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SHORT COMMUNICATION

**Masayuki Fukasawa · Mika Kimura · Sumiyo Morita
Kenichi Matsubara · Sumitaka Yamanaka
Chiaki Endo · Akira Sakurada · Masami Sato
Takashi Kondo · Akira Horii · Hiroyuki Sasaki
Izuho Hatada**

Microarray analysis of promoter methylation in lung cancers

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Abstract Aberrant DNA methylation is an important event in carcinogenesis. Of the various regions of a gene that can be methylated in cancers, the promoter is the most important for the regulation of gene expression. Here, we describe a microarray analysis of DNA methylation in the promoter regions of genes using a newly developed promoter-associated methylated DNA amplification DNA chip (PMAD). For each sample, methylated *Hpa* II-resistant DNA fragments and *Msp* I-cleaved (unmethylated + methylated) DNA fragments were amplified and labeled with Cy3 and Cy5 respectively, then hybridized to a microarray containing the promoters of 288 cancer-related genes. Signals from *Hpa* II-resistant (methylated) DNA (Cy3) were normalized to signals from *Msp* I-cleaved (unmethylated + methylated) DNA fragments (Cy5). Normalized signals from lung cancer cell lines were compared to signals from normal lung cells. About 10.9% of the cancer-related genes were hypermethylated in lung cancer cell lines. Notably, HIC1, IRF7, ASC, RIPK3, RASSF1A, FABP3, PRKCDBP, and PAX3 genes were hypermethylated in most lung cancer cell lines examined. The expression profiles of these genes correlated to the methylation profiles of the genes, indicating that the microarray analysis of DNA methylation in the promoter region of the genes is convenient for epigenetic study. Further analysis of primary tumors indicated that the frequency of hypermethylation was high for ASC (82%) and PAX3 (86%) in all tumor types, and high for RIPK3 in small cell carcinoma (57%). This demonstrates that our PMAD method is effective at finding epigenetic changes during cancer.

Keywords DNA methylation · DNA chip · Microarray · CpG island · Hypermethylation

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M. Fukasawa · M. Kimura · S. Morita · I. Hatada (✉)
Laboratory of Genome Science,
Biosignal Genome Resource Center,
Department of Molecular and Cellular Biology,
Gunma University, 3-39-15 Showa-machi,
Maebashi 371-8512, Japan
E-mail: ihatada@showa.gunma-u.ac.jp
Tel.: +81-27-2208057
Fax: +81-27-2208110

M. Fukasawa · M. Kimura · S. Morita · I. Hatada
PRESTO, Japan Science and Technology Corporation (JST),
Kawaguchi, Japan

K. Matsubara
DNA Chip Research Inc, Yokohama, Japan

S. Yamanaka · C. Endo · A. Sakurada · M. Sato · T. Kondo
Department of Thoracic Surgery, Institute of Development,
Aging and Cancer, Tohoku University,
Sendai, Japan

S. Yamanaka · A. Horii
Department of Molecular Pathology,
Tohoku University School of Medicine, Sendai, Japan

H. Sasaki
Division of Human Genetics,
Department of Integrated Genetics,
National Institute of Genetics,
Research Organization of Information and Systems,
and Department of Genetics,
School of Life Science, Graduate University for Advanced Studies,
Mishima, Japan

Introduction

In the human genome, most of the cytosine residues at CpG dinucleotides are methylated, but some remain unmethylated in specific GC-rich areas, called CpG islands (Antequera et al. 1990). Although CpG islands were traditionally considered to be located in 5′ regions of genes and to be kept consistently unmethylated, they are actually located at various positions throughout genes, such as in exons and introns, or further downstream (Takai and Jones 2002). The methylation of promoter regions is associated with a loss of gene expression and it plays an

important role in regulating gene expression. This epigenetic event is associated with the transcriptional silencing of genes involved in differentiation, genomic imprinting, and X inactivation. In cancers, aberrant methylation of 5′ CpG islands of some tumor suppressor genes has been reported (Baylin et al. 1997).

Techniques such as restriction landmark genomic scanning (RLGS) and a representational difference analysis (RDA)-based method have been developed to scan for differences in methylation in the genome in order to identify imprinted genes and aberrantly methylated genes in cancer (Hatada et al. 1993; Ushijima et al. 1997). Recently, we and others have developed microarray-based techniques to scan for differences in methylation in the genome (Hatada et al. 2002; Yan et al. 2001). Using these methods, methylated fragments in the genome are amplified and hybridized to microarrays that contain clones from libraries of CpG islands. However, methylation in cancer cells frequently occurs in CpG islands outside of promoter regions. In some cases, methylation outside the promoter induces a condensed chromatin gene structure and prevents binding of transcription factors to the promoter (Pieper 1996). However, in most cases methylation outside of promoter regions do not repress gene transcription (Ushijima 2005). To solve this problem, we cloned the promoters of genes and used them to make a microarray in this study.

Here, we describe a new method of scanning for methylation using a microarray that contains promoters of 288 cancer-related genes. We used this method to perform methylation-based analysis of lung cancers.

Materials and methods

Promoter-associated methylated DNA amplification DNA chip (PMAD) method

Each sample was used to amplify both *Hpa* II-resistant DNA and *Msp* I-cleaved DNA. The procedure is illustrated in Fig. 1a. To amplify methylated *Hpa* II-resistant DNA fragments, 0.5 μg of genomic DNA was digested

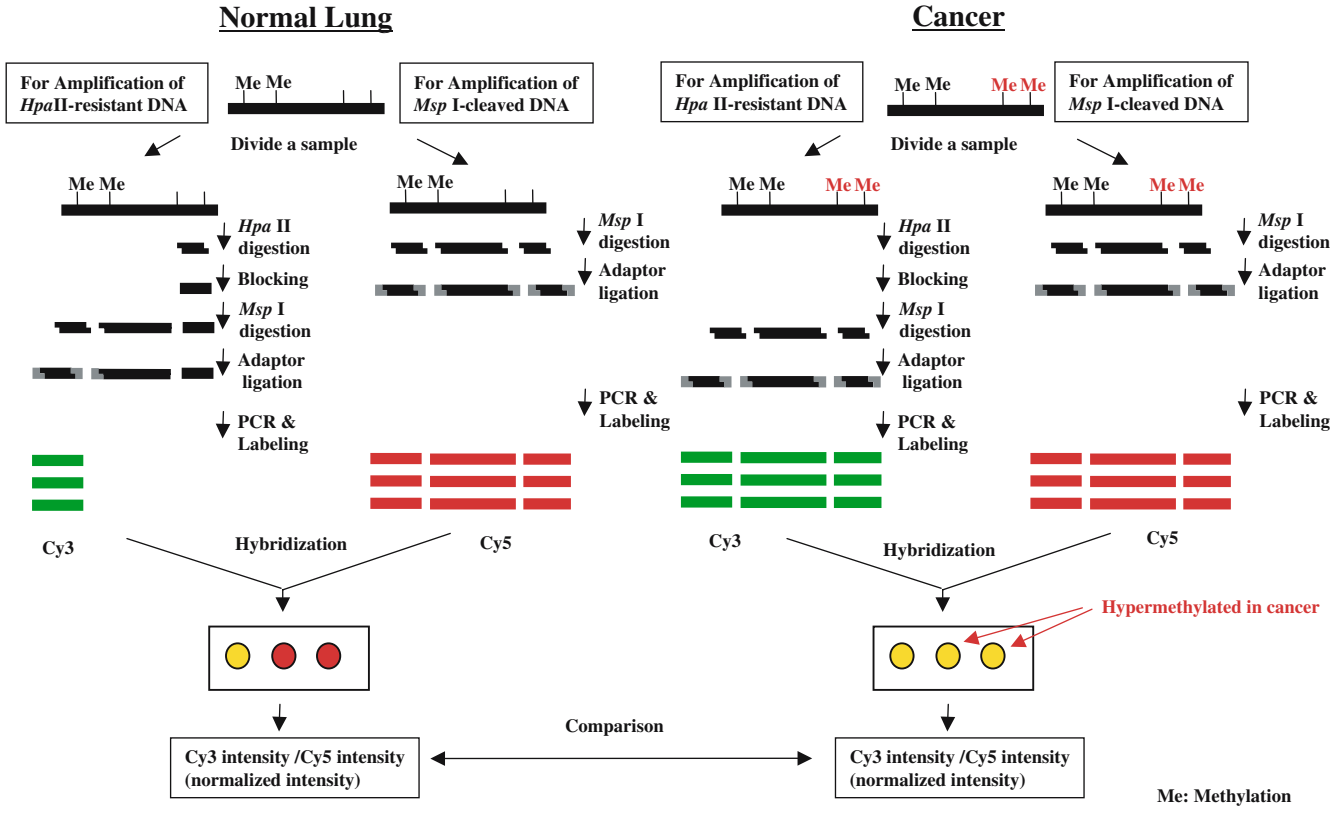

Fig. 1 Schematic flowchart for the promoter-associated methylated DNA amplification DNA chip (PMAD) method. *Me* represents a methylated *Hpa* II site. Each sample was used for both amplification of *Hpa* II-resistant DNA and *Msp* I-cleaved DNA. To amplify methylated *Hpa* II-resistant DNA fragments, genomic DNA was digested with *Hpa* II followed by treatment with alkaline phosphatase and the Klenow enzyme to block the ends of unmethylated DNA fragments. Blocked DNA was digested with *Msp* I to cleave the methylated *Hpa* II sites followed by ligation to the adaptor. PCR was performed using the adaptor primer. As a result, only *Hpa* II-resistant DNA was amplified. For the amplification of *Msp* I-cleaved (unmethylated plus methylated) DNA fragments, genomic DNA was digested with *Msp* I followed by ligation to the adaptor. PCR was performed using the adaptor primer. Amplified DNAs were labeled with Cy3 and Cy5 respectively, cohybridized to the microarray with promoter regions of 288 cancer-related genes, scanned and analyzed. Cy3 intensity (*Hpa* II-resistant DNA fragments) was normalized to Cy5 intensity (*Msp* I-cleaved DNA fragments) for comparison among samples (normalized intensity = Cy3 intensity/Cy5 intensity). Normalized intensities of cancer and normal lungs were compared

overnight with 50 units of *Hpa* II. The digests were treated with alkaline phosphatase, which was followed by a fill-in reaction using Klenow enzyme to block the ends of unmethylated DNA fragments. Blocked DNA was digested overnight with 50 units of *Map* I to cleave the methylated *Hpa* II sites, followed by ligation to 11 pmol of the adaptor. The adaptor was prepared by annealing two oligonucleotides, AGCACTCTCCAGCCTCTCACCGAC and CGGTCGGTGA. PCR was performed using 0.1 μg of each ligation mix as a template in a 100-μl volume containing 100 pmol of the primer AGCACTCTCCAGCCTCTCACCGAC and 1.25 units of GeneTaq DNA polymerase. The reaction mixture was incubated for 5 min at 72 °C and 3 min at 94 °C and subjected to cycles of amplification consisting of 10 s of denaturation at 94 °C, 30 s of annealing at 70 °C and 2.5 min extension at 72 °C. The final extension was lengthened over 9.5 min.

To amplify *Msp* I-cleaved (unmethylated + methylated) DNA fragments, 0.5 μg of genomic DNA was digested overnight with 50 units of *Msp* I followed by ligation to 11 pmol of the adaptor. PCR was performed by the same procedure as was applied to amplify methylated *Hpa* II-resistant DNA fragments.

To make the microarrays, PCR primers were selected from the promoter regions of 288 cancer-related genes (Supplementary Table 1). All of the promoter sequences are included in at least one short *Msp* I fragment. PCR products were cloned into the vector pCR2.1 (Invitrogen, Carlsbad, CA, USA) and introduced into *E. coli*. Each colony was amplified by PCR using CCAGTGT GCTGGAATTCGGC and ATGGATAT CTGCAG AATTCGGC as primers. The reaction mixture was incubated for 5 min at 94 °C and subjected to 40 cycles of amplification consisting of 10 s denaturation at 94 °C, 30 s annealing at 60 °C and 1 min extension at 72 °C. Four DNA sequences without any homology to the human genome were also amplified as control spots. Amplified DNA fragments were fixed on poly-*L*-lysine-coated microscope slides in triplicate as described (Schena et al. 1995) using a SPBIO-2000 (Hitachi Software Engineering, Tokyo, Japan) arrayer.

Amplified DNAs mixed with 10 pg of DNA complementary to control spots were labeled with Cy3 and Cy5 respectively, cohybridized to the microarray, scanned using a Scan Array Lite (Perkin Elmer, Boston, MA, USA) scanner, and analyzed with the software DNASIS Array (Hitachi Software Engineering). Labeling efficiency was normalized using the signal intensities of the control spots. Cy3 intensity (*Hpa* II-resistant DNA fragments) was normalized to Cy5 intensity (*Msp* I-cleaved DNA fragments) for comparison among samples (normalized intensity = Cy3 intensity/Cy5 intensity). The spots whose Cy5 intensities were higher than background were analyzed. We judged the spots as hypermethylated compared to normal lung when their (normalized intensity of cancer)/(normalized intensity of normal) ratios were more than 3.0 and the normalized intensity of cancer was high enough (more than 0.2).

Combined bisulfite restriction analysis (COBRA)

Genomic DNA was treated with sodium bisulfite using a CpGenome DNA Modification Kit (Intergen Co., Purchase, NY, USA) and subjected to combined bisulfite restriction analysis (COBRA). PCR products were digested with *Bsi*EI (HIC1), *Taq* I (IRF7), *Hha* I (ASC), *Hinf* I (RIPK3), *Hha* I (RASSF1A), *Hin*c II (FABP3), *Taq* I (PRKCDBP), and *Ban* III (PAX3), respectively. PCRs were performed using the following primers: HIC1, GGTAATTGTTTTTAAAAGGGTTATTG and TACCCTCTAAAATAAAAACCCAAAC; IRF7, GTAGAGTTAAGAGTTGGGGGAGTTT and TATTAAA CCAATATCCAAACCTAAC; ASC, TTTTAGTA TGTGGAATTGAGGGAGT and AAACCTCTAAA TTAAAACCCCAAAC; RIPK3, TTTTTGGT ATTTTTTAGTTTGATGT and AACTCCTAATTCT CCAATTCCTC; RASSFF1A, AGTTTTTGT ATTTA GGTTTTTATTG and AACTCAATAAACTCAAA CTCCCC; FABP3, GTTTAGAGGTTA GGAAAGGGAGAAG and CAAACTAAAA CTCACCCAAAA AAAA; PRKCDBP, AAATAGGTATATTAGGGAATTGGAG and AACTCCAACTATAACTCAAACAAAC; and PAX3, GGTTTTTGGATTAGGAAT and TAATCATCCTAAAAACAACTTC.

RT-PCR

RT-PCR was performed using the following primers: HIC1, GCTGCTGCAGCTCAACAACCA and GGCCGGTGTAGATGAAGTCCA; IRF7, TACCATCTACCTGGGCTTCG and GCTCCATAA GGAAGCACTCG; ASC, TGACGGATGAGCAGTACCAG and TCCTCCACCAGGTAGGACTG; RIPK3, CTTCCAGGAATGCCTACCAA and TCCAT TTCTGTCCCTCCTTG; RASSFF1A, CTTCAT CTGGGGCGTCGTG and CTGTGTAAGAACC GTCCTTGTTC; FABP3, CATCACTAT GGTGGACGCTTTCC and CTCATCGAACTCCAC CCCCAAC; PRKCDBP, AGCTCCA CGTTCT GCTCTTC and CGGAGGCTCTGTACCTT CTG; and PAX3, CTGGAACATTTGCCCAGACT and TAT CCAGGTGAAGGCGAAAC.

Results and discussion

We have developed a PMAD for analyzing DNA methylation in the promoter regions of genes (Fig. 1). This method can be used to amplify and compare methylated DNA fragments. A methylation-sensitive restriction enzyme *Hpa* II and its methylation-insensitive isoschizomer, *Msp* I were utilized because most CpG islands contain their recognition sequence, "CCGG". For each sample, methylated *Hpa* II-resistant DNA fragments and *Msp* I-cleaved (unmethylated plus methylated) DNA fragments were amplified and labeled with Cy3 and Cy5 respectively, then hybridized to the microarray which contains the promoter regions of 288

Table 1 Summary of the results for PMAD analysis. Genes that were hypermethylated in at least two of six lung cancer cell lines are presented. M represents hypermethylation in cancer defined as (Normalized intensity of cancer)/(Normalized intensity of normal) more than 3.0 when normalized intensity of cancer is high enough (more than 0.2). NC represents an absence of hypermethylation in cancer defined as (Normalized intensity of cancer)/(Normalized intensity of normal) less than 3.0. Results which do not meet the criteria of M and NC, such as Cy5 intensity less than background or normalized intensity of cancer less than 0.2, are represented as ND

Symbol	Position	1–87	A549	RERF-LCMS	LK79	S2	SBC-3	Hyp. Met. Cells	Description
HIC1	17p13.3	M	M	M	M	M	M	6	hypermethylated in cancer 1
IRF7	11p15.5	M	M	M	M	M	M	6	interferon regulatory factor 7
ASC	16p12–p11.2	M	M	M	M	M	M	6	apoptosis-associated speck-like protein containing a CARD
RIPK3	14q11.2	M	M	M	M	M	M	6	Receptor-interacting serine-threonine kinase 3
RASSF1A	3p21.3	M	M	ND	M	M	M	5	Ras association (RalGDS/AF-6) domain family 1
FABP3	1p33-p32	M	M	M	M	M	ND	5	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
PRKCDBP	11p15.4	M	M	M	ND	M	M	5	protein kinase C, delta binding protein
PAX3	2q35	M	M	NC	M	M	M	5	paired box gene 3 (Waardenburg syndrome 1)
HTR1B	6q13	M	M	NC	M	NC	M	4	5-hydroxytryptamine (serotonin) receptor 1B
CALCA	11p15.2-p15.1	ND	M	ND	M	M	M	4	calcitonin/calcitonin-related polypeptide, alpha
CDH13	16q24.2-q24.3	M	M	NC	M	NC	M	4	Cadherin 13, H-cadherin (heart)
DLEC1	3p22-p21.3	M	M	M	NC	ND	M	4	deleted in lung and esophageal cancer 1
SYK	9q22	M	NC	M	M	M	NC	4	spleen tyrosine kinase
CD38	4p15	M	NC	NC	M	M	M	4	CD38 antigen (p45)
MYOD1	11p15.4	M	ND	ND	M	NC	M	3	myogenic factor 3
WT1	11p13	M	ND	NC	M	ND	M	3	Wilms tumor 1
RARB	3p24	M	NC	NC	NC	M	NC	2	retinoic acid receptor, beta
PTGS2	1q25.2-q25.3	ND	NC	NC	M	NC	M	2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
LRP2	2q24-q31	NC	NC	NC	NC	M	M	2	low density lipoprotein-related protein 2
SLC5A5	19p13.2-p12	M	ND	ND	NC	ND	M	2	solute carrier family 5 (sodium iodide symporter), member 5
CTNNB1	3p21	NC	NC	NC	M	M	ND	2	catenin (cadherin-associated protein), beta 1, 88kDa
AIM1	6q21	NC	NC	M	NC	NC	M	2	absent in melanoma 1
ETV6	12p13	NC	NC	M	M	NC	NC	2	ets variant gene 6 (TEL oncogene)
MSF	17q25	NC	NC	NC	M	NC	M	2	MLL septin-like fusion
DLC1	8p22-p21.3	M	NC	ND	NC	NC	M	2	deleted in liver cancer 1
BNIP3L	8p21	NC	NC	NC	M	M	NC	2	BNIP3L
CASP3	4q34	NC	M	M	NC	NC	NC	2	Caspase 3, apoptosis-related cysteine protease

cancer-related genes. Signals from *Hpa* II-resistant (methylated) DNA (Cy3) were normalized using signals from *Msp* I-cleaved (unmethylated plus methylated) DNA fragments (Cy5).

To amplify methylated *Hpa* II-resistant DNA fragments, genomic DNA was cleaved with the methylation-sensitive restriction enzyme, *Hpa* II. This was followed by the blocking of cleaved ends by alkaline phosphatase and then a fill-in reaction. At this stage, unmethylated *Hpa* II sites were blocked. Blocked DNA was treated with the methylation-resistant isoschizomer, *Msp* I, to cleave the methylated *Hpa* II sites. At this stage, only methylated *Hpa* II sites had 5′ protruding ends that could be ligated to an adaptor. These ends were ligated to the adaptor, which was followed by PCR-amplification. To amplify *Msp* I-cleaved (unmethylated plus methylated) DNA fragments, genomic DNA was cleaved with the methylation-resistant isoschizomer *Msp* I, followed by ligation to an adaptor and PCR. As a result, both unmethylated and methylated DNA fragments were amplified. Amplified DNAs mixed with 10 pg of DNA complementary to control spots were labeled with Cy3 (*Hpa* II-resistant DNA fragments) and Cy5 (*Msp* I-cleaved DNA fragments) respectively, and cohybridized to the microarray, which contained the promoter regions of 288 cancer-related genes including 64 reported to be hypermethylated in cancers. Labeling efficiency was normalized using the signal intensities of the four control spots whose DNA sequences did not have any homology to the human genome. Cy3 intensity (*Hpa* II-resistant DNA fragments) was normalized to Cy5 intensity (*Msp* I-cleaved DNA fragments) for comparison among samples (normalized intensity = Cy3 intensity/Cy5 intensity). The spots whose Cy5 intensities were higher than the background were analyzed. We judged the spots as hypermethylated compared to normal lung when their (normalized intensity of cancer)/(normalized intensity of normal) ratio was more than 3.0 and the normalized intensity of cancer was high enough (more than 0.2).

We applied PMAD to six lung cancer cell lines (1–87, A549, RERF-LCMS, LK79, S2, and SBC-3) and a normal lung. Genes hypermethylated in at least two of six lung cancer cell lines were presented (Table 1) . On

Table 2 Proportion of hypermethylated genes in lung cancers

Types of Cancer	Adenocarcinoma			Small cell carcinoma			Average (%)
Names of cell lines	1–87	A549	RERF-LCMS	LK79	S-2	SBC-3	
Hypermethylated Genes (%)	22(9.5%)	15(6.0%)	17(8.0%)	20(8.6%)	32(14.7%)	45(18.6%)	10.9%
Average(%)	7.8%			14.0%			

average, 10.9% of the cancer-related genes were hypermethylated in these cancer cell lines (Table 2). This value is much higher than that described in a previous report by Yan et al. (2001) in breast cancer; where only 1% of regions examined were hypermethylated. There are two possible explanations. The first possibility is that these two studies reflect differences between cancers arising in the breast and the lung. Consistent with this, the rate of methylation differed among the cancer types. The average hypermethylated rate was 7.8% for adenocarcinoma (1–87, A549, RERF-LCMS) and 14.0% for small cell carcinoma (LK79, S-2, SBC-3), respectively (Table 2). The second possibility is that genes are more liable to be hypermethylated in cancers.

We further analyzed the eight genes that were hypermethylated in at least five of the six (83%) cancer cell lines that we analyzed (Fig. 2a). These were HIC1, IRF7, ASC, RIPK3, RASSF1A, FABP3, PRKCDBP, and PAX3. We confirmed these results using the COBRA method and found that 98% of the PMAD results corresponded to the COBRA results (Fig. 2). Thus, the reliability of this method was demonstrated. Next, we performed an expression analysis of these genes by RT-PCR (Fig. 3; Table 1). The expression profile of the genes correlated to the methylation profile of the genes (Figs. 2, 3). This result indicates that the microarray analysis of DNA methylation in the promoter region of the genes is convenient for detecting methylation, which is responsible for their expression. Considering that CpG islands are actually located at various positions throughout genes, such as in exons and introns, or further downstream (Takai and Jones 2002), analysis of CpG islands located in the promoter region of the genes is extremely convenient for epigenetic study. Shi et al. (2002, 2003) reported a microarray using CpG island clones that screened a cDNA library via hybridization of the 5′-end. Although 79% of the sequences are located in the promoter and first exon, others are outside of these regions. Comparing with this array, all of the genes in our array contain the promoter region of the genes.

HIC1, RASSF1A, and PRKCDBP were previously reported as hypermethylated genes in lung cancer (Issa et al. 1997; Dammann et al. 2000; Xu et al. 2001), but this is the first report indicating that IRF7, ASC, FABP3, and PAX3 are also hypermethylated in lung cancer, although these were previously known as hypermethylated genes in cancers other than that of the lung (Yu et al. 2003; Levine et al. 2003; Huynh et al. 1996; Kurmasheva et al. 2005). Receptor-interacting serine-threonine kinase (RIPK) 3, which is part of the same family as RIPK1, which contains a death domain, has never been reported to be hypermethylated in any cancers before our report. Interestingly, the locations of HIC1, RIPK3, FABP3, and PRKCDBP were reported to lose heterozygosity in lung cancer (Konishi et al. 1998; Abujiang et al. 1998; Chizhikov et al. 2001; Petersen et al. 1997).

Further methylation analyses of primary tumors were performed for IRF7, ASC, RIPK3, FABP3, and PAX3 (Fig. 4), the hypermethylation of which has not been previously reported for lung cancers. The frequency of hypermethylation was high for ASC (82%) and PAX3 (86%). The frequency of hypermethylation was not high for IRF7, RIPK3, and FABP3 compared to analysis in

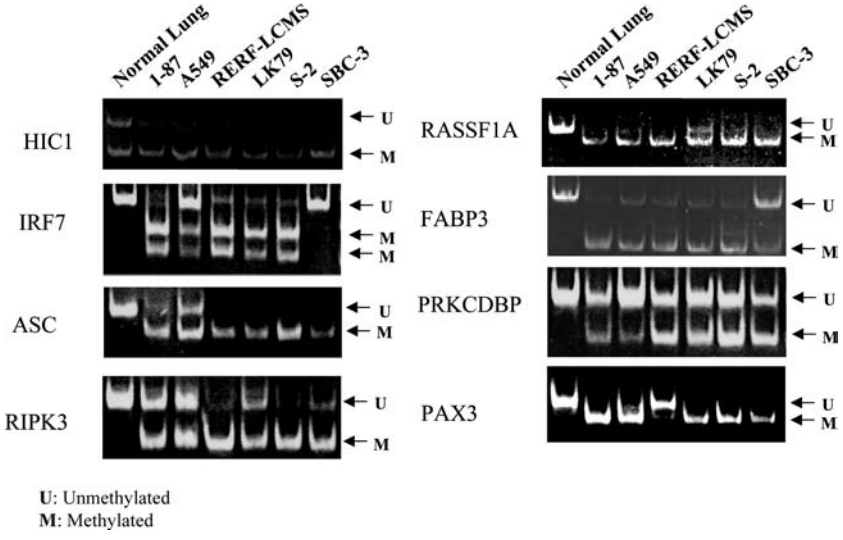

Fig. 2 Eight genes were hypermethylated in five or more of the six lung cancer cell lines analyzed. The PMAD results were confirmed by the COBRA method

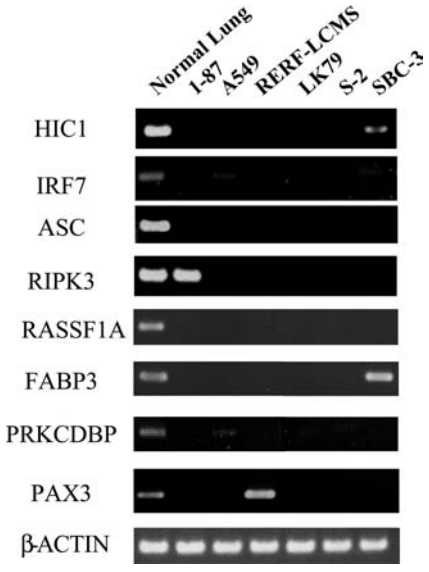

Fig. 3 RT-PCR analyses of eight hypermethylated genes in Fig. 2. Expression of *ACTB* (*β*-actin) was monitored as an internal control

cell lines. However, the frequency of hypermethylation of RIPK3 in small cell carcinoma was high (57%). Apoptosis-associated speck-like protein (ASC) is up-regulated by inflammation and apoptosis via the activation of caspase (Shinohara et al. 2002). In normal cells, this protein is localized to the cytoplasm; however, in cells undergoing apoptosis, it forms ball-like aggregates near the nuclear periphery. This gene is hypermethylated in breast cancer (Levine et al. 2003). Paired box gene 3 (PAX3) was recently reported to be hypermethylated in rhabdomyosarcoma (Kurmasheva et al. 2005). This gene is a member of the paired box (PAX) family of transcription factors. Members of the PAX family typically contain a paired box domain and a paired-type homeodomain. These genes play critical roles during fetal development. Mutations in paired box gene 3 are associated with Waardenburg syndrome, craniofacial-deafness-hand syndrome, and alveolar rhabdomyosarcoma. The translocation t (2; 13)(q35; q14), which represents a fusion of PAX3 and the forkhead gene, is a frequent finding in alveolar rhabdomyosarcoma (Shapiro et al. 1993). Interestingly, loss of 2q36, where this gene is located, was reported in non-small cell lung cancer (NSCLC) (Petersen et al. 1997).

Thus, we identified several interesting findings on PMAD analysis. One of the merits of our method is that it uses only cancer-related genes for a microarray. This enables us to detect methylation changes that occur only in cancer-related genes. If we find common epigenetic changes in cancers, we can then consider the biological meanings of those changes. However, it is true that this approach could overlook unexpected changes in other genes, so it is also important to use genome-wide microarrays. However, too many changes in genes of unknown function make it difficult to narrow down the targets in a genome-wide approach, making it time-consuming and expensive. On the other hand, our cancer-related microarray is not expensive and analysis is easy.

In summary, we have developed a PMAD and found it very useful for analyzing DNA methylation in cancers, because the microarray contains critical promoter regions of each cancer-related gene, the methylation of which is highly related to the repression of the gene. We found an unexpectedly high rate of hypermethylation in lung cancer cell lines, especially in HIC1, IRF7, ASC, RIKPK3, RASSF1A, FABP3, PRKCDBP, and PAX3. This demonstrates that our PMAD method is effective at finding epigenetic changes during cancer. Further anal-

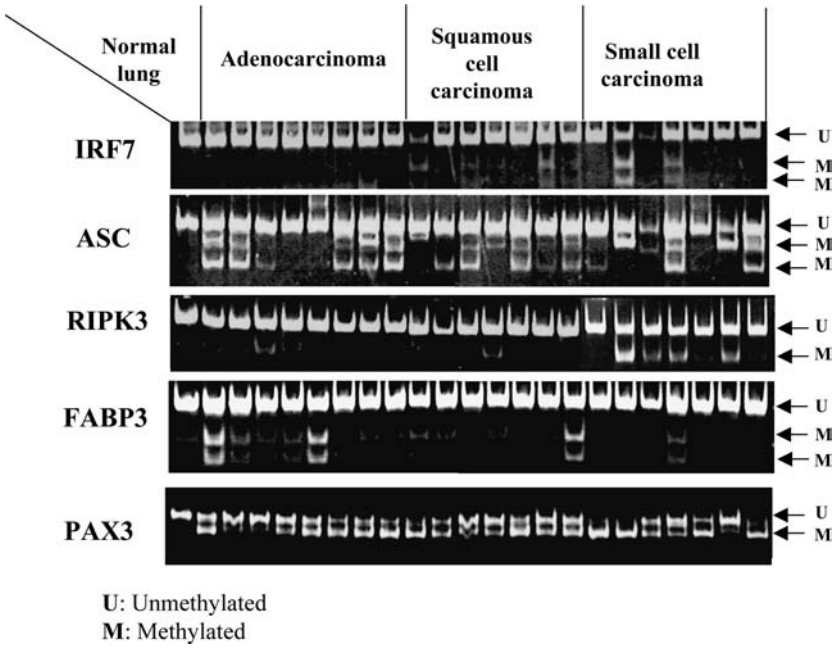

Fig. 4 Methylation analysis of five genes in primary tumors. COBRA analysis was performed for IRF7, ASC, RIPK3, FABP3, and PAX3. PCR primers and restriction enzymes used were the same as in Fig. 2. The present study was approved by the Ethics Committees of Tohoku University School of Medicine and Gunma University. Following a complete description of the research protocol, written informed consent was obtained from each participant

ysis of primary tumors indicated that the frequency of hypermethylation was high for ASC (82%) and PAX3 (86%) in all tumor types and high for RIPK3 in small cell carcinoma (57%).

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