Identification of DNA Methylation Markers for NSCLC Using *Hpa*II-*Msp*I Methylation Microarray

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*Hpa*II-*Msp*I Methylation Microarray를 이용한 비소세포폐암의 DNA Methylation Marker 발굴

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연구배경: 유전자의 후생적인 변화(epigenetic alteration)는 악성종양의 병인론에 있어서 유전자 변이와 동등한 위치를 점하고 있다. 특히 종양억제 유전자의 전사 촉진(promoter) 부위에 발생하는 비정상적인 메칠화(methylation)는 유전자의 발현을 침묵화(silencing)하고, 결과적으로 유전자의 기능 소실을 일으키게 된다. 저자들은 CpG island와 *Hpa*II site를 가지고 있으며 암화 과정에 관여할 것으로 생각되는 유전자에 대하여 *Hpa*II-*Msp*I methylation microarray를 이용하여 새로운 종양억제 유전자를 발굴하고자 하였다.

방 법: 2005년 건양대학교 병원에서 수술한 비 소세포성 폐암 환자 10명에서 폐암조직과 상응하는 암 주변의 정상조직을 얻었으며, *Hpa*II-*Msp*I methylation microarray (Methyl-Scan DNA chip®, Genomic tree, Inc, South Korea)를 이용하여 21개의 유전자에 대하여 DNA methylation profile을 분석하였다. 각각의 유전자에서 메칠화된 정도를 두 그룹에서 비교하였고, 정상 대조군으로 두 명의 젊고 건강한 기흉 환자에서 수술한 폐 조직에 대하여 methylation profile을 분석하였다.

결 과: 21개의 대상 유전자 중 10개의 유전자에서 폐암조직, 폐암 주변 정상 조직, 대조군에서 모두 공통적으로 과메칠화 되었고, 나머지 11개의 유전자 중 *APC, AR, RAR-b, HTR1B, EPHA3, CFTR*의 6개의 유전자에서 대조군에서 메칠화가 없으며, 폐암조직에서 폐암 주변 정상 조직에 비하여 더 빈번하게 과메칠화 되었다.

결 론: *HTR1B, EPHA3, CFTR*은 비소세포 폐암에서 후생적 변화로 발생하는 새로운 종양억제 유전자의 후보 유전자로서의 가능성이 있을 것으로 생각한다. *(Tuberc Respir Dis 2008;65:495-503)*

Key Words: DNA hypermethylation, Tumor suppressor gene, DNA microarray, Non-small cell lung cancer

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide attributing 1 million deaths every year[1]. There has been no effective screening tool with survival benefit[2,3], leading to search for novel, non-invasive method for early detection of lung cancers. Early detection includes the identification of lung cancer-specific biomarkers.

Pathogenesis of cancer is having been elucidated gradually as genetics and molecular biology evolves. Epigenetic alteration of genes with their rolls of tumor suppression is one of the central mechanisms of tumorigenesis other than genetic mutation. This promising field has been being researched for more than two decades, and methylation of the CpG islands in or near of promoter region of tumor suppressor genes is major composition of epigenetic mechanism. When methyl-

ation occurs, some other events such as deacetylations of histone residues readily accompany, and conformational change of DNA inhibits transcriptional factors and some proteins from binding for the transcription to be initiated. And loss of function of the tumor suppressor genes (TSGs), for example, is resulted without alterations of genetic sequences[4-6]. Epigenetic alterations are changes that could be reversed by demethylating agents such as azacitidine and some results from several studies on these agents with some kinds of cancers disclosed the effectiveness in survival rate and symptom relief, so application into clinical field was just started[5].

In the present study authors hypothesized that if a gene could be a TSG by epigenetic mechanisms, more frequent methylation profiling of that gene should be observed in tumor tissue than adjacent non-tumor tissue in a subject, or in tissue from cancer-free control group. So we investigated methylation profiles of genes in non-small cell lung cancer (NSCLC) patients and compared with corresponding adjacent non-tumor lung tissues. We tried to search for non-small cell lung cancer-specific tumor suppressor genes of which their function is inhibited by epigenetic mechanism.

Materials and Methods

1. Patients & DNA extraction

Total of 10 patients with primary NSCLC who underwent lung surgeries in 2005 in Konyang university hospital were included. This study was approved by the Bioethics Committee of Konyang University Hospital and all of the participants gave written informed consent. All of the tumor and macroscopically normal lung tissue samples were obtained at the time of surgery, and were rapidly frozen in liquid nitrogen and stored at -80°C until analysis. Also, normal lung tissue was obtained from two young healthy male patients with pneumothoraces through their bullectomies. Tissues samples were histologically confirmed by hematoxylin-eosin staining. DNA was extracted and prepared using the QiaAmp DNA mini kit[®] (Qiagen, Hilden, Germany).

2. Principle of the method

Methylation status of each promoter was detected by presence or absence of hybridization signals after PCR using samples digested with *Hpa*II (methylation-sensitive enzyme) and *Msp*I (Methylation-resistant enzyme). The hybridization signals on the Methyl-Scan DNA microarray are proportional to PCR status (Figure 1).

3. Microarray design

To make the microarray, we have chosen 50 genes (*Apaf-1, ACCN1, APC, AR, BRCA1, CALCA, CALCR, CDH13, CDKN2A, CDKN2B, CFTR, COMT, DAPK1, EBR, EDN1, EPHA3, EPO, ESR1, FHIT, H19, hMLH1, HPSE, HTR1B, IL-8, JunB, LAMA5, LDHB, LRP2, LTB4R, MDR3, MGMT, MTHFR, MUC2, PGR, PIK3CG, PLS3, PTGS2, RAR-b, RB1, S100A2, SHP1, SKT11, SLC5A5, SMARCA3, SRBC, TFF1, TP73, TUSC3, VHL,* and *WT1*) which have promoter regions for *Hpa*II searched in NCBI database.

4. Digestion of DNA

The purified DNA (100 ng for each digestion) was digested with *Hpa*II (20 units / 2 uL) and *Msp*I (40 units / 2 uL), respectively, and then genomic DNA was re-digested with same enzymes to ensure complete digestion and reduce the background noise signals. Each digestion was followed by incubation at 37°C for 2 hours and 15 hours, respectively. The second digested samples were purified with GeneClean Turbo kit[®] (Qbio, gene, Baton Rouge, LA, USA) according to the manufacturer's instructions. To ensure scanning, we used exogenous GAPDH cDNA as control.

5. Multiplex PCR and target labeling

Multiplex PCR amplification was done with un-digested and *Hpa*II-, *Msp*I-digested DNA with 9 primer sets to label 50 target promoter regions. During amplification step, fluorescent dyes were incorporated into the amplicons; Cy3-dUTP in *Msp*I-digested targets, Cy5-dUTP in *Hpa*II-digested targets, and undigested samples labeled with Cy5-dUTP by same multiplex PCR.
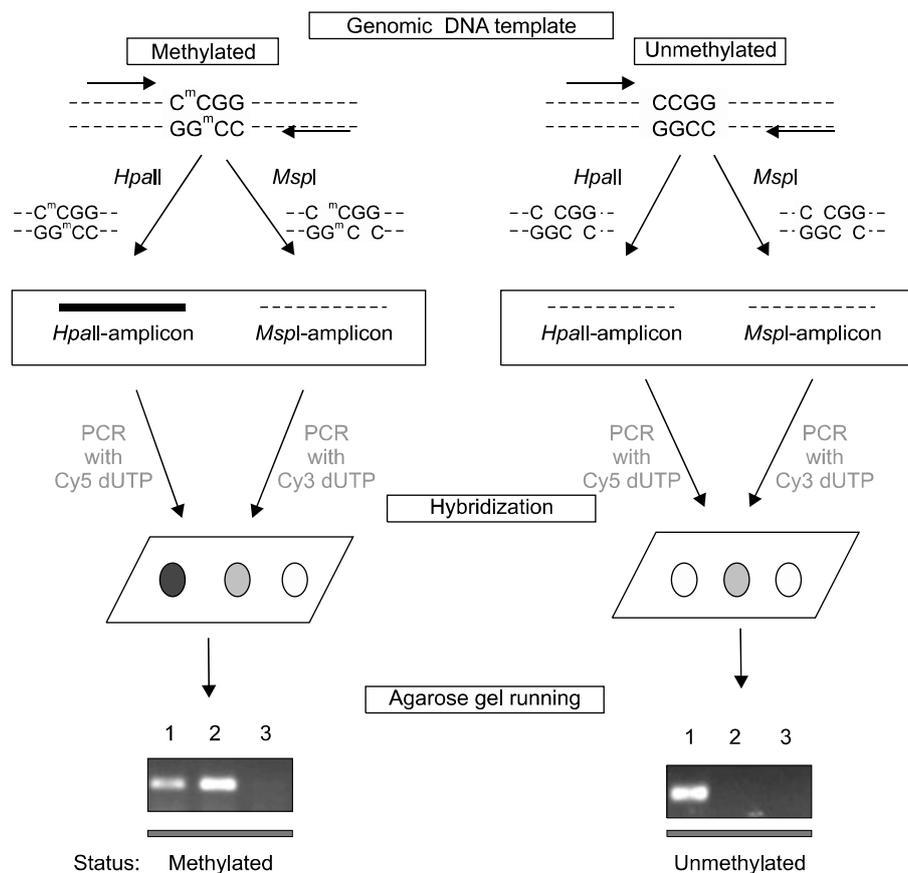
Figure 1. Schematic diagram of methylation microarray. Purified genomic DNA is digested with *Hpa*II and *Msp*I. If the genomic DNA has hypermethylation in CpG islands of promoter region, *Hpa*II is sensitive to methylation status, whereas *Msp*I is still insensitive. Multiplex PCR amplification is accomplished with un-digested and *Hpa*II-, *Msp*I-digested DNA. Each of 9 promoter regions can be amplified simultaneously in a single tube using 9 specific primer pairs, and for 50 promoters and control, 6 sets of PCR are required. During amplification step, fluorescent dyes (Cy3- or Cy5-dUTP) are incorporated into the amplicons. After PCR amplification, all amplicons are mixed and hybridized on Methyl-Scan DNA chip, on which 50 specific promoter regions are spotted. When we observe the hybridization signal of *Hpa*II-amplicon is 2.0-fold greater than *Msp*I-amplicon's signal, we considered 'methylated'.

6. Hybridization and methylation DNA microarray analysis

After PCR amplification all the amplicons were mixed, microarray hybridization was performed at 65°C for 4 hours in the humidified chamber (GenomicTree, Daejeon, South Korea). The hybridized microarray was imaged by scanner (Axon Instruments, CA, USA). If the signal intensity of *Hpa*II amplicon is 2-fold greater than that of *Msp*I amplicon, the target region was considered to be methylated, while less than 2-fold was considered to be unmethylated.

7. Validation of microarray with gel electrophoresis

Undigested, HapII-, *Msp*I-digested DNA were resolved on 1.2% Agarose Gel (SeaKem LE Agarose®, Cambrex, Rockland, USA) with voltage 100 for 30 minutes and stained with ethidium bromide for visualization under UV light.

Results

1. Patient characteristics

There were 5 male and 5 female patients, the mean

age was 62.9 years old. All of 5 male patients had smoking history with forty six of mean pack-years, two of them had squamous cell carcinoma, and the remainders had adenocarcinomas. 5 female patients were

Table 1. Patient characteristics

Patient	Age	Sex	Smoking	Amount (PY)	Histology
1	36	F	N	0	NSCLC*
2	63	M	Y	40	Squamous carcinoma
3	67	M	Y	30	Adenocarcinoma
4	75	M	Y	40	Adenocarcinoma
5	68	F	N	0	Adenocarcinoma
6	68	F	N	0	Adenocarcinoma
7	77	F	N	0	Squamous carcinoma
8	61	F	N	0	Adenocarcinoma
9	51	M	Y	40	Adenocarcinoma
10	68	M	Y	80	Squamous carcinoma

*not determined into more specific cell type.

all non-smokers, 3 of them had adenocarcinomas, one had squamous cell carcinoma, and the remainder had other kind of NSCLC, respectively (Table 1).

2. Results from microarray

The methylation microarray assay was performed for tumor tissue and adjacent normally-appearing tissue using *Hpa*II-*Msp*I methylation microarray. The PCR was successful for 21 genes (*APC, AR, BRCA1, CALCA, CDH13, CFTR, EDN1, EPHA3, H19, HTR1B, LAMA5, LDHB, LTB4R, MTHFR, MUC2, PIK3CG, PTGS2, RAR-b, S100A2, SHP1,* and *SRBC*) from 50 genes, others were not available because of PCR failure (Figure 2). *APC, AR, RAR-b, HTR1B, EDHA3, CFTR* and were hypermethylated in tumor tissue than adjacent normally appearing tissue. *CDH13, CALCA, LAMA5, MTHFR, PIC3CG, S100A2, SHP1, EDN1, H19, MUC2,* and *LTB4R* were commonly methylated in tumor and non-tumor
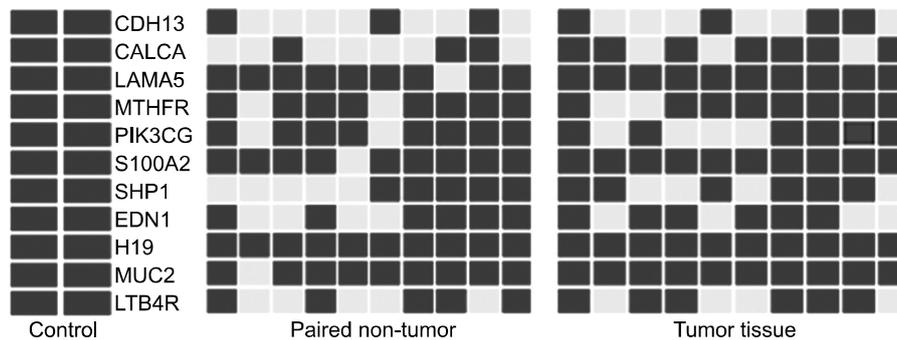
Figure 2. Genes commonly methylated in three groups. There were 11 commonly methylated genes; *CDH13, CALCA, LAMA5, MTHFR, PIC3CG, S100A2, SHP1, EDN1, H19, MUC2,* and *LTB4R.*
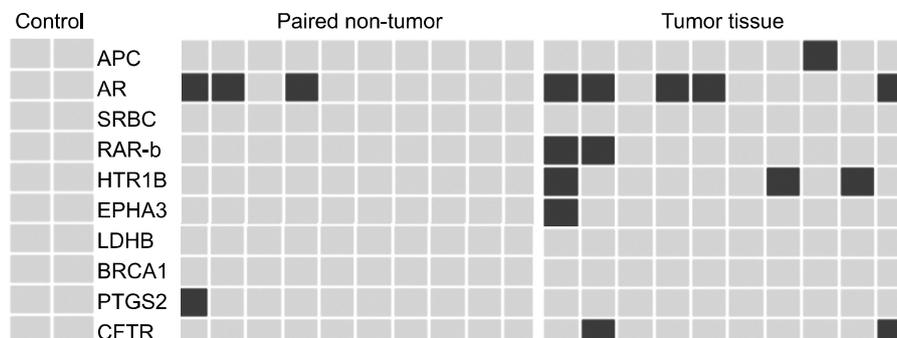

Figure 3. Genes of hypermethylation profiles in tumor tissue. Promoter regions of 6 genes of *APC, AR, RAR-b, HTR1B, EPHA3,* and *CFTR* were more frequently methylated in tumor tissue than non-tumor tissue and not methylated in control group.

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sample and we found that these genes were hyper-methylated in normal control, as well (Figure 3). Among the rest of genes excluding aforementioned eleven genes, four genes (*SRBC, LDHB, BRCA1,* and *PTGS2*) were not methylated in tumor tissue. Then six genes, *APC, AR, RAR-b, HTR1B, EPHA3,* and *CFTR,* finally turned out to be more frequently methylated in tumor tissue and unmethylated in non-tumor tissue and

control group (Table 2).

3. Results from gel electrophoresis

The results for 22 genes including control (*IFN*) from four patients among subjects with gel electrophoresis were completely identical with those of DNA microarray (Figure 4).

Discussion

Aberrant promoter hypermethylation in the field of cancer has been researched vigorously for two decades. After hematologic malignancies with this mechanism and efficacy of administration of demethylating agents were studied, many other solid tumors are having been under investigation for years. Present studies are focusing largely on identification of TSGs functioning as DNA repair genes, cell cycle regulatory genes or chromosomal loci by epigenetic mechanism, and some of them more frequently methylated in specific cancers seem to

Table 2. Frequency of methylation in 6 possible candidate genes

|	Frequency of methylation	
	Non-tumor (%) (n=10)	Tumor (%) (n=10)
APC	0 (0%)	1 (10%)
AR	3 (30%)	5 (50%)
CFTR	0 (0%)	2 (20%)
EPHA3	0 (0%)	1 (10%)
HTR1B	0 (0%)	3 (30%)
RAR-b	0 (0%)	3 (30%)
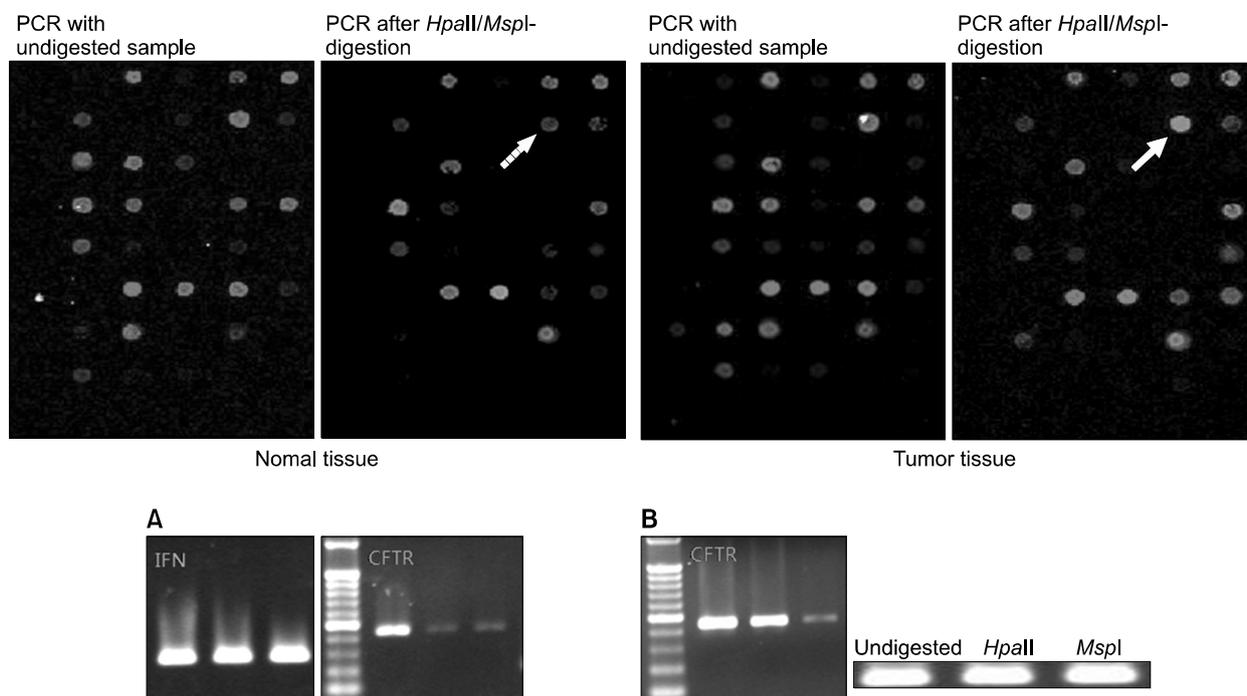
Figure 4. Validation of microarray with gel electrophoresis. An example of gel electrophoresis (patient 1). The signal of *CFTR* was revealed 'negative' in normal tissue (dashed arrow) and 'positive' in tumor tissue (solid arrow) by microarray. And the same results were seen in gel electrophoresis; undigested sample (first lane) only seen in normal tissue (A) and undigested and *Hpa*II-digested sample (second lane) seen with the absence of third lane for tumor tissue (B).

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be possible candidates[7-10]. While, hypermethylation profiles of several genes are being proposed as prognostic markers[11-13].

With the recent development of large-throughput analysis technology, assays allowing CpG island methylation to be analyzed at a genome level were developed. Oligonucleotide-based methylation assays using PCR after bisulfite treatment were developed by Adorjan et al.[14] and Shi et al.[15] Methylation assays using DNA microarray, first introduced in 2001 by Yan et al.[16], include a method comprising modifying the cytosine of genomic DNA into uracil, amplifying the modified DNA, polymerizing the amplification product into oligonucleotide or DNA-oligomer, and hybridizing the polymer in a DNA microarray[17-19]. To show the high accuracy of the method, we performed gel electrophoresis on *IFN*, which doesn't have a promoter region for *Hpa*II, as a control. The results of gel electrophoresis were exactly identical with that of microarray. We suggest that microarray could be a promising method for mass screening in the future, with extended range of genes.

In the present study, *CDH13, CALCA, LAMA5, MTHFR, PIC3CG, S100A2, SHP1, EDN1, H19, MUC2,* and *LTB4R* were commonly in all three group; Among them *CHD13, CALLA* and *EDN1* were described in previous studies to have hypermethylated promoter regions and relation with lung cancer; Takai et al. reported that DNA fragment B3 was hypermethylated in 14 of 20 (70%) primary cancers, and decreased *EDN1* (endothelin-1) expression was observed in 16 of 20 (80%)[20]. In the meanwhile, in present study, EDN1 was hypermethylated in tumor and non-tumor tissue with equal frequency of 60%, and in all of the control tissue. Hypermethylation of the promoter of *CHD13*, a gene encoding H-cadherin, was frequently associated with silencing in a few cancers, including lung cancer[21-23]. Kim et al. reported the frequency of hypermethylation in tumor and non-tumor tissue is 26 of 88 tissue samples (29.5%) and 7 of 88 tissue samples (8%), respectively[24]. In present study, frequency of methylation of tumor and non-tumor tissue was 40%, and 30%, respectively with hypermethylation in all from control group. *CALLA*

(NEP; CD10, EC 3.4.24.11) is a gene for cell surface endopeptidase inactivating the autocrine growth factors and inhibiting cell proliferation, and decreased activation of this gene have been found in lung cancers[25-27]. There could be some considerations for hypermethylation in normal tissue seen in present study; (1) the method with enzyme could have been too sensitive, that allowed false-positive results; (2) the expressions of genes are controlled by normal methylation process during the course of embryogenesis and development, as 'aging process', and the persistently functioning genes in adults are localized to specific tissue for specific functions[28-30], that some of the genes above could be normally silenced by methylation in lung tissue.

Findings from our present study showed the 6 genes of *APC, AR, CFTR, EPHA3, HTR1B,* and *RAR-b* more frequently hypermethylated in tumor tissue than corresponding normal tissue or control, which suggests that their methylation may be a tumor-restricted event. And *AR, APC* and *RAR-b* are well-known to be TSGs silenced with epigenetic alteration for NSCLC from the previous studies[7,31-35], that *CFTR, EPHA3* and *HTR1B* are worthy to be studied further as possible candidates of TSGs by epigenetic mechanism for NSCLC.

Cystic fibrosis transmembrane regulator gene (*CFTR*) is known to be related to chronic idiopathic pancreatitis, a well known risk factor of pancreatic cancer and there were several studies to show that mutation of *CFTR* (Δ F508) would be associated with malignancies, including pancreatic cancer[36,37]. While, recently a few novel functions of CFTR has been reported other than as ion channels in airway epithelial cells in cystic fibrosis[36-38]; (1) As part of a multiprotein complex at the cell surface, *CFTR* might interfere with the expression of several gene products, and participate in the signaling pathways of inflammatory response conjunctly with NF-κB, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and activated protein-1 (AP-1). And when AP-1 is acting associated with transcription co-factors which promote cell proliferation and inhibit apoptosis, this would result in respiratory epithelium carcinogenesis[39]. (2) it also permeate anti-

oxidant-reduced glutathione (GSH) out of epithelial cells, resulting in the low level of intracellular glutathione, that leads to insufficient protection from oxidative stress such as smoking, and damage and apoptosis of cells occur. This mechanism is related with high cellular level of BAX which is a pro-apoptotic member of Bcl-2 family, and in cystic fibrosis which has *CFTR* mutation, the level of BAX would be lowered.

Recently, Brantley et al.[40] reported that *EPHA2* and *EPHA3*, which are in soluble form, are thought to have a role in inhibiting angiogenesis and tumor growth, and treatment with soluble EphA2-Fc or EphA3-Fc receptors inhibited tumor angiogenesis in cutaneous window assays, and tumor growth *in vivo*, in the report of the first functional evidence for Eph A class receptor regulation of pathogenic angiogenesis induced by tumors and support the function of EPHA receptors in tumor progression. As far as we know, there is no study undertaken on hypermethylation of promoter region of *EPHA3* with decreased expression.

Takai et al.[20] reported aberrant hypermethylation of CpG island in the promoter region of *HTR1B* (hydroxy-tryptamine receptor 1B) was seen in two squamous lung cancer cell lines, EBC-a and LK-2, while it was demethylated in normal human bronchial endothelium (NHBE). And when LK-w cells were treated with 5-aza-2-deoxycytidine, H6 fragment was demethylated and expression of *HTR1B* was restored. Maybe due to the heterogeneity of clinical samples of both lung cancer tissue and normal tissue, the results of methylation profile and expression of *HTR1B* was not exactly consistent with that with lung cancer cell lines. The role of *HTR1B* silencing in lung carcinogenesis is not clear but the authors suggested that it is of great interest to examine the role of serotonin and its signaling from its type B receptors.

Some limits in this study was the small number of patients and subject genes, but this study showed hypermethylation profiles of several genes that could be TSGs of lung cancer with microarray, which is not widely used in Korea yet, with high accuracy of the method. DNA microarray has strengths of rapidity, mass analy-

sis, easy to managing and also, high accuracy, suggesting the role of screening tool for cancers with DNA hypermethylation profiles as novel biologic markers. In the present study, CpG islands of 6 genes including *APC, AR, CFTR, EPHA3, HTR1B,* and *RAR-b* were hypermethylated in tumor tissue than tumor adjacent tissue or control group which was assessed with microarray. The results from the present studies and previous reports on *CFTR, EPHA3* and *HTR1B* support that these genes possibly be novel candidates of TSGs in NSCLC. The functional study for each candidate gene is required for the confirmation of the genes as TSG by hypermethylation mechanism. Further study with this design with larger number of patients and whole human genes would be required.

Summary

Background: Epigenetic alterations in certain genes are now known as at least important as genetic mutation in pathogenesis of cancer. Especially abnormal hypermethylation in or near promoter region of tumor suppressor genes (TSGs) are known to result in gene silencing and loss of gene function eventually. The authors tried to search for new lung cancer-specific TSGs which have CpG islands and *Hpa*II sites, and are thought to be involved in carcinogenesis by epigenetic mechanism.

Methods: Tumor tissue and corresponding adjacent normal tissue were obtained from 10 patients who diagnosed with non small cell lung cancer (NSCLC) and underwent surgery in Konyang university hospital in 2005. Methylation profiles of promoter region of 21 genes in tumor tissue & non-tumor tissue were examined with *Hpa*II-*Msp*I methylation microarray (Methyl-Scan DNA chip®, Genomic tree, Inc, South Korea). The rates of hypermethylation were compared in tumor and non-tumor group, and as a normal control, we obtained lung tissue from two young patients with pneumothorax during bullectomies, methylation profiles were examined in the same way.

Results: Among the 21 genes, 10 genes were com-

monly methylated in tumor, non-tumor, and control group. The 6 genes of *APC, AR, RAR-b, HTR1B, EPHA3,* and *CFTR,* among the rest of 11 genes were not methylated in control, and more frequently hypermethylated in tumor tissue than non-tumor tissue.

Conclusion: In the present study, *HTR1B, EPHA3,* and *CFTR* are suggested as possible novel TSGs of NSCLC by epigenetic mechanism.

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