 3**). In addition to pathological characterization, further studies for estimating clinical outcome of the patients with these two groups of tumors, including responses to the TKI-therapy and possible recurrence, would be required to answer the question whether CADM1 and 4.1*B* act as epigenetic modifiers in the treatment of TKI.

As to the methylation in cancer, it should be noted that some gene promoters are often methylated not only by causative gene silencing but also by passenger mechanisms due to the methylator phenotype of cancer cells [29]. In fact, among 8 gene promoters examined, 6 genes (75%) were methylated in 2 cell lines, whereas only 2 genes (25%) were methylated in 5 cell lines, including PC-14 cells, suggesting that methylator phenotype could be involved at least in a subset of NSCLC cell lines. In PC-14 and Calu-3 cells, however, the *CADM*1 and 4.1*B* were the only genes methylated among 8 genes analyzed, suggesting that silencing of the *CADM*1 and 4.1*B* by promoter methylation would confer some biological advantages to these cancer cells. Since PC-14 carried the *EGFR* mutation, restoration of CADM1 and/or 4.1*B* expression might modulate the response of PC-14 cells to EGFR-TKI, although PC-14 is essentially sensitive to EGFR-TKI.

Mutation of the *KRAS* gene is another common alteration of driver oncogenes in various tumors, including NSCLC and HNSCC [5] [20] [30]. To understand the molecular mechanisms of NSCLC development and progression, Jackson *et al*. established an elegant mouse model, in which *Cre*-recombinase was infected into the murine airway epithelial cells through retroviral gene transfer and the mutated *KRAS* knocked-in gene was activated in the airway epithelial cells to develop lung adenocarcinoma [31] [32]. Subsequent expression profile analysis identified the *CADM*1 as one of 18 genes whose expression was significantly down-regulated in the aggressive tumors showing chromosome instability [18]. These finding suggest that loss of CADM1 expression synergistically enhances the malignant phenotype of NSCLCs carrying the *KRAS* mutation. Our study demonstrated that the mutation status of the *KRAS* and the methylation status of the *CADM*1 or 4.1*B* were independent phenomena. In fact, among 11 *KRAS*-mutated tumors, 9 tumors showed methylation of either *CADM*1 or 4.1*B*, whereas 2 tumors showed both unmethyalted *CADM*1 and the 4.1*B* (**Table A2**). However, differences in the histological differentiation and pathological stages of these two groups showed borderline or no statistical significance in this study. Further studies to check the status of CADM1-4.1*B* cascade in the *KRAS*-mutated tumors and their clinico-pathological features in larger cohort would be helpful to understand the pathobiological characterstics and clinical outcome of the *KRAS*-mutated NSCLC.

5. Conclusion

This is the first report to demonstrate that the methylation status of the *CADM*1 or the 4.1*B* gene is independent of the mutation status of the *EGFR* or the *KRAS* gene in NSCLC. Moreover, among the *EGFR*-mutated lung adenocarcinoma, methylation of both or either of the *CADM*1 or 4.1*B* was observed in tumors showing less differentiated histology and more advanced pathological stages. Since CADM1 and 4.1*B* proteins have potentials to modify the function of EGFR and KRAS signaling, methylation status of the *CADM*1 and/or 4.1*B* in tumors

could possibly provide useful information not only for deep understanding of the pathological features but also for predicting the response or recurrence of lung adenocarcinoma to the EGFR-TKI therapy.

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References

[1] Minna, J.D., Roth, J.A. and Gazdar, A.F. (2002) Focus on Lung Cancer. *Cancer Cell*, **1**, 49-52. http://dx.doi.org/10.1016/S1535-6108(02)00027-2

[2] Yokota, J. and Kohno, T. (2004) Molecular Footprints of Human Lung Cancer Progression. *Cancer Sci*, **95**, 197-204. http://dx.doi.org/10.1111/j.1349-7006.2004.tb02203.x

[3] Mitsudomi, T. and Yatabe, Y. (2007) Mutations of the Epidermal Growth Factor Receptor Gene and Related Genes as Determinants of Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors Sensitivity in Lung Cancer. *Cancer Science*, **98**, 1817-1824. http://dx.doi.org/10.1111/j.1349-7006.2007.00607.x

[4] Soda, M., Choi, Y.L., Enomoto, M., Takada, S., Yamashita, Y., Ishikawa, S., Fujiwara, S., Watanabe, H., Kurashina, K., Hatanaka, H., Bando, M., Ohno, S., Ishikawa, Y., Aburatani, H., Niki, T., Sohara, Y., Sugiyama, Y. and Mano, H. (2007) Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature*, **448**, 561-566. http://dx.doi.org/10.1038/nature05945

[5] Siddiqui, A.D. and Piperdi, B. (2010) KRAS Mutation in Colon Cancer: A Marker of Resistance to EGFR-I Therapy. *Annals of Surgical Oncology*, **17**, 1168-1176. http://dx.doi.org/10.1245/s10434-009-0811-z

[6] Bivona, T.G., Hieronymus, H., Parker, J., Chang, K., Taron, M., Rosell, R., Moonsamy, P., Dahlman, K., Miller, V.A., Costa, C., Hannon, G. and Sawyers, C.L. (2011) FAS and NF-κB Signalling Modulate Dependence of Lung Cancers on Mutant EGFR. *Nature*, **471**, 523-526. http://dx.doi.org/10.1038/nature09870

[7] Kuramochi, M., Fukuhara, H., Nobukuni, T., Kanbe, T., Maruyama, T., Ghosh, H.P., Pletcher, M., Isomura, M., Onizuka, M., Kitamura, T., Sekiya, T., Reeves, R.H. and Murakami, Y. (2001) TSLC1 Is a Tumor-Suppressor Gene in Human Non-Small-Cell Lung Cancer. *Nature Genetics*, **27**, 427-430. http://dx.doi.org/10.1038/86934

[8] Yageta, M., Kuramochi, M., Masuda, M., Fukami, T., Fukuhara, H., Maruyama, T., Shibuya, M. and Murakami, Y. (2002) Direct Association of TSLC1 and DAL-1, Two Distinct Tumor Suppressor Proteins in Lung Cancer. *Cancer Research*, **62**, 5129-5133.

[9] Tran, Y.K., Bogler, O., Gorse, K.M., Wieland, I. and Green, M.R. (1999) A Novel Member of the NF2/ERM/4.1 Superfamily with Growth Suppressing Properties in Lung Cancer. *Cancer Research*, **59**, 35-43.

[10] Kikuchi, S., Yamada, D., Fukami, T., Maruyama, T., Ito, A., Asamura, H., Matsuno, Y., Onizuka, M. and Murakami. Y. (2006) Hypermethylation of the TSLC1/IGSF4 Promoter Is Associated with Tobacco Smoking and a Poor Prognosis in Primary Non-Small Cell Lung Cancer. *Cancer*, **106**, 1751-1758.

[11] Kikuchi, S., Yamada, D., Fukami, T., Masuda, M., Sakurai-Yageta, M., Williams, Y.N., Maruyama, T., Asamura, H., Matsuno, Y., Onizuka, M. and Murakami, Y. (2005) Promoter Methylation of the *DAL*-1/4.1*B* Predicts Poor Prognosis in Non-Small Cell Lung Cancer. *Clinical Cancer Research*, **11**, 2954-2961. http://dx.doi.org/10.1158/1078-0432.CCR-04-2206

[12] Murakami, Y. (2005) Involvement of a Cell Adhesion Molecule, TSLC1/IGSF4, in Human Oncogenesis. *Cancer Science*, **96**, 543-552. http://dx.doi.org/10.1111/j.1349-7006.2005.00089.x

[13] Sun, C.X., Robb, V.A. and Gutmann, D.H. (2002) Protein 4.1 Tumor Suppressors: Getting a FERM Grip on Growth Regulation. *Journal of Cell Science*, **115**, 3991-4000. http://dx.doi.org/10.1242/jcs.00094

[14] Yamada, D., Kikuchi, S., Williams, Y.N., Sakurai-Yageta, M., Masuda, M., Maruyama, T., Tomita, K., Gutmann, D.H., Kakizoe, T., Kitamura, T., Kanai, Y. and Murakami, Y. (2006) Promoter Hypermethylation of the Potential Tumor Suppressor *DAL*-1/4.1*B* Gene in Renal Clear Cell Carcinoma. *International Journal of Cancer*, **118**, 916-923. http://dx.doi.org/10.1002/ijc.21450

[15] Masuda, M., Kikuchi, S., Maruyama, T., Sakurai-Yageta, M., Williams, Y.N., Ghosh, H.P. and Murakami, Y. (2005) Tumor Suppressor in Lung Cancer (TSLC)1 Suppresses Epithelial Cell Scattering and Tubulogenesis. *Journal of Biological Chemistry*, **280**, 42164-42171. http://dx.doi.org/10.1074/jbc.M507136200

[16] Sakurai-Yageta, M., Masuda, M., Tsuboi, Y., Ito, A. and Murakami, Y. (2009) Tumor Suppressor CADM1 Is Involved in Epithelial Cell Structure. *Biochemical and Biophysical Research Communications*, **390**, 977-982. http://dx.doi.org/10.1016/j.bbrc.2009.10.088

[17] Ishimura, M., Sakurai-Yageta, M., Maruyama, T., Ando, T., Fukayama, M., Goto, A. and Murakami, Y. (2012) Involvement of miR-214 and miR-375 in Malignant Features of Non-Small-Cell Lung Cancer by Down-Regulating CADM1. *Journal of Cancer Therapy*, **3**, 379-387.

[18] Shingai, T., Ikeda, W., Kakunaga, S., Morimoto, K., Takekuni, K., Takai, Y., *et al.* (2003) Implications of Nectin-Like Molecule-2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1 in Cell-Cell Adhesion and Transmembrane Protein Localization in Epithelial Cells. *Journal of Biological Chemistry*, **278**, 35421-35427. http://dx.doi.org/10.1074/jbc.M305387200

[19] Sweet-Cordero, A., Tseng, G.C., You, H., Douglass, M., Huey, B., Albertson, D. and Jacks, T. (2006) Comparison of Gene Expression and DNA Copy Number Changes in a Murine Model of Lung Cancer. *Genes, Chromosomes and Cancer*, **45**, 338-348. http://dx.doi.org/10.1002/gcc.20296

[20] Ebihara, Y., Iwai, M., Akashi, K., Ito, T., Omura, G., Saito, Y., Yoshida, M., Ando, M., Asakage, T., Yamasoba, T. and Murakami, Y. (2014) High Incidence of Null-Type Mutations of the *TP*53 Gene in Japanese Patients with Head and Neck Squamous Cell Carcinoma. *Journal of Cancer Therapy*, **5**, 664-671. http://dx.doi.org/10.4236/jct.2014.57075

[21] Burbee, D.G., Forgacs, E., Zöchbauer-Müller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S., Sekido, Y., Latif, F., Milchgrub, S., Toyooka, S., Gazdar, A.F., Lerman, M.I., Zabarovsky, E., White, M. and Minna, J.D. (2001) Epigenetic Inactivation of RASSF1A in Lung and Breast Cancers and Malignant Phenotype Suppression. *Journal of the National Cancer Institute*, **93**, 691-699. http://dx.doi.org/10.1093/jnci/93.9.691

[22] Herman, J.G., Graff, J.R., Myöhänen, S., Nelkin, B.D. and Baylin, S.B. (1996) Methylation-Specific PCR: A Novel PCR Assay for Methylation Status of CpG Islands. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 9821-9826. http://dx.doi.org/10.1073/pnas.93.18.9821

[23] House, M.G., Guo, M., Iacobuzio-Donahue, C. and Herman, J.G. (2003) Molecular Progression of Promoter Methylation in Intraductal Papillary Mucinous Neoplasms (IPMN) of the Pancreas. *Carcinogenesis*, **24**, 193-198. http://dx.doi.org/10.1093/carcin/24.2.193

[24] Yanagawa, N., Tamura, G., Oizumi, H., Takahashi, N., Shimazaki, Y. and Motoyama, T. (2003) Promoter Hypermethylation of Tumor Suppressor and Tumor-Related Genes in Non-Small Cell Lung Cancers. *Cancer Science*, **94**, 589-592. http://dx.doi.org/10.1111/j.1349-7006.2003.tb01487.x

[25] Guo, M., House, M.G., Akiyama, Y., Qi, Y., Capagna, D., Harmon, J., Baylin, S.B., Brock, M.V. and Herman, J.G. (2006) Hypermethylation of the GATA Gene Family in Esophageal Cancer. *International Journal of Cancer*, **119**, 2078-2083. http://dx.doi.org/10.1002/ijc.22092

[26] Suda, K., Mizuuchi, H., Maehara, Y. and Mitsudomi, T. (2012) Acquired Resistance Mechanisms to Tyrosine Kinase Inhibitors in Lung Cancer with Activating Epidermal Growth Factor Receptor Mutation-Diversity, Ductility, and Destiny. *Cancer and Metastasis Reviews*, **31**, 807-814. http://dx.doi.org/10.1007/s10555-012-9391-7

[27] Zhang, L.Q., Yin, R., Li, M., Yang, X., Jiang, F. and Xu, L. (2013) The Prognostic Value of Epigenetic Silencing of p16 Gene in NSCLC Patients: A Systematic Review and Meta-Analysis. *PLoS ONE*, **8**, e54970. http://dx.doi.org/10.1371/journal.pone.0054970

[28] Murakami, S., Sakurai-Yageta, M., Maruyama, T. and Murakami, Y. (2014) *Trans*-Homophilic Interaction of CADM1 Activates PI3K by Forming a Complex with MAGuK-Family Proteins MPP3 and Dlg. *PLoS ONE*, **9**, e82894. http://dx.doi.org/10.1371/journal.pone.0082894

[29] Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J.G., Baylin, S.B. and Issa, J.P. (1999) CpG Island Methylator Phenotype in Colorectal Cancer. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 8681-8686. http://dx.doi.org/10.1073/pnas.96.15.8681

[30] Meng, D., Yuan, M., Li, X., Chen, L., Yang, J., Zhao, X., Ma, W. and Xin, J. (2013) Prognostic Value of K-RAS Mutations in Patients with Non-Small Cell Lung Cancer: A Systematic Review with Meta-Analysis. *Lung Cancer*, **81**, 1-10. http://dx.doi.org/10.1016/j.lungcan.2013.03.019

[31] Jackson, E.L., Willis, N., Mercer, K., Bronson, R.T., Crowley, D., Montoya, R., Jacks, T. and Tuveson, D.A. (2001) Analysis of Lung Tumor Initiation and Progression Using Conditional Expression of Oncogenic K-Ras. *Genes & Development*, **15**, 3243-3248. http://dx.doi.org/10.1101/gad.943001

[32] DuPage, M., Dooley, A.L. and Jacks, T. (2009) Conditional Mouse Lung Cancer Models Using Adenoviral or Lentiviral Delivery of Cre Recombinase. *Nature Protocols*, **4**, 1064-1072. http://dx.doi.org/10.1038/nprot.2009.95

Supplemental Table A1. Sequences of the primers used for this study.

Genes and primer names	Sequences	Annealing temperature (˚C)	Product size (bp)	Primer length (bp)
EGFR				
EGFR-ex18-pF	5'-CCGGAGTTTTCAATCCAGTT-3'	64	278	20
EGFR-ex18-pR	5'-GTCAATGGCCCCTTTCATAA-3'			20
EGFR-ex19-pF	5'-TGGATGAAATGATCCACACG-3'	64	250	20
EGFR-ex19-pR	5'-AGTGCTGGGTAGATGCCAGT-3'			20
EGFR-ex20-pF	5'-TCCGACTCCTCCTTTATCCA-3'	64	337	20
EGFR-ex20-pR	5'-GATGGGACAGGCACTGATTT-3'			20
EGFR-ex21-pF	5'-AAGTTCAAGCCCAGGTCTCA-3'	58	346	20
EGFR-ex21-pR	5'-TCATTCACTGTCCCAGCAAG-3'			20
KRAS				
Kras-ex1-pF	5'-AAAGGTACTGGTGGAGTATTTG-3'	58	265	22
Kras-ex1-pR	5'-TCTGTATCAAAGAATGGTCCTG-3'			22
Kras-ex2-pF	5'-TCTTTGGAGCAGGAACAATG-3'	58	400	20
Kras-ex2-pR	5'-TGCATGGCATTAGCAAAGAC-3'			20
RASSF1A				
RASSF1A-M-pF	5'-CGAGAGCGCGTTTAGTTTCGTT-3'	58	169	22
RASSF1A-M-pR	5'-CGATTAAACCCGTACTTCGCTAA-3'			23
RASSF1A-U-pF	5'-GGGGGTTTTGTGAGAGTGTGTTT-3'	58	169	23
RASSF1A-U-pR	5'-CCCAATTAAACCCATACTTCACTAA-3'			25
CDKN2A				
CDKN2A-M-pF	5'-GGGTTGTTTTCGGTTGGTGTTTTC-3'	68	150	24
CDKN2A-M-pR	5'-TCTAATAACCAACCAACCCCTCC-3'			23
CDKN2A-U-pF	5'-GTGAGGGTTGTTTTTGGTTGGTGTTTTT-3'	68	151	28
CDKN2A-U-pR	5'-TCTAATAACCAACCAACCCCTCC-3'			23
APC				
APC-5-Ex-pF	5'-TGGGYGGGGTTTTGTGTTTTATT-3'	56	136	23
APC-5-Ex-pR	5'-TACRCCCACACCCAACCAATC-3'			21
APC-5-M-pF	5'-TATTGCGGAGTGCGGGTC-3'	61	98	18
APC-5-M-pR	5'-TCGACGAACTCCCGACGA-3'			18
APC-5-U-pF	5'-GTGTTTTATTGTGGAGTGTGGGTT-3'	58	108	24
APC-5-U-pR	5'-CCAATCAACAAACTCCCAACAA-3'			22
CDH1				
CDH1-M-pF	5'-GGTGAATTTTTAGTTAATTAGCGGTAC-3'	55	204	27
CDH1-M-pR	5'-CATAACTAACCGAAAACGCCG-3'			21
CDH1-U-pF	5'-GGTAGGTGAATTTTAGTTAATTAGTGGTA-3'	55	211	29
CDH1-U-pR	5'-ACCCATAACTAACCAAAAACACCA-3'			24
GATA-4				
GATA-4-M-pF	5'-GTATAGTTTCGTAGTTTGCGTTTAGC-3'	60	136	26
GATA-4-M-pR	5'-AACTCGCGACTCGAATCCCCG-3'			21
GATA-4-U-pF	5'-TTTGTATAGTTTTGTAGTTTGTGTTTAGT-3'	60	142	29
GATA-4-U-pR	5'-CCCAACTCACAACTCAAATCCCCA-3'			24
GATA-5				
GATA-5-M-pF	5'-AGTTCGTTTTTAGGTTAGTTTTCGGC-3'	60	140	26
GATA-5-M-pR	5'-CCAATACAACTAAACGAACGAACCG-3'			25
GATA-5-U-pF	5'-TGGAGTTTGTTTTTAGGTTAGTTTTTGGT-3'	60	147	29
GATA-5-U-pR	5'-CAAACCAATACAACTAAACAAACAAACCA-3'			29

Supplemental Table A2. Methylation status of the CADM1 and the 4.1*B* and pathological charactersitcs of KRAS-mutated lung adenocarcinoma.

Charactersitics	*CADM*1 Methylation		4.1*B* Methylation		*CADM*1 *or* 4.1*B* Methylation		Total
	(+)	(−)	(+)	(−)	(+)	(−)	
Histological differetiation							
Total	4	7	8	3	9	2	11
Well differentiated	0	1	0	1	0	1	1
Moderately differentiated	2	6	6	2	7	1	8
Poorly differentiated	2	0	2	0	2	0	2
		NS		NS		NS	
Pathological stages							
Total	4	7	8	3	9	2	11
I	1	3	3	1	4	1	5
II	1	4	3	2	3	1	4
III	2	0	2	0	2	0	2
IV	0	0	0	0	0	0	0
		NS		NS		NS	

NS: not significant.