Genome-Wide Analysis of DNA Methylation and the Gene Expression Change in Lung Cancer

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Introduction: The recent DNA methylation studies on cancers have revealed the necessity of profiling an entire human genome and not to restrict the profiling to specific regions of the human genome. It has been suggested that genome-wide DNA methylation analysis enables us to identify the genes that are regulated by DNA methylation in carcinogenesis.

Methods: So, we performed whole-genome DNA methylation analysis for human lung squamous cell carcinoma (SCC), which is strongly related with smoking. We also performed microarrays using 21 pairs of normal lung tissues and tumors from patients with SCC. By combining these data, 30 hypermethylated and down-regulated genes, and 22 hypomethylated and up-regulated genes were selected. The gene expression level and DNA methylation pattern were confirmed by semiquantitative reverse-transcriptase polymerase chain reaction and pyrosequencing, respectively.

Results: By these validations, we selected five hypermethylated and down-regulated genes and one hypomethylated and up-regulated gene. Moreover, these six genes were proven to be actually regulated by DNA methylation by confirming the recovery of their DNA methylation pattern and gene expression level using a demethylating agent. The DNA methylation pattern of the *CYTL1* promoter region was significantly different between early and advanced stages of SCC.

Conclusion: In conclusion, by combining the whole-genome DNA methylation pattern and the gene expression profile, we identified the six genes (*CCDC37*, *CYTL1*, *CDO1*, *SLIT2*, *LMO3*, and *SERPINB5*) that are regulated by DNA methylation, and we suggest their value as target molecules for further study of SCC.

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The clinical, pathologic, and genomic characteristics of lung cancer are very diverse, and the most decisive treatment for it is to perform a curative resection at its earliest detection. Nevertheless, lung cancer still has been reported to be one of the most common malignant diseases with a poor prognosis.[1] Therefore, to increase the cure and survival rates, developing various diagnostic and treatment methods by using genomic studies of lung cancer might improve this situation. In this context, there have been various genomic studies of lung cancer for the last few decades, but any brilliant solution has not been found. Accordingly, epigenetic studies represented by DNA methylation and histone modification have been receiving much attention. This is because DNA methylation is related to the gene expression at the very early stage of tumorigenesis and DNA methylation is a reversible reaction.[2,3] It was found that abnormal DNA methylation of specific genes can cause various cancers to have a different response to chemotherapeutic drugs.[4] For example, the hypermethylation of the *MGMT* gene, and this gene is related to DNA repair, decreases the sensitivity to alkylating agents in glioma patients by lowering the DNA repair function, and the hypermethylation of the *WRN* gene (DNA helicase) increases the sensitivity to the topoisomerase inhibitor irinotecan in patients with colorectal cancer.[5–8] Similarly, analyzing the DNA methylation of individual genes could be crucial index for selective treatment. Many genes such as *APC*, *CADM1*, *CDH1*, *CDH13*, *CDKN2A/p14*, *CDKN2A/p16*, *DAPK*, *FHIT*, *GSTP1*, *MGMT*, *MLH1*, and *RASSFA1* have been proven to be hypermethylated in non-small cell lung cancer (NSCLC).[1] Despite these achievements, there has been a limitation for not covering the whole genome. So, whole-genome scale analyses for a comprehensive understanding of DNA methylation have recently been performed. For example, the MeDIP-seq analysis with breast cancer cell lines has allowed us to understand the abnormal DNA methylation pattern of various genes involved in the development of breast cancer, and MeDIP-tilling array with testicular germ cell tumor has enabled us to identify the epigenetically regulated genes and ncRNAs.[9,10] DNA methylation analyses

of lung cancer on a whole-genome scale have also been tried.[11] Although similar studies have been conducted on renal cell carcinoma and breast cancer,[12,13] our approach is the first trial to combine the genome-wide methylation pattern and the gene expression profile to identify the genes regulated by DNA methylation in squamous cell carcinoma (SCC). Herein, we examined the DNA methylation of SCC of the lung on a whole-genome scale by using the methylated CpG island recovery assay (MIRA) and an Illumina Genome Analyzer (GA), which is a next-generation sequencer. The MIRA technique we used herein was recently developed, and it has the advantage that MBD3L1 can enhance the specific recognition of methylated CpG dinucleotides by the MBD2b protein.[14–16] The Illumina GA, which sequences methylated DNA, seems to measure cytosine methylation on a genome-wide scale more sensitively than the chip-based methods.[17] We used tissue samples of SCC of the lung, and this malignancy is strongly related to smoking.[18,19] DNA methylation data were obtained by the technique mentioned earlier, and then we analyzed this data combined with the gene expression profile. Therefore, this study has implications for using a whole-genome DNA methylation analysis to find the genes in lung cancer that are regulated by DNA methylation.

PATIENTS AND METHODS

Patients and Tissue Samples

We used a total of 50 tissue samples from 50 patients with lung cancer who underwent surgery at Korea Cancer Center Hospital without preoperative treatment. The histology of all the specimens was SCC. The mean age of patients was 62.6 years (42–82 years), and 48 persons (96%) had the history of smoking. We used another 21 samples for the microarray, three samples for the whole-genome DNA methylation assay, and 30 samples for semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) and pyrosequencing. All the specimens were stored in liquid nitrogen immediately after surgical resection. The clinical information was obtained from the medical records. The pathologic stage was judged according to the criteria of the American Joint Committee on Cancer (7th edition). The Institutional Review Board of Korea Cancer Center Hospital approved this study.

Microarray

Total RNA from the frozen tissues of patients with lung cancer was isolated using Trizol (Invitrogen, Carlsbad, CA) as per manufacturer's instruction. Two micrograms of RNA was used to produce cRNA, which was hybridized to CodeLink Expression Arrays. We used the fold change of the average gene expression value for the definition criteria. When $\log_2$ (T = gene expression level of SCC/N = gene expression level of normal lung tissue) is above 1 or below -1, we regarded this as up-regulation or down-regulation, respectively.

Whole-Genome DNA Methylation Analysis by the GA II (Illumina Inc., San Diego, CA)

We purified the genomic DNA from the normal lung and tumor tissue of three patients with SCC by using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Three genomic DNAs from each group (normal and tumor) were then pooled at the same concentration. MIRA was carried out as described previously.[14] The GST-tagged MBD2b and His-tagged MBD3L1 proteins were prepared as described previously.[15] We used 10 ng of the DNA obtained from MIRA for the Illumina GA sequencing. Sequence tags were mapped to the human genome (the University of California Santa Cruz [UCSC] hg18 database based on the NCBI Build 36.1 assembly) using the Solexa Analysis Pipeline (version 0.3.0). The sequenced reads of 34 bp (excluding the first and last nucleotides) were obtained. We converted the output to browser extensible data files for visualization in the UCSC genome browser (http://genome.ucsc.edu). The methylation score (MES) was calculated as the $\log_2$ of (target read number/target size)/(total read number/genome size). To evaluate the measured hypermethylation or hypomethylation of the promoters, we introduced one arbitrary term called the difference of MES (dMES) (dMES = [SCC MES] − [normal MES]). Using this term, we regarded the genes of dMES more than 1.5 as hypermethylated genes and the genes of dMES less than -1.5 as hypomethylated genes.

Semiquantitative RT-PCR

We selected the hypermethylated and down-regulated genes and the hypomethylated and up-regulated genes by combining the microarray data with whole-genome DNA methylation pattern. Then, we examined the expression levels of selected genes by semiquantitative RT-PCR. The total RNA was isolated from 30 SCC frozen tissues using the RNeasy Mini Kit (Qiagen Science, MD) and following the manufacturer's protocol. The concentration of RNA was measured by spectrophotometry. A total of 1 μg RNA from each sample was subjected to reverse transcription to produce single-stranded complementary DNAs with using an oligo dT primer (Bioneer, Seoul, Korea) and Superscript II (Invitrogen). Semiquantitative RT-PCR experiments were carried out with the following sets of synthesized primers that were specific to 52 representative genes. They include 30 hypermethylated and down-regulated genes and 22 hypomethylated and up-regulated genes in SCC. We used GAPDH-specific primers as an internal control. The PCR reactions were optimized for the number of cycles and the annealing temperature to obtain a DNA band of the exact product size.

Pyrosequencing

The genomic DNA was isolated from 20 frozen SCC tissues and two SCC cell lines (HCC-95 and HCC-1588) using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and from 110 SCC formalin-fixed and paraffin-embedded tissues using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), per the manufacturer's instructions. The DNA was quantified using a NanoDrop ND-100 Spectrophotometer (Thermo Fisher Scientific, MA) before bisulfite treatment. The DNA (2 μg/reaction) was bisulfite treated using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The bisulfite-treated DNA was eluted in 20 μl of the manufacturer's Elution Buffer (20 μl final volume). The bisulfite-

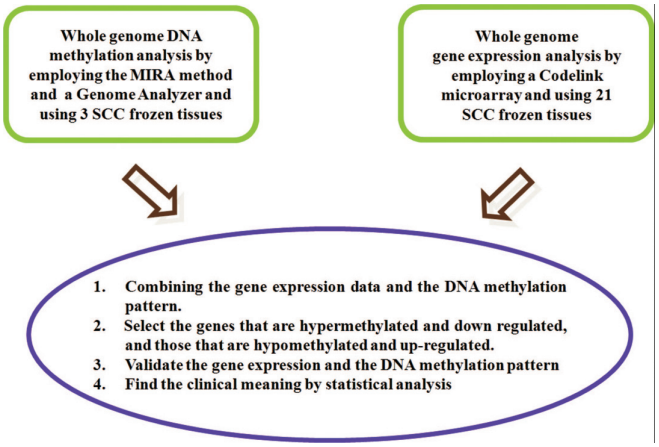

FIGURE 1. Schematic diagram showing all the steps to identify the target genes. The outline of the procedures to find the genes regulated by DNA methylation in squamous cell carcinoma (SCC). Two independent experiments (whole-genome DNA methylation assay and microarray) were performed, and then the results were combined. According to this flowchart, we selected the target genes from the whole-genome scale combining assay, and we confirmed the data of the clinical samples. At the end, we can obtain the clinical meanings by statistical analysis.

converted sequences were examined by using Assay Design Software version 1.0.6 (Biotage, Uppsala, Sweden). We considered that the promoter region of each gene is 1000 bp upstream and 500 bp downstream from the transcription start site. Each 50 μl PCR contained 2.5 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphate, 0.05 U/μl EF-*Taq*DNA polymerase (Solgent, Korea) and 0.2 μM each of the forward and reverse primers (Table 4), and 200 ng of bisulfite-treated DNA. All the primers were synthesized at Bioneer (Seoul, Korea). In this study, we used the following PCR cycling conditions. One cycle of 95°C for 2 minutes for the initial denaturation steps was followed by 55 cycles of denaturation at 95°C for 30 seconds, various annealing temperatures (55–63°C) for 30 seconds, and then extension at 72°C for 30 seconds. PCR was terminated after a final cycle at 72°C for 5 minutes. Pyrosequencing was performed according to the manufacturer's instructions.

Demethylation Test In Vitro

The human SCC cell lines HCC-1588 and HCC-95 were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2 in air. The other human SCC cell lines Calu-1 and SK-MES-1 were maintained in ATCC-formulated McCoy's 5a Medium Modified Catalog No. 30-2007 and Eagle's Minimum Essential Medium, Catalog No. 30-2003 supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2 in air, respectively. Another human SCC cell line SW900 was maintained in Leibovitz's L-15 Medium, Catalog No. 30-2008 supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in 100% air; 2×10^6 cells of each cell line were treated with 20 μM 5-aza-2′-deoxycytidine (Sigma, St. Louis, MO), and they were grown for 4 days. The gene expression and DNA methylation pattern before and after treatment with demethylating agent were examined by semiquantitative RT-PCR and pyrosequencing, respectively. Cell lines HCC-1588 and HCC-95 were obtained from Korean Cell Line Bank on March 23, 2010. The other cell lines Calu-1, SK-MES-1 and SW900 were obtained from ATCC on May 5, 2011. The cell lines have been characterized using DNA fingerprinting analysis by the cell bank.

Statistical Analysis

Student's *t* tests were used to examine the difference of the DNA methylation levels between the normal tissues and the SCC tissues. One-way analysis of variance was applied to determine the correlation of the DNA methylation level with the SCC stages, and *p* values less than 0.05 were regarded as statistically significant. The statistical analysis was done using GraphPad Prism software version 5.03 for Windows (San Diego, CA, www.graphpad.com).

RESULTS

Selection of Candidate Genes

In an effort to find the genes regulated by DNA methylation in SCC, we performed a microarray and a genome-wide DNA methylation sequencing. All the steps to identify the target genes are briefly depicted in Figure 1. We selected 52 candidate genes after combining the gene expression data with the whole-genome DNA methylation pattern as listed in Table 1. At first, we chose 26 genes, including *CDO1*, with the criteria of a dMES more than 1.5 and a $\log_2$ (T/N) less than −1, and 17 genes, including *SERPINB5*, with the criteria of a dMES less than −1.5 and a $\log_2$ (T/N) more than 1. Second, we included two genes (*CCDC37* and *DLEC1*) that were extremely hypermethylated (dMES >2.5) and five genes, including *ELMO3*, that were extremely hypomethylated (dMES <−2.1) in this screening list regardless of the change of gene expression. Finally, the highly down-regulated genes *MYH2* and *DNM3* ($\log_2$ (T/N) <−2.5) were added to the list. In this screening procedure, we focused on DNA methylation of the promoter region. Moreover, we tried to screen all the promoters without consideration of the existence of CpG islands because it was reported that the promoters not having CpG island also have an effect on gene expression.[20]

The Genes Selected by the Validation Steps: CCDC37, CYTL1, CDO1, LMO3, SLIT2, and SERPINB5

We attempted to verify the gene expression pattern of 52 genes by performing semiquantitative RT-PCR with using another 30 SCC tissue samples listed in Table 2. We found 12 down-regulated genes and one up-regulated gene in more than 57% of the SCC samples, as listed in Table 3. From screening to validation, we used one gene, *DLEC1*, as a positive control to improve the reliability. As *DLEC1* had been reported to be down-regulated by DNA methylation,[21]

TABLE 1. Selected Genes by Combining the Microarray Data with the Whole-Genome DNA Methylation Patterns

Classification	No.	Gene	dMES(T-N)	Log_2(T/N)
Hypermethylation	1	*CCDC37*	3.441251143	−0.438497777
	2	*DLEC1*	2.797680143	(-)
	3	*MYH2*	1.215407714	−3.987329902
	4	*DNM3*	1.311622857	−2.665170126
	5	*PAX9*	3.29980875	−1.108395547
	6	*CDO1*	3.226884714	−1.985933328
	7	*HIST1H3A*	2.782833571	−1.264196556
	8	*PPP1R14A*	2.5877785	−1.440936626
	9	*SLIT2*	2.489663125	−1.817988624
	10	*CTSE*	2.421966571	−1.154048118
	11	*ADAM33*	2.300755429	−1.221087063
	12	*KLRC4*	2.130859375	−1.451254932
	13	*CYTL1*	2.11463975	−1.165674222
	14	*AGTR1*	2.01972575	−1.356755098
	15	*CLTCL1*	2.009838863	−1.023127859
	16	*RGS5*	2.005905988	−1.434346574
	17	*NID1*	1.998283186	−1.272255513
	18	*TCAP*	1.95850975	−1.012605722
	19	*PGC*	1.91369675	−2.417429465
	20	*ASTN2*	1.904766	−2.116127773
	21	*TMEM146*	1.863058571	−1.029065264
	22	*LMO3*	1.845670163	−1.188834946
	23	*SLC1A2*	1.8171925	−1.129283017
	24	*APOB48R*	1.781701143	−1.809420667
	25	*HOPX*	1.757325125	−1.099664166
	26	*GUCA2B*	1.750145286	−1.17527637
	27	*KANK2*	1.736910125	−1.182291311
	28	*COL13A1*	1.728430757	−1.470238996
	29	*CA10*	1.662334943	−2.829902911
	30	*HIST1H1B*	1.622941043	−2.52113352
Hypomethylation	1’	*KRTCAP3*	−3.2628938	1.552826764
	2’	*NETO2*	−2.797227	1.125125323
	3’	*ELMO3*	−2.550139125	0.859621462
	4’	*MT1B*	−2.521117188	0.832941395
	5’	*NSDHL*	−2.43747225	1.037793283
	6’	*COL1A1*	−2.350527429	0.84662518
	7’	*LASP1*	−2.237798571	1.026014302
	8’	*PSMA6*	−2.235948875	0.964107805
	9’	*ADSSL1*	−2.182571286	1.172602594
	10’	*EDN2*	−2.18182875	3.577795253
	11’	*CBS*	−2.152533629	0.854992104
	12’	*CCL7*	−2.149588125	1.128776661
	13’	*PDCL2*	−2.027630429	4.248027683
	14’	*SPC25*	−2.970506286	2.701452195
	15’	*PAGE4*	−2.1768414	3.211556392
	16’	*GBX2*	−1.637031629	2.88765218
	17’	*PRIM1*	−1.64499125	2.185488422
	18’	*SERPINB5*	−1.784078125	4.121717307
	19’	*CDCA5*	−1.820698857	2.514666762
	20’	*SLC35F3*	−1.99832255	2.02506441
	21’	*KCNC1*	−1.952510671	2.427335724
	22’	*HOXD11*	−1.83193375	2.72750794

Thirty genes that were hypermethylated and down-regulated and 22 genes that were hypomethylated and up-regulated are presented. Arbitrary term called dMES means (dMES = [SCC MES] − [normal MES]), and the fold-change in the expression levels was calculated by log_2 (tumor/normal lung). The expression level of each gene in normal lung and tumor was represented by the average of 21 normal tissues and tumors, respectively. The symbol (-) means that the gene expression data for DLEC1 in the microarray do not exist.

SCC, squamous cell carcinoma.

TABLE 2. Clinical Data of the Patients Whose Tissues were Used for RT-PCR and Pyrosequencing

No.	Gender	Age (yr)	Smoking (Pack per Year)	Cell Type	Stage
1	M	78	75	SCC	T2aN0M0, IB
2	M	76	40	SCC	T2aN0M0, IB
3	M	72	20	SCC	T2bN0M0, IIA
4	M	68	150	SCC	T2aN0M0, IA
5	M	69	35	SCC	T2bN0M0, IIA
6	M	65	40	SCC	T2aN0M0, IB
7	M	70	40	SCC	T2aN0M0, IB
8	M	67	45	SCC	T1bN1M0, IIA
9	M	61	40	SCC	T1aN1M0, IIA
10	M	60	50	SCC	T2aN1M0, IIA
11	M	58	30	SCC	T2aN1M0, IIA
12	M	73	25	SCC	T2aN1M0, IIA
13	M	53	60	SCC	T2aN1M0,IIA
14	M	47	35	SCC	T2aN1M0, IIA
15	M	84	60	SCC	T2aN2M0, IIIA
16	M	63	30	SCC	T2aN2M0, IIIA
17	M	66	20	SCC	T3 N2M0, IIIA
18	M	68	60	SCC	T2aN2M0, IIIA
19	M	59	60	SCC	T2aN2M0, IIIA
20	M	62	80	SCC	T2aN2M0, IIIA
21	M	68	40	SCC	T2bN2M0, IIIA
22	M	70	20	SCC	T4N0M0, IIIA
23	M	65	10	SCC	T2aN1M0, IIA
24	M	47	20	SCC	T2aN0M0, IB
25	M	64	0	SCC	T4N1M0, IIIA
26	M	71	60	SCC	T2aN2M0, IIIA
27	M	58	40	SCC	T3N0M0, IIB
28	M	74	50	SCC	T2bN0M0, IIB
29	M	66	20	SCC	T2bN2M0, IIIA
30	M	69	50	SCC	T3N0M1a, IV

RT-PCR, reverse-transcriptase polymerase chain reaction; SCC, squamous cell carcinoma.

TABLE 3. Down- or Up-Regulated Genes in >57% of the SCC Tissue Samples

Classification	No.	Gene	The Number of Samples Down- or Up-Regulated
Hypermethylation	1	*CCDC37*	17 (57%)
	2	*DLEC1*	15 (50%)
	3	*MYH2*	18 (60%)
	6	*CDO1*	20 (67%)
	8	*PPP1R14A*	22 (73%)
	9	*SLIT2*	22 (73%)
	13	*CYTL1*	18 (60%)
	17	*NID1*	19 (63%)
	19	*PGC*	25 (83%)
	22	*LMO3*	23 (77%)
	25	*HOPX*	17 (57%)
	27	*KANK2*	20 (67%)
	28	*COL13A1*	23 (77%)
Hypomethylation	18’	*SERPHINB5*	26 (87%)

Thirty tissue samples were used for semiquantitative RT-PCR, and the number (ratio) of tissue samples where each gene was down- or up-regulated is shown.

RT-PCR, reverse-transcriptase polymerase chain reaction; SCC, squamous cell carcinoma.

TABLE 4. Pyrosequencing Profile of Six Selected Genes

Gene	Amplicon (bp)	PCR Primer (5′–3′)	Sequencing Primers	Position of CpG Island
SLIT2	100	FW: GGAGGAGGAGGGGAAAGAT RV: CACCCCTACCATACCACCTTA	**GGTTGGTAGATGTTGTTTTT** GTYGTTGGGGTTAGTGTTGGYGATTTT GAATAA GGTGGTATYGTAGGYGTGT	1, +239; 2, +257; 3, +278; 4, +284
CDO1	236	FW: AGTAGTTAAAGTGGGGGAGAGAT RV: CTCCCCAACCCCTTTTAAAC	**AAGTGGGGGAGAGATT** GYGYGGAGTTTAYGYGATTTTTGGGAYGTYGGAGATAAYGGGG TTTTTGGGAAGGYGYGGAGTTYGGGGAAGTYGGGGATGTGYGY GTGAGTYGTGTTYGTAGGGTTTTTTYGTTTTYGTTATTTTTTTT GGGTGGTTTTTYGTTTYGTTTTTTTTTYGAGGGTYGTTGGTATA TTTTTAGTGATTTTAAGYGTTTAAAAGGGGTTYGGGAG	1, −172; 2, −170; 3, −161; 4, −159; 5, −147; 6, −144; 7, −135; 8, −118; 9, −116; 10, −109; 11, −100; 12, −90; 13, −88; 14, −81; 15, −75; 16, −62; 17, −56; 18, −32; 19, −27; 20, −16; 21, −9; 22, +18; 23, +33
CYTL1	70	FW: GGTTTTTTTTTTGTTATTTTGGATG RV: CACTCCACCCCTCCACTTCCTATA	**TTGTTATTTTGGATGG** ATYGTAGTTTGYGTTGGTTTTATAGGAAGTGGAGGYGTGGAGTY	1, −81; 2, −72; 3, −48; 4, −40
	107	FW: ATGAGGGTTTTGAGTTAGGAGATT RV: TAAATCTACCATACACCCCTATC	**GTTTTGAGTTAGGAGATTAT** TYGYGATTTTAATTTTTTGTAGGTTTYGGAGTTTTYGGTGAG TTTTTYGTTYGTTTAGGATAGGGGTGTATGGTAGATTTA	1, +143; 2, +145; 3, +168; 4, +177; 5, +189; 6, +193;
LMO3	227	FW: TGGTGGGGGTTAGTATTAGTGAAT RV: ACACACAACCCTCCCCTTTAA	**TTTTGAATTAGGTTTGTGTGG** YGGATTTGYGGAGTAGYGATTTTAGAGAGTTTATGAATGG AAGTTATAGATAGATTAGYGATTGTAGTTATTTTTAGGTATTTTTT AAAGGGGAGGGTTGTGTGT	1, −626; 2, −618; 3, −610; 4, −568
CCDC37	256	FW: TTGGGGAGAGGATTGAAAATTT RV: CCCAATTTCTTCCTCTAAAATTCA	**TTAAAAGTATTTATTTTGTA** YGTTTTTTTTAGTGTTTGGTAYGTGGYGAATAAATTAATTAGGAT GAAAGGAGTTAATTTAAGTGYGGAGTGTTGAATTTTAGAGGAAG AAATTYGYGTTTATTA	1, −328; 2, −307; 3, −302; 4, −263; 5, −234; 6, −232
SERPHINB5	226	FW: AGAGGTTTGAGTAGGAGAGGAGTG RV: CAACCCTACTACCCCACCTTACTT	**TTGAGTAGGAGAGGAGTGT** YGTYGAGGYGGGGYGGGGYGGGGYGTGGAGTTGGGTTGGTA GTGGGYGTGGYGGTGTTGTTTAGGTGAGTTATYGTTGTTTTTGTTT AGATAYGGTYGTTTTTATATTTAGGTTTTTGTGTTTTTYGTTTGTTTG TTTTTTTTTTAYGTATTTTTTAGGATAATTGTGATTTTAGGTAA GTAAGGTGGGGTAGTAGGGTTG	1, −40; 2, −37; 3, −32; 4, −27; 5, −22; 6, −17; 7, +6; 8, +11; 9, +33; 10, +52; 11, +56; 12, +85; 13, +106

The gene name, the PCR product size for pyrosequencing, the sequences of the forward and reverse primers, the sequences of the sequencing primer for pyrosequencing, and the tested CpG position are listed.
PCR, polymerase chain reaction.

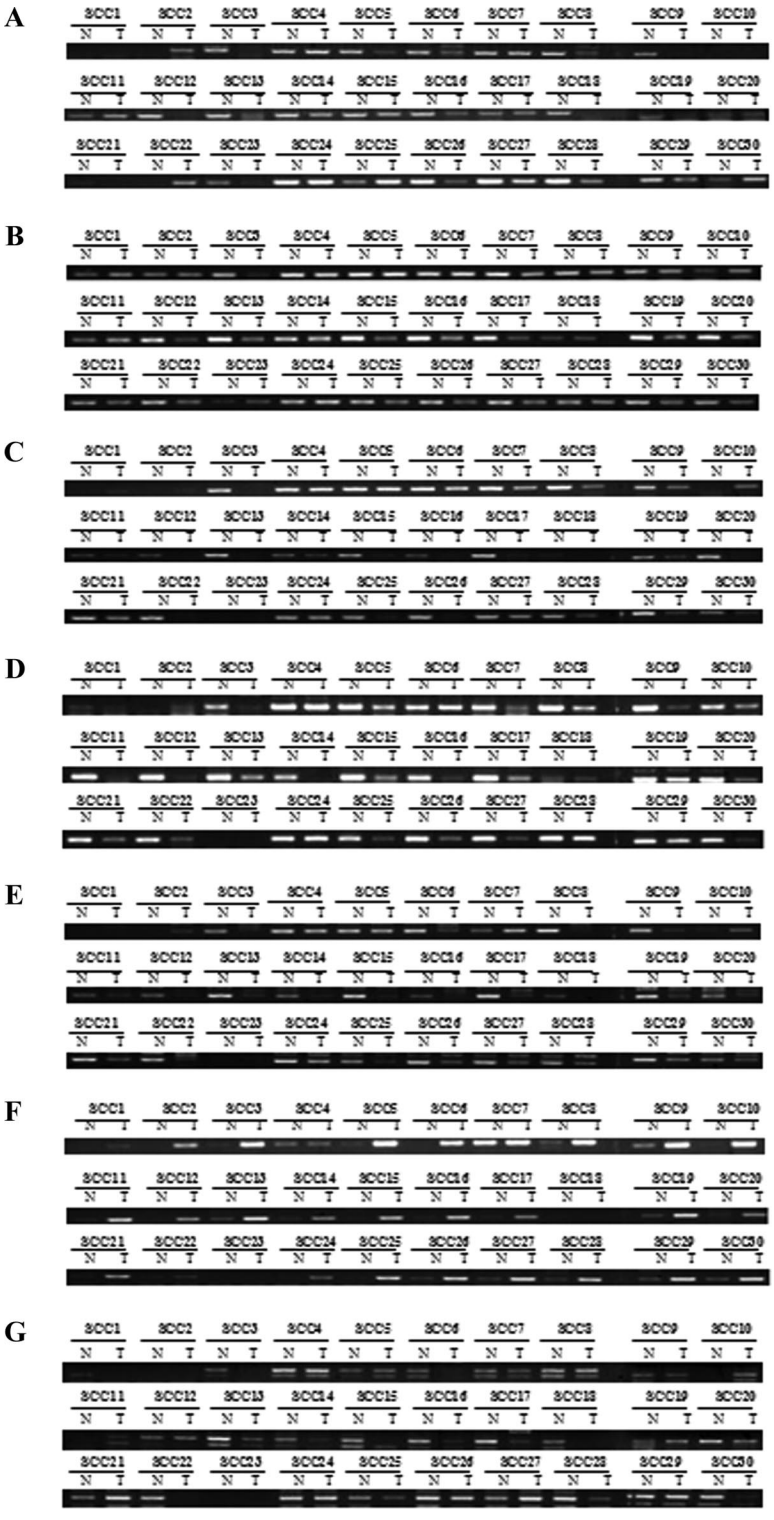

FIGURE 2. Genes confirmed to be in accordance with the whole-genome scale combining analysis in the 30 squamous cell carcinoma (SCC) samples by semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR). *CCDC37* (*A*), *CYTL1* (*B*), *CDO1* (*C*), *LMO3* (*D*), and *SLIT2* (*E*) are hypermethylated and down-regulated genes in the whole-genome scale combining assay. *SERPINB5* (*F*) is a hypomethylated and up-regulated gene in the same assay. *DLEC1* (*G*) is a positive control for the hypermethylated and down-regulated genes. SCC1–30 is frozen tissues from patients with squamous cell carcinoma. N, normal lung tissue; T, tumor.

we added *DLEC1* to Table 3. As we pooled the frozen tissues from only three patients with SCC, the whole-genome DNA methylation pattern should be also validated in a number of tissue samples by methods such as methylation-specific PCR and pyrosequencing. Subsequently, we validated the DNA methylation pattern by pyrosequencing under the conditions listed in Table 4 with 20 SCC samples in which the candidate genes were significantly down or up-regulated. Therefore, we have narrowed the list down to five genes (*CCDC37*, *CYTL1*, *CDO1*, *LMO3*, and *SLIT2*) that were hypermethylated and

down-regulated and one gene that was hypomethylated and up-regulated in specific CpG sites of the promoter, as in Figure 2 and Table 5. The down-regulated genes were proven to be hypermethylated at their promoter regions in more than 75% of the tested tissue samples. We could identify specific CpG sites of the promoter that showed apparent differences of DNA methylation between normal lung and SCC. In addition, with the aid of pyrosequencing, we could obtain the data reflecting the quantitative differences of DNA methylation of specific promoter regions between normal lung and SCC. As listed in Table 5, we additionally performed the pyrosequencing using the genomic DNA from 110 SCC paraffin blocks, and we analyzed these data with Student's *t* test. Through these analyses, we have also confirmed that the DNA methylation patterns of these six genes were significantly different between the normal and tumor tissues. Figure 3 represents the degree of DNA methylation of all the tested tissues, and we can see the definite differences of the DNA methylation level between the normal and tumor tissues.

We compared the level of DNA methylation of these six genes according to the pathologic stage. We found that the DNA methylation level of the *CYTL1* promoter region was increased with an advanced stage, as shown in Figure 4. According to ***TRANSFEC*** database, *CYTL1* (+168) has YY1 transcription binding site (from +169 to +173, GGAGC). Around *CYTL1* (+168) region, from +180 to +190, many Sp1 binding sites exist. YY1 interacts with Sp1 and histone deacetylases (HDACs), which increases repression activity.[22] Moreover, *CYTL1* (+176 and +192) shows similar pattern in terms of relationship between disease progression and methylation. As a matter of fact, the methylation of *CYTL1* (+176 and +192) increased between stages 1 and 2 but not increased between stages 2 and 3 (data not shown). Nevertheless, *CYTL1* (+168) hypermethylation seems not to have an effect on the repression activity by HDACs through the direct interaction with YY1, because YY1 binding is not affected by CpG methylation.[23] Rather, *CYTL1* (+168) hypermethylation in SCC is considered to be involved in the regulation by HDACs themselves, as DNA methylation can affect the HDACs' binding to a promoter and their functional roles.[24,25] Accordingly, *CYTL1* (+168) hypermethylation is likely involved in the repression activity by HDACs and further related to the disease progression of SCC.

DNA Methylation Regulates the Expression of Selected Genes in Lung Cancer

We examined the gene expression of these six genes in the SCC cell lines using RT-PCR. We found that the results were consistent with that from the tissue samples (Figure 5*A*). To verify whether these genes are regulated by DNA methylation in specific regions of their promoter, we tested the demethylation effect on the expression of the five down-regulated genes. When the cell lines were treated with 5-aza-2′-deoxycytidine, the DNA methylation level of these five genes was restored, and their expressions were recovered (Figure 5*B*). Table 6 lists the DNA methylation level of those genes before and after treatment with the demethylating agent in the two SCC cell lines. In this experiment, we could confirm that the expressions of *CCDC37*, *CYTL1*, *CDO1*, *LMO3*, and *SLIT2* in SCC are regulated by DNA methylation on their specific promoter region.

DISCUSSION

DNA methylation is one of the epigenetic modifications that does not change the DNA sequence, but it is able to determine the gene expression by regulating the chromatin organization.[26–28] Epigenetic changes that induce an aberrant gene expression have been suggested as a cause of tumorigenesis.[29] DNA methylation analysis provides the opportunity for discovering the genes related to tumorigenesis and developing new target therapies for restoring epigenetic alteration. It is no surprise that many researchers are interested in this area. Costello et al.[30] reported a global analysis of the methylation status of 1184 unselected CpG islands in each of 98 tumor samples using restriction landmark genomic scanning. Since this report, many researchers have investigated the genome-wide analyses of methylation. Ruike et al.[9] performed methylated DNA immunoprecipitation sequencing (MeDIP-seq) and they obtained the whole-genome DNA methylation profiles for human breast cancer cells. As an another example, Cheung et al.[10] have used methylated DNA immunoprecipitation and whole-genome tiling arrays (MeDIP-chip) to identify the genes and ncRNA regulated by DNA methylation in human testicular cancer.

There have also been whole-genome DNA methylation analyses of lung cancer. Using restriction landmark genomic scanning, Brena et al.[31] found 47-gene methylation signatures that together could distinguish two lung cancer subgroups, adenocarcinoma and SCC, and they demonstrated that the expression of OLIG1 was significantly correlated with the overall survival of patients with NSCLC. Other frequently used assays for whole-genome methylation analysis include expression microarray analysis of cell lines before and after treatment with DNMT inhibitor and with HDAC inhibitors ("pharmacological reactivation"), BeadArray-based methylation analysis of a panel of cancer-related genes (Illumina GoldenGate methylation assay) and microarray analysis in combination with immunoprecipitation of methylated DNA (5-methylcytidine antibody; MeDIP-chip).[3] By using the pharmacological reactivation method, Shames et al.[32] reported that *ALDH1A3*, *BNC1*, *CCNA1*, *CTSZ*, *LOX*, *MSX1*, and *NRCAM* could be methylated only in lung cancer. Meanwhile, using a sodium bisulfite conversion approach combined with the BeadArray technology, Bibikova et al.[33] analyzed the methylation profiles of 1536 CpG sites from 371 cancer-related genes in cancer cell lines and in lung adenocarcinomas. Weber et al.[34] established a strategy to isolate methylated DNA fragments by immunoprecipitation (MeDIP) using an anti-5-methylcytidine antibody and they combined the MeDIP assay with the microarray technology (MeDIP-chip). Using these high throughput approaches, they identified genes that were not known to be methylated in lung cancer, but they realized that in some genes the methylation pattern by single-gene methylation analysis was different from those methylation patterns by these high-throughput approaches. A possible explanation for the differences be-

TABLE 5. *CCDC37*, *CYTL1*, *CDO1*, *LMO3*, *SLIT2*, and *SERPINB5* were Confirmed to be Differently Methylated in Specific Promoter Regions in the SCC Tissue Samples

Hypermethylation

	CCDC37			*CYTL1*					
	−307	**−302**	**−263**	**−72**	**+143**	**+168**	**+176**	**+188**	**+192**
Paraffin	47/61 (77%)	52/61 (85%)	49/61 (80%)	86/110 (78%)	86/110 (78%)	110/110 (100%)	91/110 (83%)	96/110 (87%)	71/110 (64%)
N	14	47	34	7	13	18	14	21	14
T	55.8 (27)	72.9 (11.3)	68.8 (18.4)	19.6 (9.8)	30.1 (14.8)	54.6 (18.3)	29.1 (10.4)	38 (13)	25.6 (11.9)
Difference	41.8	25.9	34.8	12.6	17.1	36.6	15.1	17	11.6
Frozen	14/16 (88%)	13/16 (81%)	13/16 (81%)	14/18 (78%)	12/18 (67%)	16/18 (89%)	14/18 (78%)	12/18 (67%)	9/18 (50%)
N	13.2 (2.9)	55.8 (3.7)	35.5 (4)	8.1 (2.6)	10.9 (2.4)	23.8 (3.9)	13.3 (3.2)	24.6 (5.2)	14.1 (3)
T	35.6 (20)	70.1 (10)	58.5 (16.2)	19.7 (9.8)	27.4 (15)	37.2 (13.1)	27.6 (15.4)	26 (12.4)	32.7 (21.1)
Difference	22.4	14.3	23	11.6	16.5	13.4	14.3	1.4	18.6

Hypermethylation

	LMO3				*SLIT2*		
	−626	**−618**	**−610**	**−568**	**+239**	**+257**	**+284**
Paraffin	94/106 (89%)	94/106 (89%)	92/106 (87%)	94/106 (89%)	56/83 (67%)	55/83 (66%)	14/83 (17%)
N	28	29	22	19	68	42	93
T	63 (16.1)	60.4 (14.3)	53.5 (15.8)	51.8 (15.7)	82.7 (7.6)	64.2 (13.8)	100 (0)
Difference	35	31.4	31.5	32.8	14.7	22.2	7
Frozen	19/19 (100%)	19/19 (100%)	19/19 (100%)	15/19 (79%)	17/20 (85%)	15/20 (75%)	9/20 (45%)
N	87.5 (2)	31.8 (6.5)	22.3 (2.9)	27.3 (11.4)	35.8 (6.4)	22.6 (3.9)	89.1 (0.8)
T	94.2 (2.5)	68.3 (11.3)	58.6 (14.8)	52.3 (11.7)	53.4 (16.9)	38.5 (19.1)	91.4 (1.1)
Difference	6.7	36.5	36.3	25	17.6	15.9	2.3

(*Continued*)

Each number in the N and T line means the methylation level in each CpG site. The numbers below the gene names represent the CpG position. –, + mean upstream and downstream from transcription start site (TSS), respectively. The differences of the DNA methylation level between normal lung and SCC were determined by (tumor score − normal score) and (normal score − tumor score) in the case of hypermethylation and hypomethylation, respectively. Also, the ratio (hypermethylated or hypomethylated in SCC/tested samples) was depicted as a percentage. Normal bronchial epithelial cells were used as a counterpart of the 110 SCC paraffin blocks (the control). Therefore, all the DNA methylation levels from the tissues samples are presented as a mean value except the normal bronchial epithelial cell. In the paraffin block data, the +51 and +55 regions of *SERPINB5* were missed due to pyrosequencing failure. The standard deviation is shown in parenthesis.

SCC, squamous cell carcinoma.

TABLE 5. (*Continued*)

CDO1													
−172	**−170**	**−161**	**−159**	**−146**	**−143**	**−134**	**−117**	**−115**	**−108**	**−99**	**−89**	**−87**	**−80**
35/105 (33%)	22/105 (21%)	99/105 (94%)	97/105 (92%)	71/105 (68%)	43/105 (41%)	80/105 (76%)	74/105 (70%)	79/105 (75%)	94/105 (89%)	83/105 (79%)	81/105 (77%)	42/105 (40%)	80/105 (76%)
39	7	71	24	52	50	42	46	18	75	61	49	24	64
44.1 (3.7)	14 (10.2)	85.1 (4)	29.15 (4)	73.3 (10.6)	58.1 (6.3)	59.7 (7.5)	52.4 (4.7)	25.5 (5.7)	98.5 (9)	77 (10)	54.57 (5.9)	32.9 (7)	78.7 (8.9)
5.06	7	14.1	5.15	21.31	8.07	17.7	6.38	7.5	23.5	16	5.57	8.9	14.7
13/20 (65%)	14/20 (70%)	12/20 (60%)	15/20 (75%)	12/20 (60%)	14/20 (70%)	13/20 (65%)	12/20 (60%)	16/20 (80%)	11/20 (55%)	12/20 (60%)	14/20 (70%)	15/20 (75%)	12/20 (60%)
27.4 (3.4)	3.5 (0.8)	60.7 (14.2)	18.4 (3.8)	36.3 (12.3)	29.2 (6.4)	30.1 (9.9)	34.8 (6.8)	14.2 (2.9)	51.2 (19.8)	42.3 (13.8)	32.8 (8.6)	11.2 (10.6)	49.4 (17.7)
33.1 (4.7)	8.1 (4)	73.3 (8.3)	26.6 (7.2)	58.5 (12.4)	44.4 (10.6)	44.2 (9.7)	49.1 (7.5)	22.9 (8)	81.8 (17.7)	66.6 (14.6)	49.2 (10.1)	31.9 (9.7)	72.9 (9.9)
5.7	4.6	12.6	8.2	22.2	15.2	14.1	14.3	8.7	30.6	24.3	16.4	20.7	23.5

Hypomethylation

SERPINB5										
−40	**−37**	**−32**	**−27**	**−22**	**−17**	**+6**	**+11**	**+33**	**+51**	**+55**
109/110 (99%)	106/110 (96%)	110/110 (100%)	47/110 (43%)	19/110 (17%)	20/110 (18%)	110/110 (100%)	110/110 (100%)	104/110 (95%)	—	—
55	52	50	60	61	59	43	36	70	—	—
18.3 (11.6)	18.3 (13.3)	13.2 (8.6)	33.3 (12)	36.8 (13.7)	38.6 (14.4)	9.9 (8.9)	6.5 (7.8)	29.3 (20.7)	—	—
36.7	33.7	36.8	26.7	24.2	20.4	33.1	29.5	40.7	—	—
17/20 (85%)	13/20 (65%)	18/20 (90%)	17/20 (85%)	18/20 (90%)	17/20 (85%)	18/20 (90%)	17/20 (85%)	17/20 (85%)	17/20 (85%)	19/20 (95%)
89.7 (8.6)	78.1 (3.1)	42.8 (6.1)	64.7 (6.4)	77.3 (4.3)	73.3 (3.3)	37.4 (5.5)	35.4 (5.3)	73.8 (4.4)	47.2 (5)	45.7 (4.7)
46.8 (20.5)	68 (5.8)	25.1 (9.9)	34.8 (14.2)	42.7 (18.3)	39.2 (15.8)	22.1 (7.9)	18.8 (7.2)	42.3 (14)	27.8 (8.3)	24.6 (11.6)
42.9	10.1	17.7	29.9	34.6	34.1	15.3	16.6	31.5	19.4	21.1

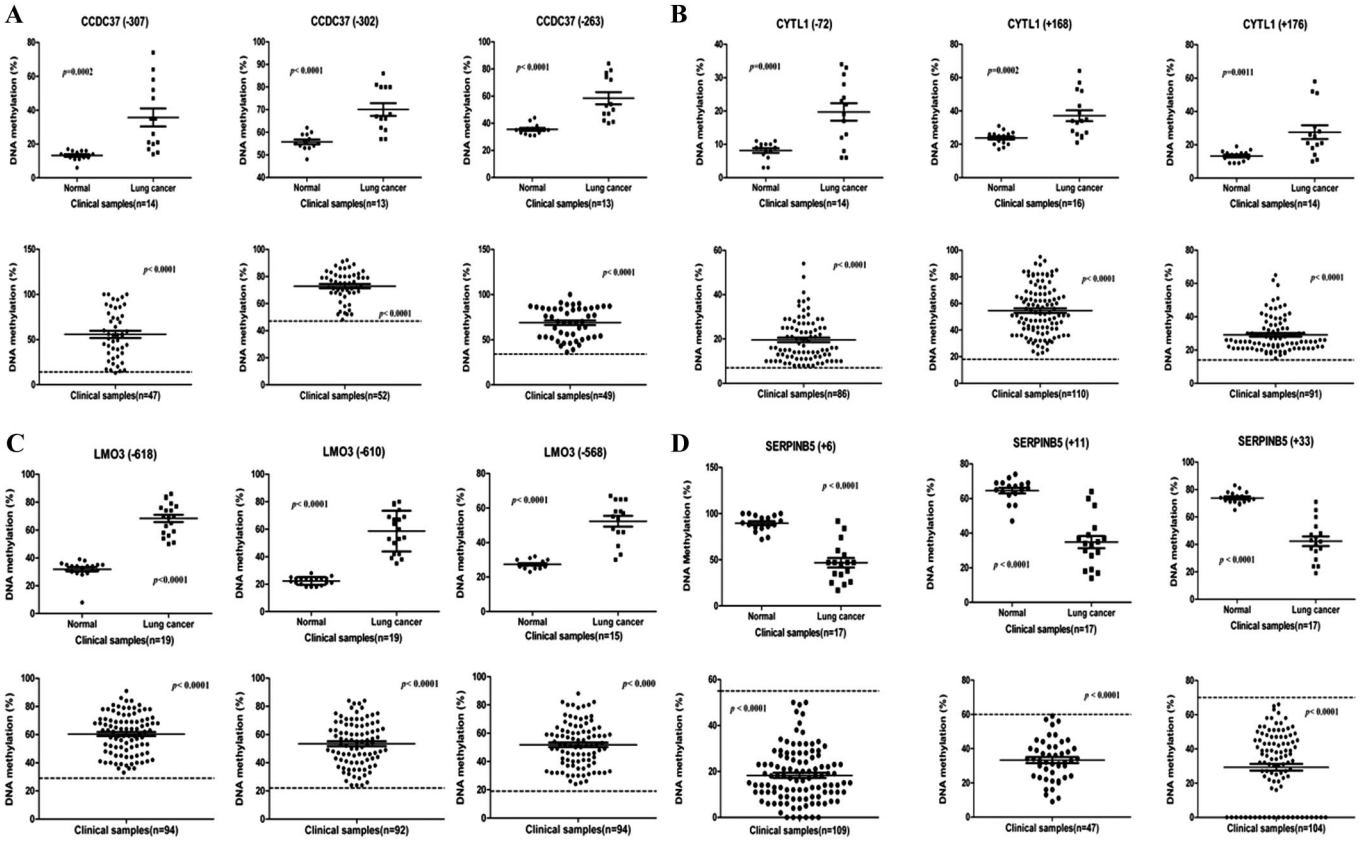

FIGURE 3. Differences in DNA methylation between normal lung and squamous cell carcinoma (SCC). These graphs show the DNA methylation pattern of *CCDC37* (*A*), *CYTL1* (*B*), *LMO3* (*C*), and *SERPINB5* (*D*) in both normal lung and SCC. Four genes among the selected six genes were confirmed to have different DNA methylation patterns as compared between normal lung and SCC. In detail, *CCDC37*, *CYTL1*, and *LMO3* were hypermethylated, and *SERPINB5* was hypomethylated in SCC. Each dot represents the DNA methylation of each sample, which was from frozen tissue or a paraffin block. For statistical analysis, Student's *t* test was applied, and *p* values <0.05 were considered to be significant. The promoter region (the transcription start site is +1) was examined, and the mean value and standard error of the mean of the normal lung and lung cancer are also depicted. Each graph displays the number of samples used and qualified by pyrosequencing. Although the DNA methylation of 20 SCC samples was examined by pyrosequencing, each graph has different sample numbers because of the quality check for pyrosequencing. In each panel, the upper three graphs and lower three graphs are the results from the frozen tissues and SCC paraffin blocks, respectively. In the lower three graphs in each panel, the dotted line means the DNA methylation level of the purified epithelial cells of the representative normal lung.

tween the two analyses could be that mainly a small number of samples were used in the high-throughput analyses.[3] Therefore, to more accurately reflect the DNA methylation status, additional validation procedures should be considered for the whole-genome DNA methylation analysis when using small numbers of samples.

In this study, by combining the DNA methylation pattern and the gene expression data, we performed screening of genes that are possibly regulated by DNA methylation. To increase the fidelity of the screening results from the whole-genome scale analysis, we applied a very strict validation process and we attempted to discover the epigenetically regulated genes in lung cancer. In addition, we examined the gene expression level and the methylation pattern of *DLEC1*, which had been reported to be hypermethylated in approximately 40% of NSCLC.[21] We also tried to use as many tissue samples as possible in the validation steps. First, we validated each gene expression by semiquantitative RT-PCR using 30 tissue samples of SCC and regarded 13 genes as significant candidates. Compared with methylation-specific PCR, pyrosequencing has advantages that it is able to comprehend the quantitative difference of DNA methylation, and it also examines all the CpG sites within 100 bp simultaneously. Therefore, we decided to perform pyrosequencing with 13 genes to more accurately examine the quantitative differences of DNA methylation. Nevertheless, this technique has disadvantage that examination of DNA methylation is impossible if PCR for a specific region fails. Further, as the quantity of the specific PCR product is critical for pyrosequencing, some samples with a PCR product less than the minimal requirement were excluded from the pyrosequencing. As a result, we were able to identify six genes (*CCDC37*, *CYTL1*, *CDO1*, *SERPINB5*, *LMO3*, and *SLIT2*) and find which CpG site of the promoter region was significantly hyper or hypomethy-

lated. After that, to increase the accuracy of the results, we performed additional pyrosequencing for these six genes with 110 paraffin block samples. In this experiment, we used normal bronchial epithelial cells as a normal control for the SCC paraffin block samples, and we used the method we reported in 2008.[35] With this process, we could confirm the similar DNA methylation pattern with those from the 20 frozen tissue samples in four genes: *CCDC37*, *CYTL1*, *LMO3*, and *SERPINB5*. On the other hand, two genes, *CDO1* and *SLIT2*, showed different results from that of the 20 frozen tissue samples, as is presented in Table 5. *CDO1* had different ratios at each CpG site between the frozen tissue samples and the paraffin block samples, whereas *SLIT2* had reduced ratios at all the CpG sites compared with that of the frozen tissue samples. This might have resulted from the fact that the 20 frozen tissue samples were not enough to reflect the accurate ratio of hypermethylation of those two genes.

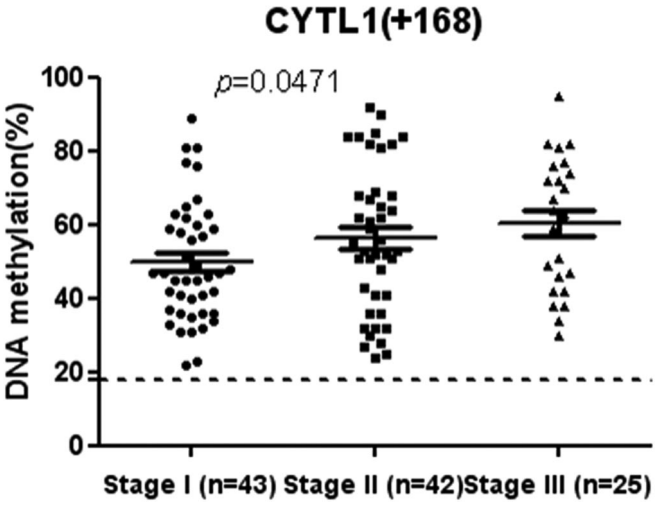

FIGURE 4. The correlation of DNA methylation of *CYTL1* with the squamous cell carcinoma (SCC) stages. The DNA methylation level of *CYTL1* (+168) was compared after dividing the samples according to their pathologic stage. The difference of the DNA methylation levels was 6.49 between stage I and stage II and 4.38 between stage II and stage III. Their *p* value of one-way analysis of variance (ANOVA) analysis was 0.0471. Therefore, the CpG site (+168) of the *CYTL1* promoter region was recognized as a hot spot that has correlation with the SCC stage. The dotted line is the DNA methylation level of normal lung.

We used the SCC cell lines to examine whether the expression level and DNA methylation pattern of the five genes are restored by a demethylating agent. As we treated these cell lines with 5-aza-2′-deoxycytidines, we could see the restoration of DNA methylation of the five genes that were originally hypermethylated. Therefore, we could identify that DNA methylation of specific CpG sites of the promoter region affects the gene expression of the five genes, and the five genes showed the difference of the methylation pattern between normal individuals and patients with SCC, as they are shown in Figure 2 and Table 5, respectively.

Herein, we identified six genes regulated by DNA methylation. Among them, three genes (LMO3, SERPINB5, and SLIT2) have been reported to be related to lung cancer. LMO3 belongs to the LIM-only protein family, and it is known to be down-regulated in SCC of the lung.[36,37] Our data also showed that *LMO3*, which was down-regulated in 23 of 30 SCC samples, was hypermethylated in SCC. LMO3 has been reported to regulate the expression of p53 dependent genes in neuroblastoma by interacting with p53,[38] and it is involved in cell proliferation by interacting with HEN2, a neuronal transcription factor, within a nucleus. Nevertheless, since nothing has been revealed about the function of LMO3 and its binding partner in lung cancer, it is necessary to unveil the role of LMO3 and define the signaling pathway involved with DNA methylation of *LMO3*. SERPINB5 is a tumor suppressor protein that has been shown to inhibit tumor progression and metastasis.[39] According to our data, *SERPINB5* was hypomethylated and up-regulated in SCC. In addition, the aberrant hypomethylation of *SERPINB5* is

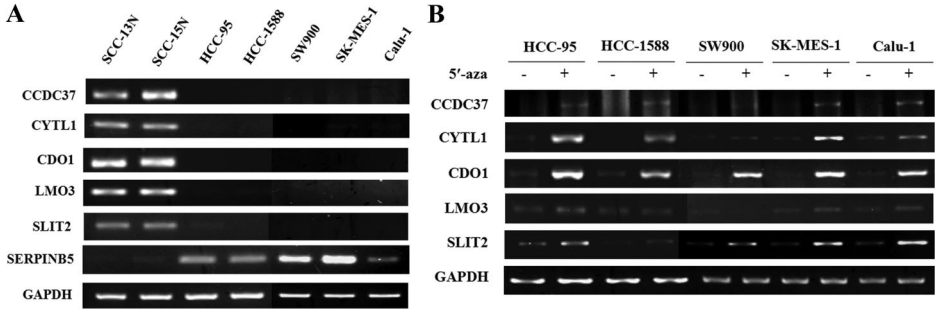

FIGURE 5. Restoration of the gene expression by 5-aza-2′-deoxycytidine treatment in the SCC cell lines. *A*, The total RNA was extracted from the normal lung tissues and two SCC cell lines, and the complementary DNAs (cDNAs) were synthesized. By using the cDNA as a template, semiquantitative RT-PCR was done, and *GAPDH* was used as a loading control. *CCDC37*, *CDO1*, *LMO3*, *CYTL1*, and *SLIT2* were down-regulated, and *SERPINB5* was up-regulated in the SCC cell lines in accordance with the data from the tissue samples. *B*, To validate whether these results were caused by DNA methylation, 5-aza-2′-deoxycytidine treatment was performed in the SCC cell lines. The SCC cell lines were treated with a demethylating agent (20 μM 5-aza-2′-deoxycytidine) for 4 days, and the expression levels of the five genes that were found to be hypermethylated in the previous experiment were recovered to their original expression level in the SCC cell lines. SCC-13N and SCC-15N: normal lung tissue; HCC-95, HCC-1588, Calu-1, SK-MES-1, and SW900: squamous cell carcinoma cell lines.

TABLE 6. Restoration of the DNA Methylation Level in the Specific CpG Sites Residing in the Promoter Region of Each Gene After 5-Aza-2′-Deoxycytidine Treatment in the Two SCC Cell Lines

	Hypermethylation												
	CCDC37				*CYTL1_1*			*CYTL1_3*					
Cell Line	**−324**	**−307**	**−302**	**−263**	**−81**	**−72**	**−48**	**+143**	**+145**	**+168**	**+176**	**+188**	**+192**
95−	8	39	4	88	32	21	39	94	83	100	93	90	44
95+	6	40	3	78	25	15	36	77	68	69	75	77	20
Difference	2	−1	1	10	7	6	3	17	15	31	18	13	24
1588−	86	92	80	85	29	24	35	67	57	95	82	82	30
1588+	59	78	50	81	17	15	27	61	54	67	74	62	22
Difference	27	14	30	4	12	9	8	6	3	28	8	20	8

	Hypermethylation *CDO1*												
Cell Line	**−172**	**−170**	**−161**	**−159**	**−146**	**−143**	**−134**	**−117**	**−115**	**−108**	**−99**	**−89**	**−87**	**−80**
95−	39	40	62	40	54	71	49	59	30	96	73	59	50	100
95+	36	34	60	37	48	65	46	52	27	89	65	54	40	61
Difference	3	6	2	3	6	6	3	7	3	7	8	5	10	39
1588−	40	18	67	35	56	63	50	58	25	88	82	54	33	76
1588+	35	13	53	23	43	44	42	51	17	73	67	46	28	63
Difference	5	5	14	12	13	19	8	7	8	15	15	8	5	13

	Hypermethylation							
	LMO3				*SLIT2*			
Cell Line	**−626**	**−618**	**−610**	**−568**	**+239**	**+257**	**+278**	**+284**
95−	89	91	89	82	30	38	60	68
95+	69	72	69	55	25	29	57	62
Difference	20	19	20	27	5	9	3	6
1588−	94	93	94	92	21	28	55	69
1588+	66	75	67	63	18	23	55	65
Difference	28	18	27	29	3	5	0	4

In the two SCC cell lines, HCC-95 and HCC-1588, the DNA methylation levels of all five genes were restored by treatment with a demethylating agent 5-aza-2′-deoxycytidine. These results provided the evidence that these five genes could be regulated by DNA methylation and the information about which CpG sites are methylated is critical for that regulation.

SCC, squamous cell carcinoma.

frequently observed in human melanoma cell lines.[40] Taken together, it seems that *SERPINB5*, when hypomethylated and up-regulated, functions as a tumor suppressor in SCC of the lung. This is supported by the fact that SERPINB5 reduces the phosphorylation of Akt.[39] SERPINB5 has been reported to be an endogenous inhibitor of histone deacetylase 1 (HDAC1).[41] We assume that it might induce the expression of other tumor suppressor genes. In case of SLIT2, several reports have shown similar results as our data. Dallol et al. reported that SLIT2 is frequently inactivated in breast cancer, colorectal cancer, lung cancer, and glioma by hypermethylation of the CpG islands in its promoter region,[42–44] and Jin et al.[45–47] demonstrated that it might be useful as a therapeutic target for the treatment of human hepatocellular carcinoma. It was reported that the expression of SLIT2 was restored in hepatoma cell lines with a low expression after treatment with 5-aza-2′-deoxycytidine.[46] Our results suggest that deregulation of *SLIT2* by hypermethylation might be associated with the development and progression of SCC. It was recently revealed that SLIT2 attenuation during lung cancer progression deregulates beta-catenin and E-cadherin, and this is associated with a poor prognosis.[47,48] We need more studies on the functions of SLIT2 and its related pathways to more concretely understand how it functions in lung cancer development.

On the other hand, three genes (CDO1, *CYTL1*, and CCDC37) have not been well studied in lung cancer. There was a report that methylation of *CDO1* was a predictive factor for distant metastasis in patients with lymph node-positive/estrogen receptor-positive breast cancer who underwent anthracycline-based chemotherapy.[49] Similarly, our data showed that *CDO1* was hypermethylated and down-regulated in lung cancer. In addition, we found its expression level and methylation pattern were recovered after treatment with a demethylating agent.

In summary, *SLIT2*, *DLEC1*, and *LMO3* showed down-regulation and hypermethylation, and *SERPINB5* showed up-regulation and hypomethylation in SCC. Nevertheless,

two genes, CCDC37 and *CYTL1*, have not been well studied as to their functions in lung cancer. So, we think we need further study of those genes. *CYTL1* is known to control chondrogenesis of mesenchymal cells, and its expression was also reported to be quite decreased in mesenchymal cells.[50] Accordingly, we can speculate that *CYTL1* might be involved in the tumorigenesis of lung cancer as its expression is suppressed during the epithelial to mesenchymal cell transition. We are attempting to get some solid clues about the function of CCDC37 by examining cell proliferation and invasion when this gene is overexpressed (the data are not shown here).

We combined the whole-genome DNA methylation pattern and the gene expression profile specific to SCC of the lung. Through this analysis, we proved this method is very effective in finding genes regulated by DNA methylation, and we found six genes (*CCDC37*, *CYTL1*, *CDO1*, *SLIT2*, *LMO3*, and *SERPINB5*) in SCC of the lung. We hope that this result can contribute to the development of a new method for the early detection and prevention of lung cancer.

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