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**Title: 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 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 plus 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 plus 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 profile of these genes correlated to the methylation profile 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 tumours 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 for finding epigenetic changes in cancer.

**Keywords**

DNA methylation, DNA chip, microarray, CpG island, hypermethylation

## 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 consistently kept 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 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 tumour 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 promoter regions. In some cases, methylation outside the promoter induces a condensed chromatin structure of the gene and prevents binding of transcription factors to the promoter (Pieper 1996). However in most cases methylation outside 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 which contains promoters of 288 cancer-related genes. We used this method for a methylation-based analysis of lung cancers.

## Materials and Methods

### Promoter-associated Methylated DNA Amplification DNA chip (PMAD) method

Each sample was used for both amplification of *Hpa* II-resistant DNA and *Msp* I-cleaved DNA. The procedure is illustrated in Fig. 1A. For the amplification of methylated *Hpa* II-resistant DNA fragments, 0.5 μg of genomic DNA was digested overnight with 50 units of *Hpa* II. The digests were treated with Alkaline phosphatase followed by fill in reaction by Klenow enzyme to block the ends of unmethylated DNA fragments. Blocked DNA was digested overnight with 50 units of *Msp* 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$^{o}$C and 3 min at 94$^{o}$C and subjected to cycles of amplification consisting of 10 s denaturation at 94$^{o}$C, 30 s annealing at 70$^{o}$C and 2.5 min extension at 72$^{o}$C. The final extension was lengthened over 9.5 min.

For the amplification of *Msp* I-cleaved (unmethylated plus 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 for the amplification of 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 the promoter

sequences are at least included in one short *Msp* I fragment. PCR products were cloned into the vector pCR2.1 (Invitrogen) and introduced into *E. coli*. Each colony was amplified by PCR using CCAGTGTGCTGGAATTCGGC and ATGGATATCTGCAGAATTCGGC as primers. The reaction mixture was incubated for 5 min at 94$^{o}$C and subjected to 40 cycles of amplification consisting of 10 s denaturation at 94$^{o}$C, 30 s annealing at 60$^{o}$C and 1 min extension at 72$^{o}$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 Co., Ltd) arrayer.

Amplified DNAs mixed with 10 pg of DNA complementary to control spots were labeled with Cy3 and Cy5 respectively, cohybridzed to the microarray, scanned using a Scan Array Lite (PerkinElmer) scanner, and analyzed with the software DNASIS Array (Hitachi Software Engineering). Labeling efficiency was normalized by 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) were more than 3.0 and 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) 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), *Hinc* II (FABP3), *Taq* I (PRKCDBP), and *Ban* III (PAX3),

respectively. PCRs were performed using the following primers: HIC1, GGTAATTGTTTTTAAAAGGGTTATTG and TACCCTCTAAAATAAAAACCCAAAC; IRF7, GTAGAGTTAAGAGTTGGGGGAGTTT and TATTAAACCAATATCCAAACCTAAC; ASC, TTTTAGTATGTGGAATTGAGGGAGT and AAACCTCTAAATTAAAACCCCAAAC; RIPK3, TTTTTGGTATTTTTTAGTTTGATGT and AACTCCTAATTCTCCAATTCCTC; RASSFF1A, AGTTTTTGTATTTAGGTTTTTATTG and AACTCAATAAACTCAAACTCCCC; FABP3, GTTTAGAGGTTAGGAAAGGGAGAAG and CAAACTAAAACTCACCCAAAAAAAA; 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 GCTCCATAAGGAAGCACTCG; ASC, TGACGGATGAGCAGTACCAG and TCCTCCACCAGGTAGGACTG; RIPK3, CTTCCAGGAATGCCTACCAA and TCCATTTCTGTCCCTCCTTG; RASSFF1A, CTTCATCTGGGGCGTCGTG and CTGTGTAAGAACCGTCCTTGTTC; FABP3, CATCACTATGGTGGACGCTTTCC and CTCATCGAACTCCACCCCCAAC; PRKCDBP, AGCTCCACGTTCTGCTCTTC and CGGAGGCTCTGTACCTTCTG; and PAX3, CTGGAACATTTGCCCAGACT and TATCCAGGTGAAGGCGAAAC.

**Results and Discussion**

We have developed a Promoter-associated Methylated DNA Amplification DNA chip (PMAD) for analysis of DNA methylation in the promoter regions of genes (Fig.1). Using this method, methylated DNA fragments can be amplified and compared. 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 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).

For the amplification of 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 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 followed by PCR-amplification. For the amplification of *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 by 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 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 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 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 and Table 1).

The expression profile of the genes correlated to the methylation profile of the genes (Fig.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 the microarray using CpG island clones screening by hybridization of 5' end cDNA library. Although 79% of the sequences located in the promoter and first exon, others are out of these regions. As comparison with this array, all genes on 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 of RIPK1 containing 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 tumours were performed for IRF7, ASC, RIPK3, FABP3, and PAX3 (Fig. 4) whose hypermethylation in lung cancers has not been previously reported. 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 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 using only cancer-related genes for a microarray. This enables us to detect methylation changes occurring only in cancer-related genes. If we find common epigenetic changes in cancers, it is easy to think the biological meaning of those changes. However, it is true this approach miss the changes in unexpected genes. So it is also important to use genome-wide microarray. However, too many changes of the genes of unknown function make it difficult to narrow down the targets in genome-wide approach. So it is a time-consuming and expensive analysis. On the other hand, our cancer-related microarray is not expensive to perform and analysis is easy.

In summary, we developed a Promoter-associated Methylated DNA Amplification DNA chip (PMAD) and found it very useful for analyzing DNA

methylation in cancers because the microarray contains critical promoter regions of each cancer-related gene whose methylation 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 for finding epigenetic changes in cancer. Further analysis of primary tumours 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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**Legends to figures and tables**

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. For the amplification of 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 by 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 by using the adaptor primer. Amplified DNAs were labeled with Cy3 and Cy5 respectively, cohybridzed 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.

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.

Fig. 4 Methylation analysis of five genes in primary tumours. 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.

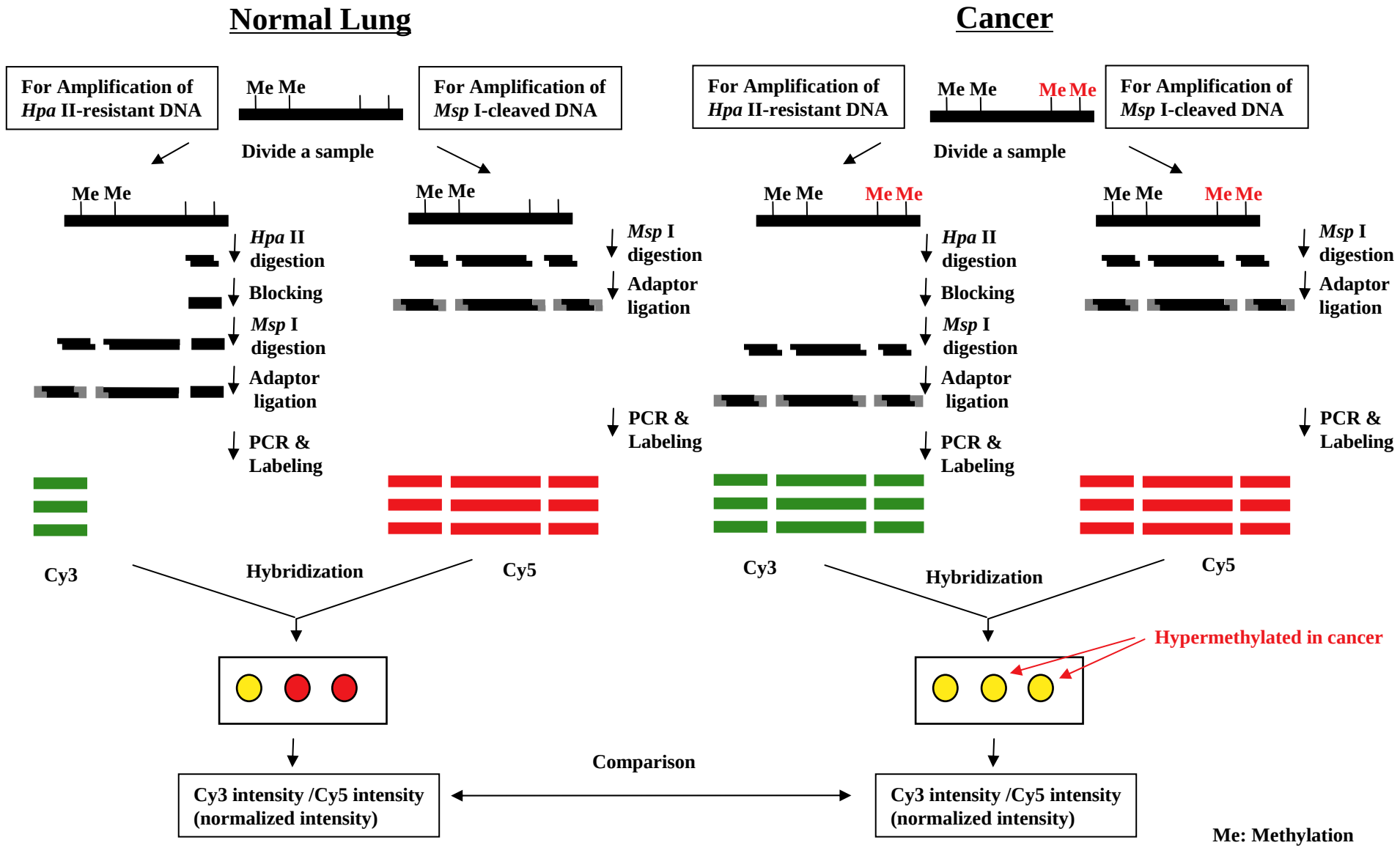
Table 1 Summary of the results for PMAD analysis. Genes that were hypermethylated in at least two of six lung cancer cell lines are presented. *M* represents hypermethylation in cancer defined as (Normalized intensity of cancer) / (Normalized intensity of normal) more than 3.0 when normalized intensity 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.

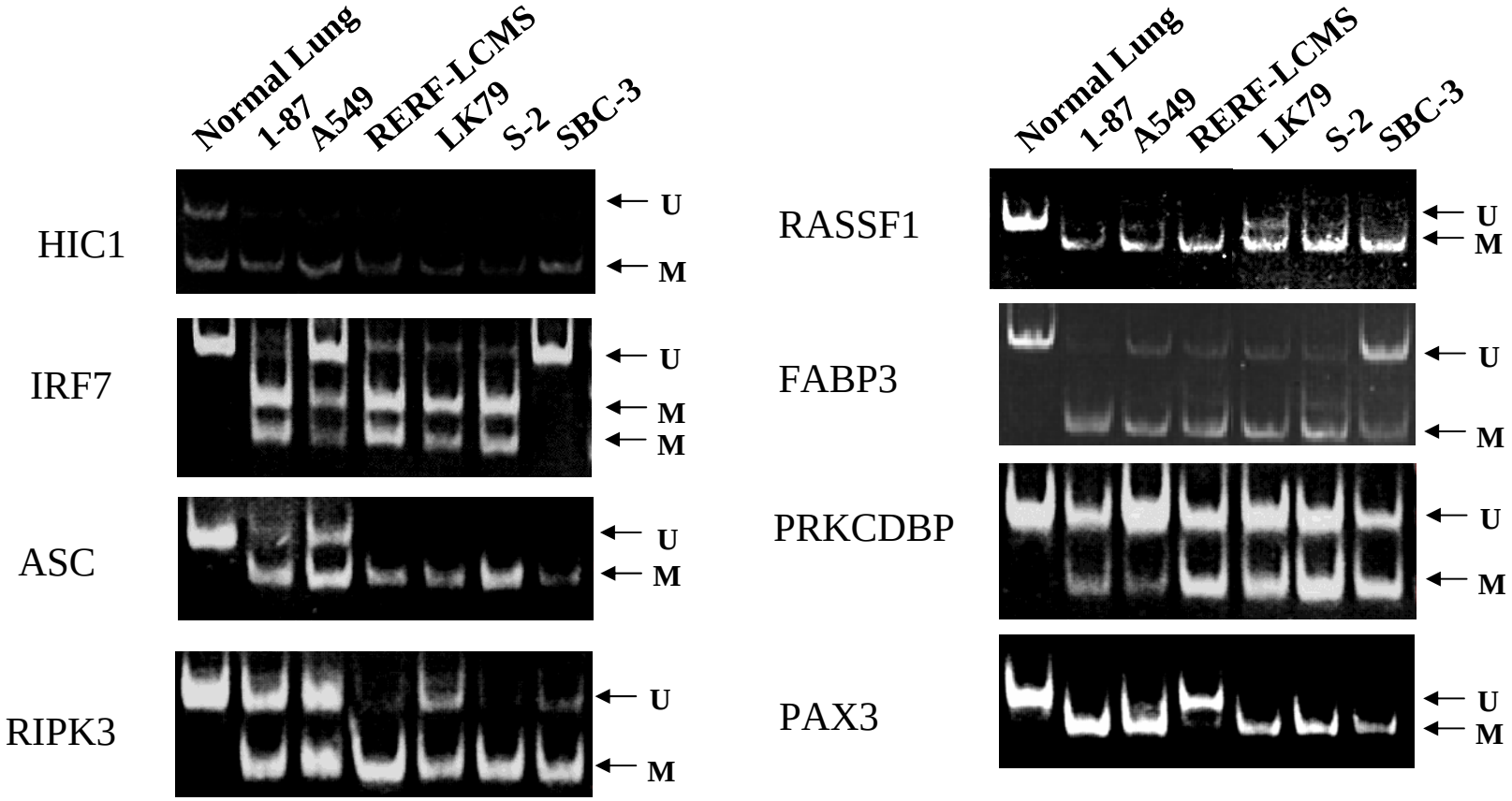
Table 2 Proportion of hypermethylated genes in lung cancers.

Supplementary Fig. 1 CpG maps of eight genes analyzed in Fig. 2. Probe regions are presented. Black and red circles indicate CpG and *Hpa* II sites, respectively. Black filled circles indicate the sites analyzed by COBRA analysis.

# Fig.1

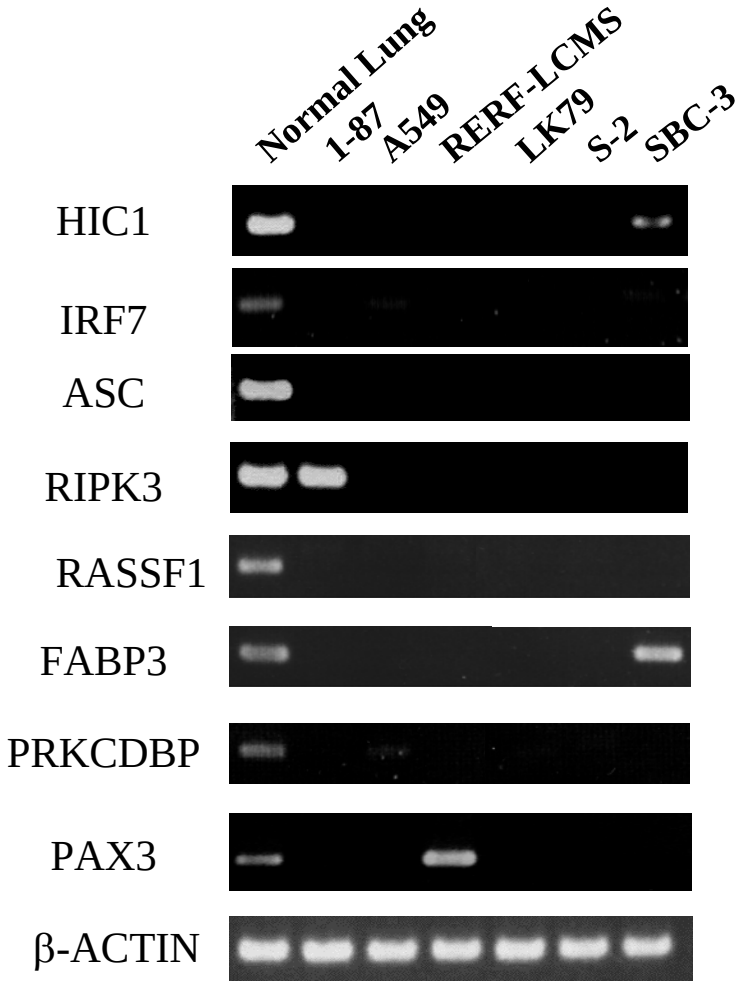

Normal Lung
Cancer
For Amplification of *Hpa* II-resistant DNA
Me Me
For Amplification of *Msp* I-cleaved DNA
Divide a sample
*Hpa* II digestion
Blocking
*Msp* I digestion
Adaptor ligation
PCR & Labeling
*Msp* I digestion
Adaptor ligation
PCR & Labeling
Cy3
Cy5
Hybridization
Hypermethylated in cancer
Comparison
Cy3 intensity /Cy5 intensity (normalized intensity)
Me: Methylation

# Fig.2

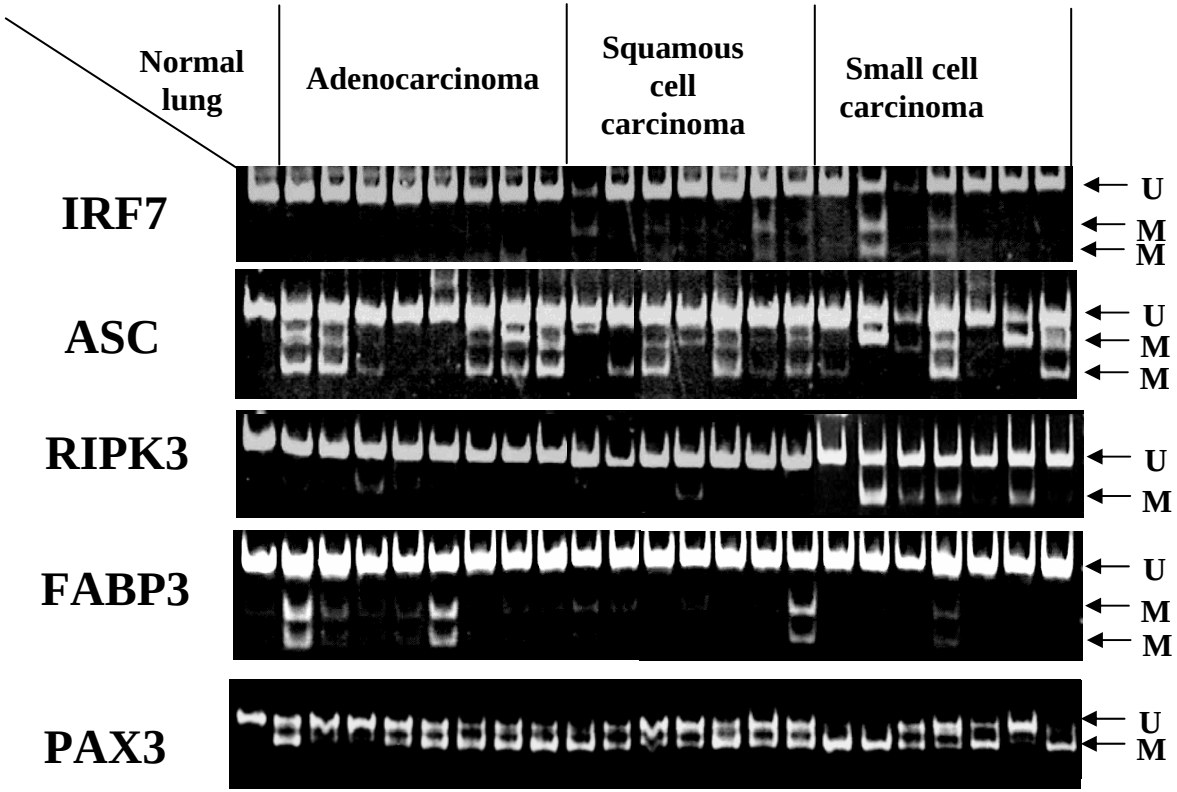



**U**: Unmethylated
**M**: Methylated

# Fig.3


Normal Lung
1-87
A549
RERF-LCMS
LK79
S-2
SBC-3
HIC1
IRF7
ASC
RIPK3
RASSF1
FABP3
PRKCDBP
PAX3
β-ACTIN

# Fig.4




**U**: Unmethylated
**M**: Methylated