SHORT COMMUNICATION

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Microarray analysis of promoter methylation in lung cancers

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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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 con-

densed 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
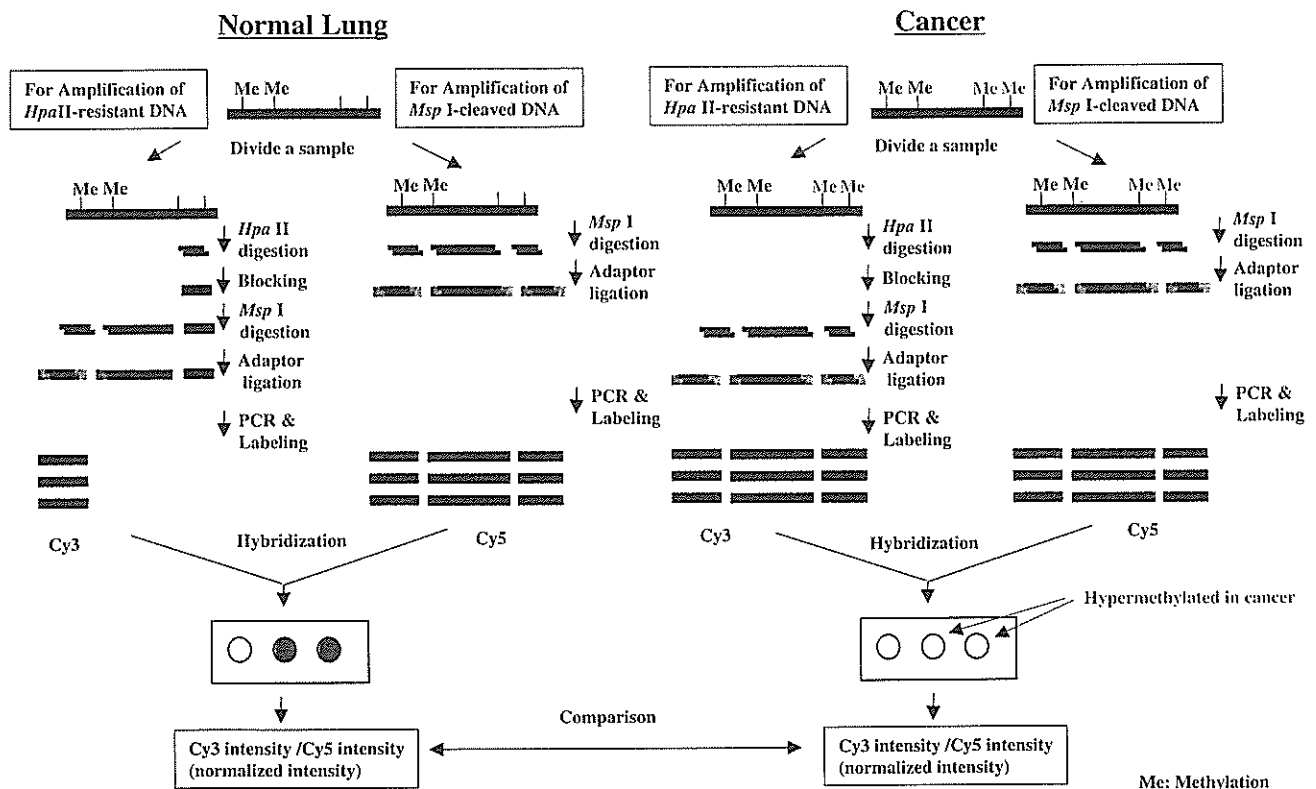

Fig. 1 Schematic flowchart for the promoter-associated methylated DNA amplification DNA chip (PMAD) method. *Me* represents a methylated *Hpa* II site. Each sample was used for both amplification of *Hpa* II-resistant DNA and *Msp* I-cleaved DNA. To amplify methylated *Hpa* II-resistant DNA fragments, 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 un-methylated 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, AGCACTCTCCAGCCTCTCACC-GAC and CGGTCGGTGA. PCR was performed using 0.1 μg of each ligation mix as a template in a 100-μl vol-ume containing 100 pmol of the primer AG-CACTCTCCAGCCTCTCACCGAC 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 + methy-lated) 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 (Invitro-gen, 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 Soft-ware Engineering, Tokyo, Japan) arrayer.

Amplified DNAs mixed with 10 pg of DNA com-plementary to control spots were labeled with Cy3 and Cy5 respectively, cohybridized to the microarray, scan-ned using a Scan Array Lite (Perkin Elmer, Boston, MA, USA) scanner, and analyzed with the software DNASIS Array (Hitachi Software Engineering). Labeling effi-ciency 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 sam-ples (normalized intensity = Cy3 intensity/Cy5 inten-sity). The spots whose Cy5 intensities were higher than background were analyzed. We judged the spots as hy-permethylated 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., Pur-chase, NY, USA) and subjected to combined bisulfite restriction analysis (COBRA). PCR products were di-gested 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 TAC-CCTCTAAAATAAAAACCCAAAC; IRF7, GTAGA-GTTAAGAGTTGGGGGAGTTT and TATTAAA CCAATATCCAAACCTAAC; ASC, TTTTAGTA TGTGGAATTGAGGGAGT and AAACCTCTAAA TTAAAACCCCAAAC; RIPK3, TTTTTGGT ATTTTTTAGTTTGATGT and AACTCCTAATTCT CCAATTCCTC; RASSFF1A, AGTTTTTGT ATTTA GGTTTTTATTG and AACTCAATAAACTCAAA CTCCCC; FABP3, GTTTAGAGGTTA GGAAAGG-GAGAAG and CAAACTAAAA CTCACCCAAAA AAAA; PRKCDBP, AAATAGGTATATTAGGGA-ATTGGAG and AACTCCAACTATAACTCAAA-CAAAC; and PAX3, GGTTTTTGGATTAGGAAT and TAATCATCCTAAAAACAACTTC.

RT-PCR

RT-PCR was performed using the following primers: HIC1, GCTGCTGCAGCTCAACAACCA and GGC-CGGTGTAGATGAAGTCCA; IRF7, TACCATC-TACCTGGGCTTCG and GCTCCATAA GGAAGC-ACTCG; ASC, TGACGGATGAGCAGTACCAG and TCCTCCACCAGGTAGGACTG; RIPK3, CTTCCA-GGAATGCCTACCAA and TCCAT TTCTGTCC-CTCCTTG; RASSFF1A, CTTCAT CTGGGGCGT-CGTG and CTGTGTAAGAACC GTCCTTGTTC; FABP3, CATCACTAT GGTGGACGCTTTCC and CTCATCGAACTCCAC CCCCAAC; PRKCDBP, AGCTCCA CGTTCT GCTCTTC and CGGAGGCT-CTGTACCTT CTG; and PAX3, CTGGAACATTTG-CCCAGACT 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.
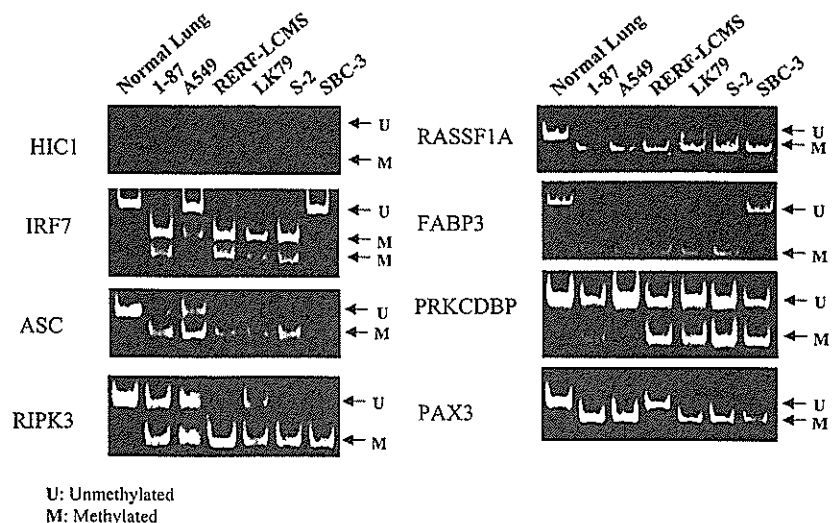
We further analyzed the eight genes that were hypermethylated in at least five of the six (83%) cancer cell lines that we analyzed (Fig. 2a). These were HIC1, IRF7, ASC, RIPK3, RASSF1A, FABP3, PRKCDBP, and PAX3. We confirmed these results using the CO-BRA method and found that 98% of the PMAD results 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
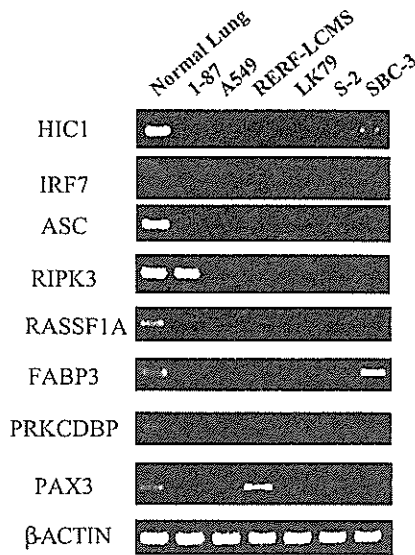

U: Unmethylated
M: Methylated

Fig. 3 RT-PCR analyses of eight hypermethylated genes in Fig. 2. Expression of *ACTB* (β-actin) was monitored as an internal control
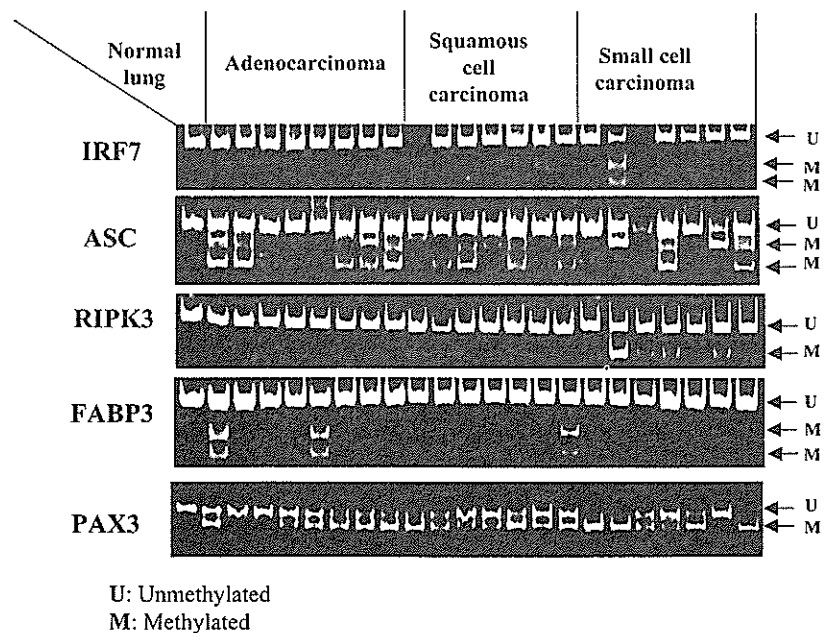
family typically contain a paired box domain and a paired-type homeodomain. These genes play critical roles during fetal development. Mutations in paired box gene 3 are associated with Waardenburg syndrome, craniofacial-deafness-hand syndrome, and alveolar rhabdomyosarcoma. The translocation t (2; 13)(q35; q14), which represents a fusion of PAX3 and the fork-head 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-

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

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

U: Unmethylated
M: Methylated

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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The 21st Lung Cancer Mass Screening Seminar

肺癌集団検診

―喀痰細胞診をめぐって―

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要旨 ―― 喀痰細胞診は，肺門部早期肺癌の発見と大人数を対象としたスクリーニング法として，他の方法では代用できない重要な役割を有している．喀痰細胞診の成績についてかつて大規模な RCT が行われた1970年代および80年代の成績と1990年代の宮城県の成績とを比較すると，時代を追うにつれて，発見成績，治療成績が向上していることが明らかとなった．一方，宮城県のがん登録の成績からは，1990年代の男性喫煙者における扁平上皮癌の罹患は腺癌の罹患を凌駕していた．男性全体では，扁平上皮癌と腺癌の罹患はほぼ同数であった．日本における喫煙率は，アメリカの約2倍であり，今後も長期にわたって喫煙の影響が存続することとを合わせて考えると，喀痰細胞診の役割は今後も重要であると考えられた．その一方で，都道府県によって，喀痰細胞診による肺癌発見率に著しい差異がみられ，検診の効果を十分に発揮するには，精度管理の重要性が改めて痛感される現状にあることも明らかであった．（肺癌．2006;46: 863-870）

索引用語 ―― 喀痰細胞診，肺癌検診，扁平上皮癌，早期発見

Lung Cancer Mass Screening with Sputum Cytology in Japan

Masami Sato[1]; Yasuki Saito[2]; Satomi Takahashi[1]; Zenichi Nishino[3]

ABSTRACT ―― Sputum cytology is the only method that can detect early hilar type lung cancer. No other method such as CT or bronchoscopy could be an alternative for huge mass screening. According to the results of famous randomized control studies conducted in 1970s and 1980s in the US and those of Miyagi Prefecture in 1990s, sputum cytology showed that higher sensitivity for lung cancer detection and better prognosis in the 1990s. In addition, Miyagi Cancer Registry revealed that the incidence of lung squamous cell carcinoma was higher than that of adenocarcinoma in male smokers in 1990s. Also the Registry showed that the number of male squamous cell carcinoma patients of the lung was almost equal to that of male adenocarcinoma patients. The ratio of male smokers in Japan is double that of the US. These findings suggest that lung cancer screening with sputum cytology is important in Japan. However, the detection ratio of lung cancer with sputum cytology varied in prefectures in Japan. Thus, it is evident that quality control for sputum cytology is necessary in each prefecture. (*JJLC*. 2006;46:863-870)

KEY WORDS ―― Sputum cytology, Lung cancer mass screening, Squamous cell carcinoma, Early detection

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はじめに

喀痰細胞診は，検診において，肺門部早期癌，とくに肺門部早期扁平上皮癌を発見する方法として非侵襲的で他の検査法では代用できない重要な位置を占めている．たとえば，気管支鏡あるいは蛍光気管支鏡などは，高価なばかりではなく，最初からスクリーニングとして実施するには被験者に肉体的苦痛を与える，あるいは大人数に実施することは不可能であるなどの問題点がある．このため，住民検診など，大人数を対象とする肺癌検診には適さない．一方，CT では，肺門部の早期扁平上皮癌を画像として捉えることは不可能であり，PET においても同様で，CT，PET 両者とも肺門部早期肺癌のスクリーニングには不適である．従って，現時点では，大人数を対象とした肺門部早期肺癌のスクリーニングとして現実的に実施可能な方法として，喀痰細胞診に代わる方法は他にはない．

しかるに，アメリカで 1970 年代から 80 年代にかけて行われた無作為化比較試験（RCT）では喀痰細胞診の有効性を示すことができなかった．[1-11] しかし，我々が，現在日本で行っている喀痰検診と過去にアメリカで行われた喀痰検診を比較すると，染色方法が異なる，[12] 早期肺癌細胞の診断基準が異なる，など，同一検査法として一括しえないほど，大きな相違点がある．

そこで，本稿では，大きく 3 つの検討を行った．はじめに，1）過去に行われた RCT[1-11,13,14] と宮城県の 1990 年代の成績を比較することを試みた．ついで，2）喀痰細

胞診の主たる対象である扁平上皮癌の推移について，宮城県がん登録の成績で検討した．日本の喫煙率はアメリカの約 2 倍であり，今後も喫煙の影響が長期間にわたることが予想される．現時点での組織型別の肺癌の罹患および死亡の推移をみることは，日本における喀痰細胞診の担うべき役割を明らかにしうると考え，この検討を行った．さらに，喀痰細胞診をめぐる日本国内の問題点として，3）診断基準のばらつきや各都道府県の発見率の差異などについて言及する．

1）喀痰細胞診の成績は年代によって変化したか？

肺癌検診時，喀痰細胞診による発見率などの成績の比較を試みた．現在まで，報告のある大規模な肺癌検診の成績として，チェコスロバキアのもの，[13,14] さらに有名なアメリカの 3 つの RCT[1-11] を取り上げた．一方，日本の成績として，1990 年代に宮城県で行われた喀痰細胞診を併用した肺癌検診の成績[15-17]を集計した．しかしながら，それぞれの検診の背景や対象などが異なるため，単純な比較は不可能である．そこで，可及的に共通な背景とするため，今回はそれぞれの検診において，レントゲン写真と喀痰細胞診の両者を受診した症例でなおかつ，はじめて検診を受診した群における成績を比較した．検討した項目はそれぞれの検診における肺癌発見例において，喀痰細胞診，胸部レントゲン写真，および両者の陽性率，さらに発見肺癌例における stage 0〜I 期の比率，切除率，5 年生存率である．

Table 1，2 にその結果を示した．はじめて検診を受診し，肺癌が発見された症例において，喀痰細胞診がどの

Table 1. Lung Cancer Prevalence Detected Among Screenees Who Underwent Both Screening Methods

	Miyagi	MAYO	JHLP	MSKLP	Czech Rep.
Chest X-ray film	26 (26.3%)	59 (64.8%)	20 (51.3%)	14 (46.7%)	18 (94.7%)
Sputum cytology	58 (61.1%)	17 (18.7%)	11 (28.2%)	9 (30.0%)	0 (0.0%)
Both methods	11 (11.6%)	15 (16.5%)	8 (20.5%)	7 (23.3%)	1 (5.3%)
Total	95	91	39	30	19
Year	1990s	1970s to early 1980s			1970s

Table 2. Comparison of Stage 0-I, Resection Ratio and 5-year Survival Among Prevalent Lung Cancer Patients Who Received Both Screening Methods

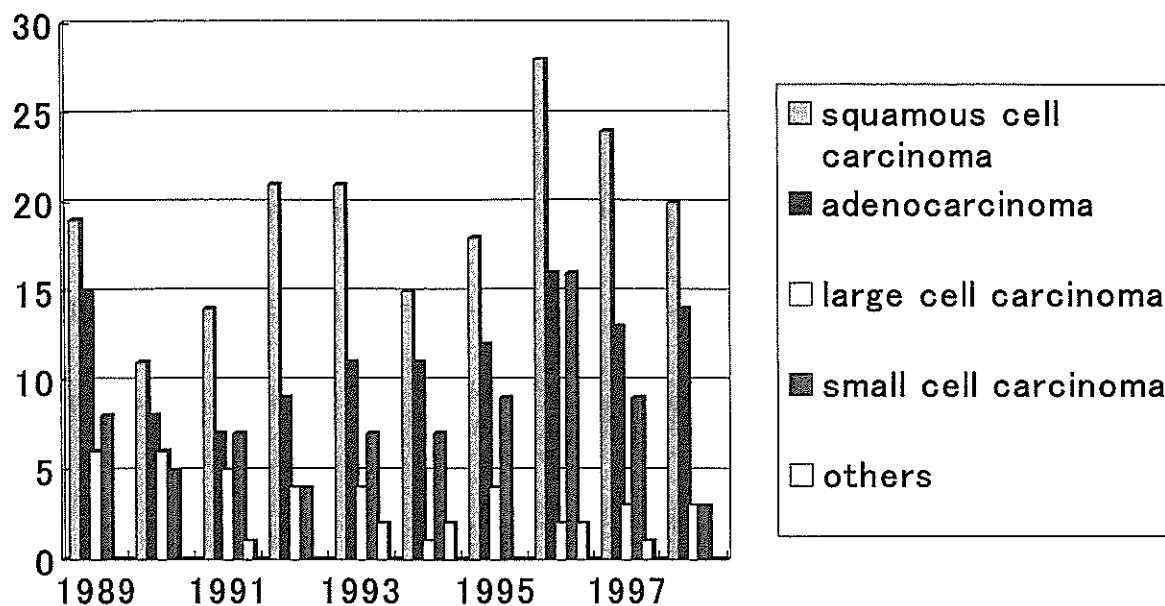|	Miyagi	MAYO	JHLP	MSKLP	Czech Rep.
Stage 0-I	74.7%	45%	61.5%	46.7%	27.8%
Resection	69.5%	54%	66.7%	60.0%	33.3%
Five-year survival	67.4%	40%	58%	48%	28%
Year	1990s	1970s to early 1980s			1970s

Figure 1. Annual numbers of lung cancer cases during 1989 to 1998 among male smokers who received screening with X-ray film and sputum cytology in 1989.

程度発見に寄与しえたかを示したものが Table 1 である．歴史的に最も古いチェコスロバキアの成績では，喀痰細胞診による発見例は 1 例に留まり，しかもその 1 例は，レントゲン写真でも所見のある症例であった．その後，アメリカで行われた検診では，喀痰細胞診のみによる発見例がみられるようになった．しかし，検診発見肺癌例においては，レントゲン写真による発見例が半数以上を占めていた．一方，1990 年代に宮城県で行われた検診では，いわゆる prevalence 例で，レントゲン写真および喀痰細胞診の両者を受けて発見された肺癌例の 72.7％が喀痰細胞診で陽性または疑陽性とされていた．時代の推移とともに，喀痰細胞診の精度が向上した可能性が示唆される．Table 2 には，いわゆる prevalence 例の stage 0〜I 期の比率，切除率，さらに 5 年生存率を示した．いずれの指標においても，年代を経るにつれて治療成績が向上していることが伺える．

この成績に関しては，誰でもが容易に指摘しうるように，それぞれの検診の背景が異なるため，このような比較そのものが妥当か否か，議論のあるところであろう．たとえば，宮城県では，通常とはやや異なる染色法を用いているので，それがこのような結果の違いをもたらしたのかもしれない．また，それぞれに時代，地域における喫煙率も異なることを指摘することもできる．しかし，喀痰細胞診のスクリーニングの対象が喫煙者であることを考慮すれば，ほぼ近似した集団であろうと推測することも可能かもしれない．その一方で，あの有名なサコマ

Table 3. Detection Ratio by Sputum Cytology in Patients Who Received Both Chest X-ray Film Examination and Cytologic Examination by Sputum from 1989 to 1999

Squamous cell carcinoma	185/227	(81.5%)
Adenocarcinoma	18/92	(19.6%)
Large cell carcinoma	4/22	(18.2%)
Small cell carcinoma	8/29	(27.6%)
Others	3/7	(42.9%)
Total	218/377	(57.8%)

ノが，日本からの訪問者に対して，MAYO の前半の喀痰細胞診の精度はお粗末で，信用してはならないという非公式な発言も行ったといわれている．そのような視点でアメリカの 3 つのトライアルを比較するとたしかに MAYO の値が一番低いのも事実である．このような事実を考慮すると，時代の変遷とともに，喀痰細胞診の精度は向上してきたと捉えることもできよう．その一方で，アメリカやチェコスロバキアなどの検討は，あまりにも先進的すぎて，十分に喀痰細胞診の能力を引き出しえないままに，早計な結論を出してしまった可能性を否定しえないのではなかろうか？

今，時代は CT 検診の時代である．しかし，その CT 検診を 21 世紀の今，1980 年代の CT で行おうとする人間は，1 人として存在しないであろう．喀痰細胞診とて同様である．20 年以上も前の喀痰細胞診の精度で，現在の
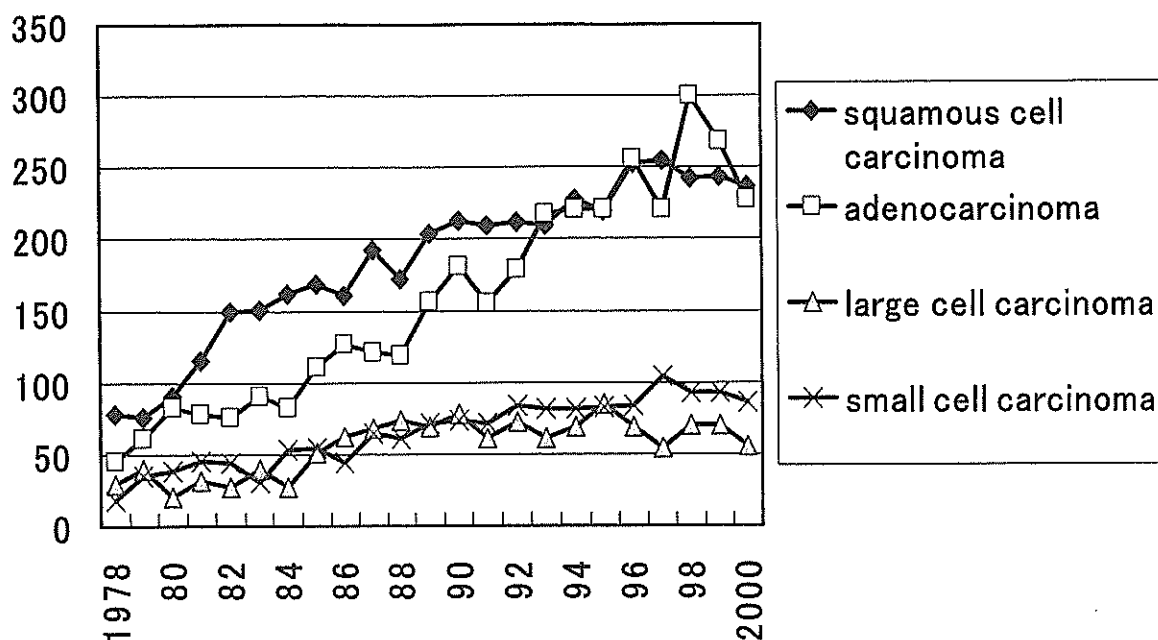
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Figure 2. Trends in male lung cancer patients in Miyagi prefecture by histologic subtype.
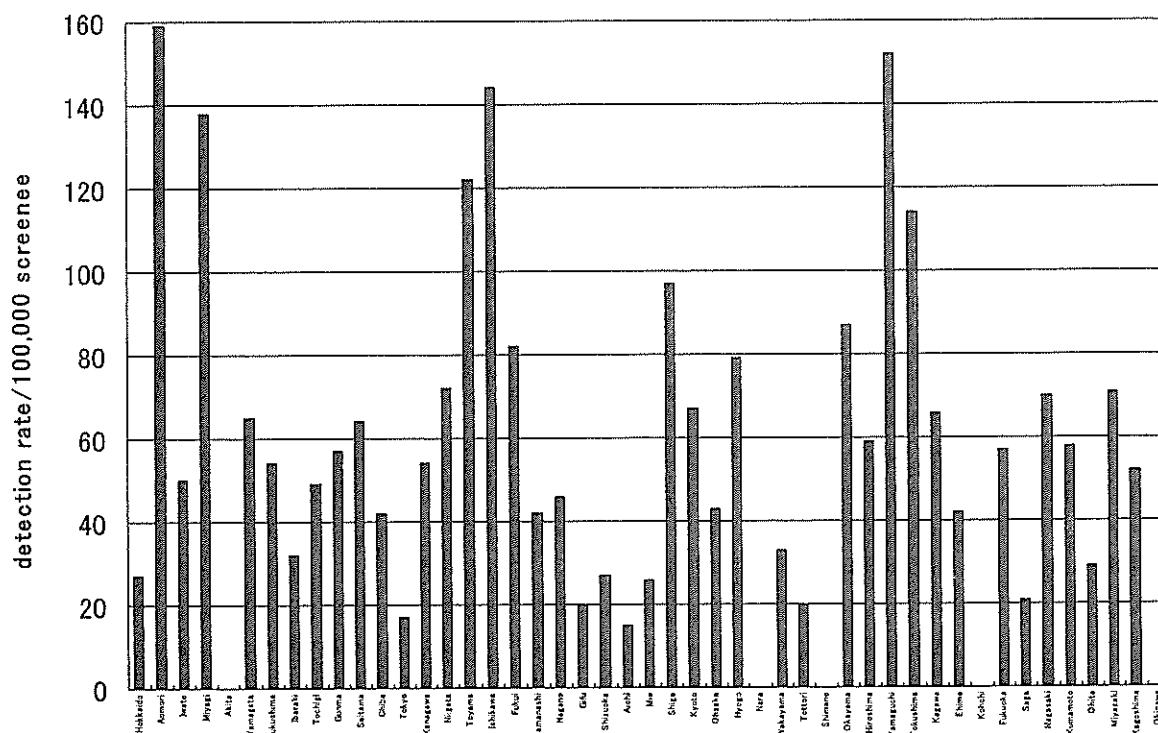

Figure 3. Lung cancer detection rate by sputum cytology/100,000 screennes in each prefecture, Japan.

検診を論じることは現実的に無理がありすぎると考える. 現在の検診は現在の精度で行われるようになってからの成績で論じる必要があろう.

Table 3 に宮城県で 1990 年代に肺癌検診で発見され

た肺癌症例のうち，検診時に胸部レントゲン写真と喀痰細胞診の両者を受けていた症例での喀痰細胞診の陽性率を示した．発見された扁平上皮癌例 227 例のうち，喀痰細胞診で陽性または疑陽性とされた症例は 185 例で
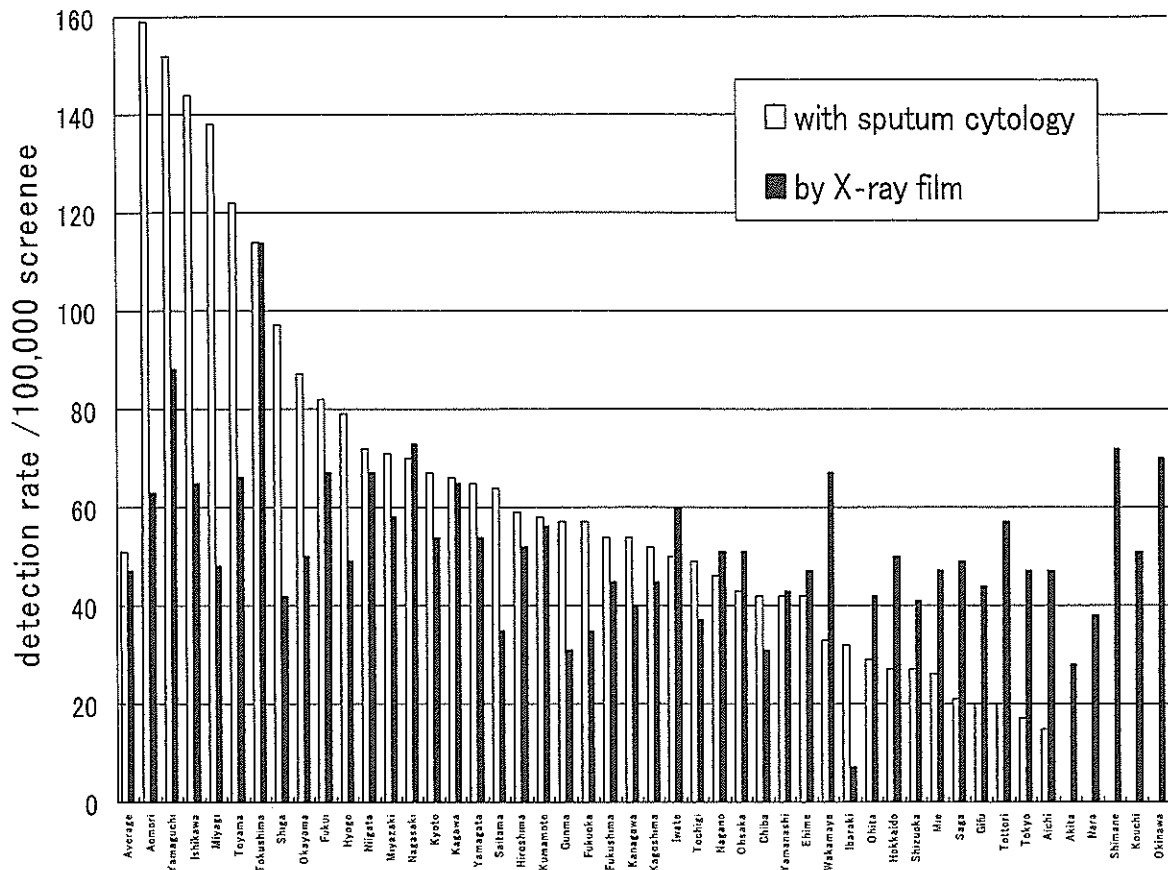
Figure 4. Lung cancer detection rate in each prefecture, Japan.

81.5％ を 占 め て い た. 一方 腺 癌 例 の 陽 性 率 は 18/92 (19.6％), 大細胞癌では 4/22 (18.2％), 小細胞癌 8/29 (27.6％)で全体では 377 例の肺癌例のうち, 218 例 57.8％ で陽性または疑陽性を示した. 喀痰細胞診の検出能力に 関しては, 様々な報告があるが, 日本における大規模な 検診での検出能力の一成績として, 参考となれば幸いで ある.

2) 宮城県がん登録による肺癌例の組織型別推移

世界的にみて腺癌例の増加や, 一部先進国では, 肺癌 死亡の減少が観察されている. しかし, 日本の成人男性 における喫煙率は, アメリカの約 2 倍であり, 喫煙率の 漸減が報告されている一方で, タバコの消費量そのもの には著明な減少はみられていない. 喫煙の影響は 20 年以 上にわたり長期の影響があることは良く知られた事実で ある. 今後, 日本の肺癌罹患が, アメリカやイギリスと 同様の経過をたどって, 減少すると楽観的に予想できな い現状にある. このような観点からは, 現在の日本の肺 癌罹患状況を正確に知ることが最も重要であろう. そし て, 組織型別の推移を知ることは, 今後の肺癌検診のあ るべき姿に示唆を与えてくれるものと考えられる. そこ

で, 宮城県における肺癌罹患の状況を組織型別に調査し た.

まず最初に, 平成元年に喫煙歴が正確に把握できた男 性 10421 人を対象にその後の 10 年間の肺癌罹患を組織 型別に検討した. 具体的には平成元年に肺癌検診で, 喀 痰細胞診を受診した男性例で, 申告した喫煙指数が 600 以上の症例を対象とし, 肺癌罹患を宮城県がん登録で照 合把握した.

その結果, いずれの年度においても, 扁平上皮癌の罹 患が腺癌の罹患を上回っていた (Figure 1). 一方, 宮城 県の男性全体でみた場合, 扁平上皮癌の罹患が先行して いたが, 腺癌の罹患が追いつき, 両者の罹患がほぼ, 同 数になってきている (Figure 2). これらの事実から男性 および喫煙者においては, 扁平上皮癌と腺癌の両組織型 を念頭において肺癌検診を進めることが必要であること が明らかである. いずれか一方のみでは, 片手落ちとい わざるをえないと考えられる. 都道府県により喫煙率の 多少の差異がみられることを考慮すると, 今後, 宮城県 のみではなく, 日本の複数の都道府県において同様の検 討報告がなされることが望ましいと思われた.

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Case	Histology	Number of cytopathologists who diagnosed the case as having no malignancy	Number of cytopathologists who diagnosed the case as having malignancy	Number of cytopathologists who diagnosed the case as having no lesions which need bronchoscopic examination
1	No malignancy	14	5	2
2	No malignancy	10	6	1
3	Cancer	15	3	
4	Cancer	10	6	4
5	No malignancy	7	8	2
6	Cancer	15	2	
7	No malignancy	3	12	6
8	Cancer	17	2	
9	Cancer	8	10	3
10	No malignancy	5	8	1
11	No malignancy	1	16	7
12	Cancer	16	2	
13	No malignancy	9	6	1
14	No malignancy	11	6	
15	Cancer	14	3	
16	No malignancy	1	14	6
17	Cancer	11	5	1
18	Cancer	18	1	
19	No malignancy	3	12	6
20	No malignancy	6	2	3

Figure 5. Difference in final diagnosis among cytopathologists in cases of early lung cancer or of dysplasia.

3) 喀痰細胞診の診断基準のばらつきと各都道府県の発見率について

Figure 3 に厚生労働省の 2003 年版のがんの統計のホームページより算出した各県の受診者 10 万対の喀痰細胞診による肺癌の発見率を示した．さらに Figure 4 では，レントゲン写真による肺癌の発見率と喀痰細胞診による肺癌の発見率を併記した．これらの図からも明らかなように，喀痰細胞診による肺癌発見率には都道府県により著しい差異がみられる．その一方で Figure 4 にみられるように，レントゲン写真による肺癌発見率は日本全国でみた場合，ほぼ一定の水準を示しており，喀痰細胞診による発見率のばらつきと比較すると大きな差異がみられている．この原因には様々な要因が関与していると思われるが，少なくとも，喀痰細胞診をめぐる日本の肺癌発見率の現状を如実に表わしていると思われる．

その一因を明らかにしうる傍証として，喀痰細胞診の診断基準のばらつきの問題がある．Figure 5 に平成 17 年に宮城県で開催した喀痰細胞診セミナーでの診断結果のばらつきを示した．この中で，ケース 4，9，17 は肺癌が存在した症例であるが，一部の人間は C 判定と診断していた．C 判定と診断された場合，気管支鏡の検査の対象

とはならず，喀痰中に異型細胞が出現していながら，細胞判定の段階で，これを見落とすことになる．同様の検討は，金子班でも行った．この時も同じように，診断施設により，診断結果に食い違いのある症例が散見されている．[18]

ここで，興味ある文献を紹介しておきたい．コロラドのグループから報告されたもので，彼らのいう moderate atypia の症例で気管支鏡を施行すると高率に早期扁平上皮癌が発見された[19] というものである．従来は severe atypia を気管支鏡の対象としていたものをそれより 1 ランク細胞異型の軽度なものを対象とすることによって，より多くの早期癌が発見された．喀痰細胞診の判定により気管支鏡検査の要否を決定していることは洋の東西を問わず同様である．日本の場合は D，E 判定が気管支鏡検査の対象とされている．そこで問題になるのは，ある症例を D 判定とするか，C 判定とするか，迷うような場合であろう．このような場合には，D 判定にすることを肺癌学会では薦めている．また，コロラドの事例が示すように，喀痰細胞診の能力を遺憾なく発揮するためには，気管支鏡の適応となる症例の喀痰上の細胞異型を如何に適切に捉えるかが，重要であることを示している．

今後は，このような事実を念頭におきながら精度管理の徹底を図る必要があろう．これらのほかにも，気管支鏡検査の水準，[20-23] 精査結果の把握の徹底，診断未確定例の経過観察などが各都道府県で温度差がある可能性がある．早期発見や肺癌死亡率の減少を図るためには，ひとつひとつの精度管理の積み上げが重要であることを強調しておきたい．精度管理の参考となる文献[24-27]を文末に引用したので参考とされたい．

最後に，宮城県では，年に1回，全国の希望の方々を対象に，喀痰細胞診の勉強会を開催している．これは，宮城県の肺癌検診で発見された早期扁平上皮癌の標本を直接，顕鏡する機会を設けているのと同時に，喀痰細胞診に関わる現場の人間の意見交換や情報交換などの場も提供したいと考えてのことである．日本の各県の現状をみるに，孤軍奮闘しているスクリーナーの方々や指導医，気管支鏡施行医などが見受けられる．胸部レントゲン写真やCTに関しては，年に1～2回の講習など，勉強や情報交換の機会が長年にわたって，設定されてきたが，喀痰細胞診に関しては，そのような機会が継続的に開催されたことはなかった．喀痰細胞診の精度を向上させるために，少しでもお役に立てれば幸いである．宮城県対がん協会検診センターの呼吸器部門が窓口になっていることを付け加えたい．

まとめ

第21回肺癌集検セミナーにおいて肺癌集団検診の今後—科学的評価を踏まえた問題点への対応—喀痰細胞診をめぐってとのテーマで発表を行った．喀痰細胞診は，肺門部早期肺癌の発見と大人数を対象としたスクリーニング法として，他の方法では代用できない重要な役割を有している．

喀痰細胞診の成績について，かつて大規模なRCTが行われた1970年代および80年代の成績と1990年代の宮城県の成績を比較すると，時代を追うにつれて，発見成績，治療成績が向上していることが明らかとなった．

一方，宮城県のがん登録の成績からは，1990年代の男性喫煙者における扁平上皮癌の罹患は腺癌の罹患を凌駕していた．男性全体では，扁平上皮癌と腺癌の罹患はほぼ，同数であった．日本における喫煙率は，アメリカの約2倍であり，今後も長期にわたって，喫煙の影響が存続することとを合わせて考えると，喀痰細胞診の役割は今後も重要であると考えられた．その一方で，都道府県によって，喀痰細胞診による肺癌発見率に著しい差異がみられ，検診の効果を十分に発揮するには，精度管理の重要性が改めて痛感される現状にあることも明らかであった．

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［症 例］

結節性陰影を呈した胃癌肺転移の2症例

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要　旨

最近10年間に当センターで手術施行した転移性肺腫瘍は全59例で，胃癌からの転移はわずかに2例であった．消化器癌のうち，大腸癌からの転移に対する切除例は多く術後成績も良好であるのに対し，胃癌からの転移は癌性リンパ管症や癌性胸膜炎の形をとることが多いため外科治療の適応となりにくく治療成績も不良である．今回経験した胃癌からの肺転移症例について，文献的な考察を加えて報告した．症例1：76歳，男性．胃癌の診断で胃全摘術施行．2年後の胸部X線で結節影がみられ，胸部CTで左S10に腫瘍状陰影を認めた．腫瘍の増大がみられたため，原発性肺癌を疑い左下葉切除術施行，病理組織検査にて胃癌からの転移と考えられた．症例2：82歳，男性．胃癌の診断で幽門側胃切除術施行．2年後の腹部CTにて左肺に異常陰影を指摘され，原発性肺癌を疑い手術施行．病理組織検査で胃癌の肺転移と診断された．

は じ め に

肺は全身の静脈血の還流を受けるため，他臓器に発症した悪性腫瘍からの血行性転移を最も来しやすい臓器である．近年，血行性の肺転移に対して外科治療の有効性が述べられ，特に結腸・直腸癌からの転移についての良好な術後成績が報告されている．一方で，胃癌からの肺転移は癌性リンパ管症や癌性胸膜炎の形で発症することが多いため，手術の適応となることは極めて稀である．今回，我々は，胃癌手術後に胸部レントゲン写真上結節影を来たし，手術可能であった肺転移症例2例を経験したので報告する．

症　例

症例1：76歳，男性．
主　訴：胸部異常陰影．
既往歴：急性虫垂炎，1947年　手術．白内障，2003年　手術．胃癌，2002年3月　胃全摘術．

宮城県立がんセンター　呼吸器外科

家族歴：父，胃癌．
喫煙歴：20本/日，54年間．
現病歴：2002年3月胃癌のため当センター外科で手術を施行した［胃全摘術およびRoux-en-Y再建，肉眼的進行度：MU前，50×40mm，0（IIa）型，T2（MP），N1，H0，P0，CY0，M（－）　Stage II，組織学的診断：adenocarcinoma，tub1，ssβ，INFβ，ly1，v1，pm（－），dm（－），n（＋）；＃3，根治度B］．以後，外来経過観察中であったが，2004年3月胸部単純X線にて左肺の異常陰影を指摘された．3月の胸部CTでは，左肺下葉に下行大動脈に接する結節影を認めた．4月呼吸器外科紹介となり，気管支鏡検査を実施したが，確定診断は得られなかった．5月胸部CTで陰影の増大を認め原発性肺癌を疑われ，手術目的で入院となった．

入院時現症：身長157.8cm，体重48.5kg．胸部，腹部ともに打聴診・触診上異常なし．上腹部正中に開腹手術の術創あり．表在リンパ節触知せず．

入院時検査所見：末梢血液像，生化学検査とも異常はなかった．血液ガスはpH 7.430，PCO2 41.2mmHg，PO2 92.8mmHgと正常．腫瘍マーカーは，CEA 4.4ng /mlと軽度高値．呼吸機能検査では％VC 101.9％，
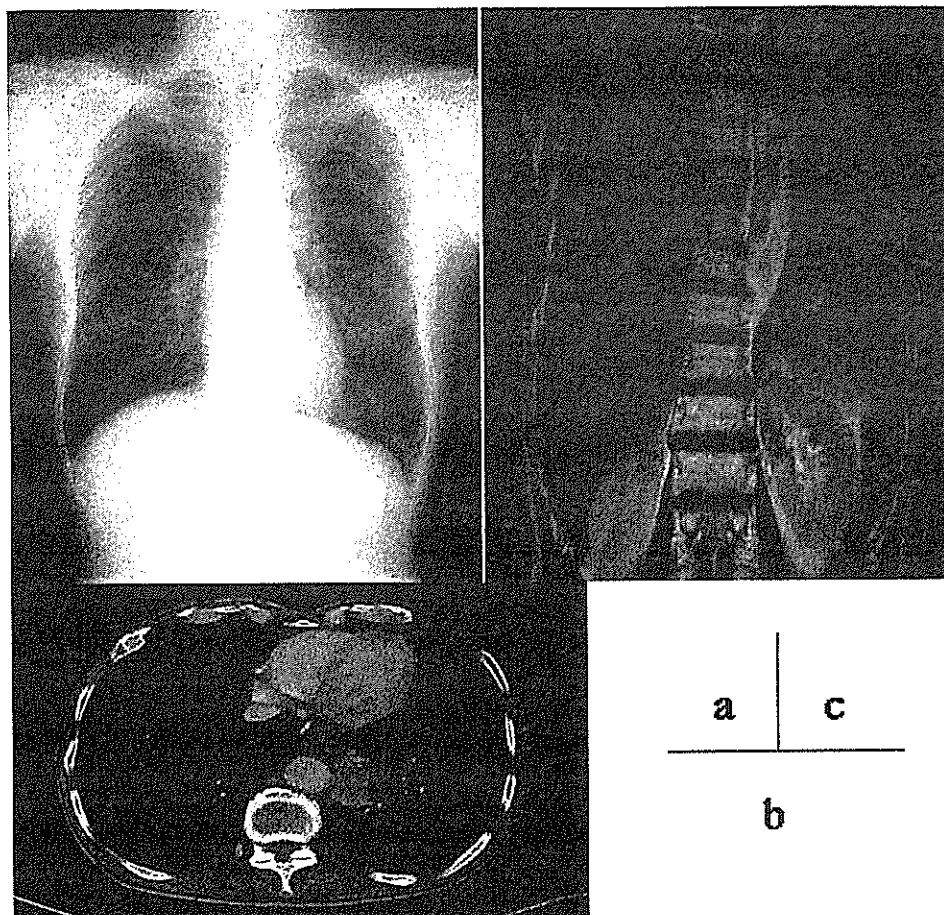
Fig. 1a　Chest X-ray film taken 2 years after the abdominal section showed a mass (46×22mm) hidden in the cardiac shadow.

Fig. 1b　Chest CT showed an irregular-shaped heterogeneous mass adjacent to the thoracic aorta and the esophagus at the left hilum. No lymph node was detected.

Fig. 1c　The coronary section of chest MRI indicated an irregular low signal mass on T1 weighted image. A thin fat layer between the mass and the thoracic aorta was found.

FEV1.0 2.10*l*, FEV1.0% 72.91％と正常範囲であった.

　胸部単純 X 線所見：心陰影後方で下行大動脈に接する結節影を認めた（Fig. 1a）.

　胸部 CT 所見：下行大動脈および食道（挙上された胃管）に接する結節影を認めた. 肺門・縦隔リンパ節の腫大はみられなかった（Fig. 1b）. 大動脈外膜との境界が不明瞭で浸潤の可能性も考えられたため, MRI 検査を行った.

　胸部 MRI 所見：CT の所見と同様に, 下行大動脈に接する結節影が認められたが, 浸潤の可能性は低い, と考えられた（Fig. 1c）.

　その他の検査で遠隔転移の所見もみられなかった.

以上の所見により臨床病期 cT2N0M0, stage IB の原発性肺癌を疑い, 6 月手術を施行した.

　手術所見：左後側方切開, 第 6 肋間で開胸すると少量の胸水がみられた. 腫瘍は S10a に存在しており, 大動脈や縦隔への直接浸潤は見られなかったものの, 腫瘍の表面は大動脈の形状に一致して窪んでいた. 定型的な左下葉切除術とリンパ節郭清（ND2a）を行った. 腫瘤は大きさ45×34×25mm で, sT2N0M0, stage IB と思われた.

　病理組織学的所見：層状の背の高い円柱上皮細胞からなる分化型の腺癌であり, 腫瘍内部に広範な壊死像もみられることから, 胃癌からの肺転移と考えられた
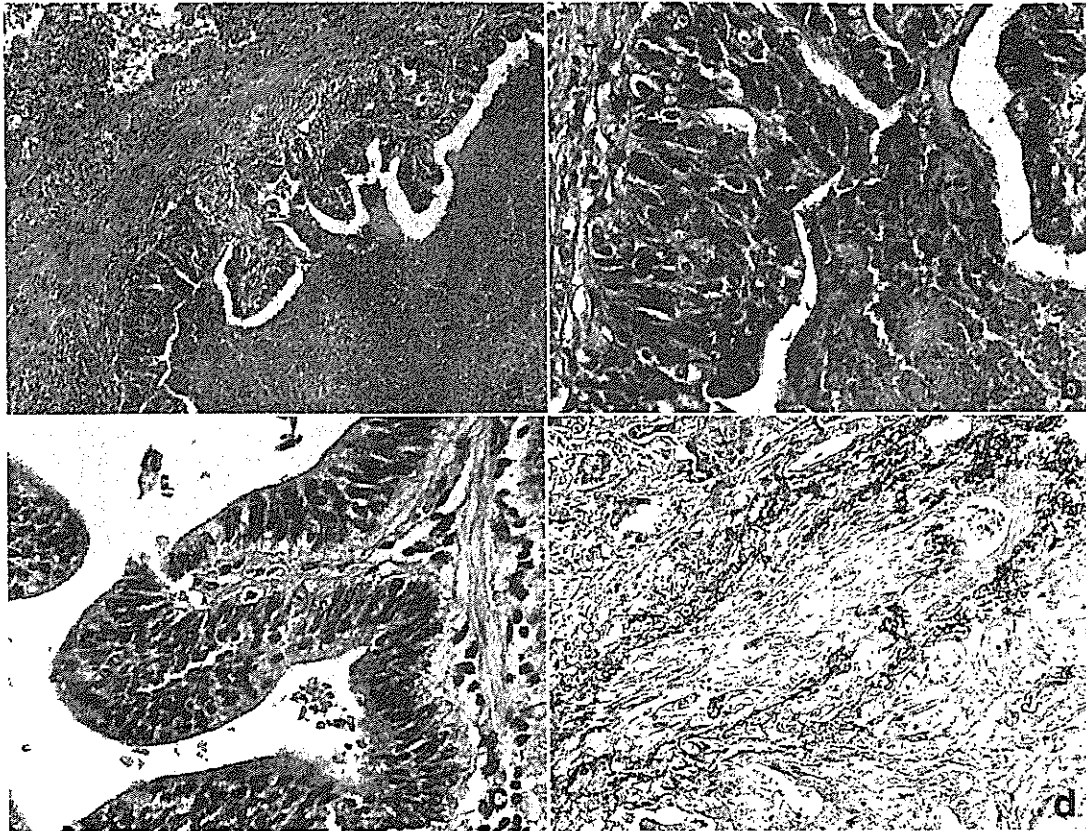
Fig. 2a Photomicrograph shows the structure of well differentiated adenocarcinoma with marked central necrosis. Under low-powered magnification.

Fig. 2b Photomicrograph shows the structure of well differentiated adenocarcinoma with necrosis. Under high-powered magnification. These findings suggested that the mass was the metastasis from the gastric cancer.

Fig. 2c Photomicrograph shows well differentiated adenocarcinoma (tub1) of primary gastric cancer.

Fig. 2d Tumor invasion to the vessel lumen (v1) was observed in the lesion of primary gastric cancer by Elastica-Masson staining.

（Fig. 2a, 2b）．これは，前回の胃癌の組織像（Fig. 2c）に類似しており，同原発巣には静脈侵襲像が確認されていた（Fig. 2d）．胸水の細胞診は陰性であった．気管支断端陰性でリンパ節転移はみられず，腫瘍は肺胸膜表面に到達していなかった．

　術後経過：術後3日目より乳糜胸となるが，自己血注入による癒着療法を施し治癒した．その後の経過は良好で術後27日目に退院した．術後2ヵ月目から2年間の予定でTS-1の内服を開始した．現在までに再発や転移の所見はみられていない．

　症例2：82歳，男性．

　主　訴：胸部異常陰影．

　既往歴：糖尿病・高脂血症，1984年〜．狭心症，1992

年頃〜．胃癌，2002年5月　幽門側胃切除術．白内障，2002年　手術．

　家族歴：特記すべきことなし．

　喫煙歴：20本/日，60年間．

　現病歴：2002年5月胃癌のため当センター外科で手術施行した［幽門側胃切除術，肉眼的進行度：L後，30×15mm，1型，T1，N0，H0，P0，CY0，M（−）Stage IA，組織学的診断：adenocarcinoma，tub1，sm2，INFα，ly0，v1，pm（−），dm（−），n（−），根治度A］．以後，外来経過観察中であったが，2004年8月腹部CTにて左下肺野に異常陰影を指摘され，9月呼吸器外科紹介された．胃癌からの肺転移の可能性は低いと考え，手術目的で入院となった．