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Oncogene (2002) 21, 5450–5461
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www.nature.com/onc

DNA methylation analysis: a powerful new tool for lung cancer diagnosis

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Carcinoma of the lung is the most common cause of cancer death worldwide. The estimated 5-year survival ranges from 6–16%, depending on the cell type. The best opportunity for improving survival of lung cancer patients is through early detection, when curative surgical resection is possible. Although the subjects at increased risk for developing carcinoma of the lung (long-term smokers) can be identified, only 10–20% of this group will ultimately develop the disease. Screening tests of long-term smokers employed to date (radiography and sputum cytology) have not been successful in reducing lung cancer mortality. The application of molecular markers specific for lung cancer offers new possibilities for early detection. Hypermethylation of CpG islands in the promoter regions of genes is a common phenomenon in lung cancer, as demonstrated by the analysis of the methylation status of over 40 genes from lung cancer tumors, cell lines, patient sputum and/or serum. Determination of the methylation patterns of multiple genes to obtain complex DNA methylation signatures promises to provide a highly sensitive and specific tool for lung cancer diagnosis. When combined with the development of non-invasive methods to detect such signatures, this may provide a viable method to screen subjects at risk for lung cancer.
Oncogene (2002) **21,** 5450–5461. doi:10.1038/sj.onc.1205605

Keywords: lung cancer; methylation; CpG island; diagnosis; hypermethylation

Lung cancer is the leading cause of cancer mortality in most countries, causing over 1 million deaths worldwide each year (Parkin *et al*., 2001). Approximately 90% of such cases are attributable to exposure to cigarette smoke (Williams and Sandler, 2001). In the United States, over 150 000 men and women die of lung cancer each year (Ries *et al*., 1999). Lung cancer is divided into four major histological subtypes as proposed by the World Health Organization (WHO, 1982): Adenocarcinoma (AD), Squamous Cell Carcinoma (SCC), Large Cell Lung Carcinoma (LC), and Small Cell Lung Carcinoma (SCLC), accounting for approximately 30, 30, 10 and 15% of lung cancers respectively (reviewed by Travis *et al*., 1996). Since the prognosis and treatment of SCLC is markedly different from non-SCLC (NSCLC), the four histological subtypes are clinically divided into SCLC and NSCLC (comprising AD, SCC, LC and other minor forms) (Travis *et al*., 1996). Early NSCLC is routinely resected (Naruke *et al*., 1988), with survival rates of 35 to 85%, depending on tumor stage. Unfortunately, most lung cancers are detected late, so that the overall five-year survival rate for NSCLC is only 16% (Ries *et al*., 1999). The standard therapy for SCLC consists of chemotherapy (Elias, 1997), which elicits a response in over 60% of SCLC patients. However, the cancer usually returns within a few months, resulting in an abysmal overall five-year survival rate (6%) for SCLC (Ries *et al*., 1999).

The survival rates for SCLC and NSCLC have changed little over the past two decades. A major factor in the high mortality of lung cancer patients is the presence of metastatic tumors in approximately two-thirds of patients at time of diagnosis (Ries *et al*., 1999). Detection of cancer in these patients at earlier stages could potentially increase survival rates 10–50-fold (Figure 1 (Ries *et al*., 1999)). Lung cancer screening by chest X-ray and sputum cytology have proven ineffective in increasing patient survival (Ellis and Gleeson, 2001; Marcus, 2001), leading to the search for more sensitive and specific tests. One promising approach is the identification of lung cancer-specific biomarkers, and non-invasive methods for the detection of these biomarkers at an early stage. While proteins or other small molecular markers derived from cancers have proven useful in some cases (e.g. in prostate cancer; Ward *et al*., 2001), markers based on changes in the genetic material have the powerful advantage of allowing signal amplification by polymerase chain reaction (PCR), thus increasing sensitivity. One very promising DNA-based alteration commonly occurring in cancer is DNA methylation, an epigenetic modification of DNA. Studies of DNA methylation in lung cancer to date strongly suggest that the analysis of DNA methylation patterns could become a powerful tool for accurate and early lung cancer diagnosis, with unparalleled specificity and sensitivity.

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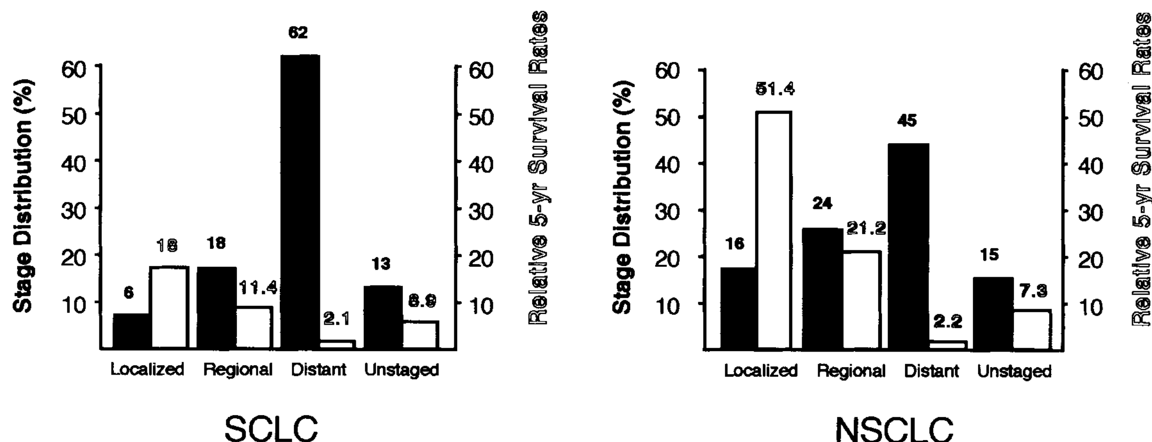

Figure 1 Stage distribution and relative five year survival rates for SCLC (left) and NSCLC (right), based on the Surveillance, Epidemiology and End Results (SEER) study (Ries *et al*, 1999). The stage distribution (black bars) is based on SEER study numbers from 1983–1996. The stages are defined as follows: Localized: a tumor confined entirely to the organ of origin; Regional: a tumor that has extended beyond the organ of origin directly into surrounding organs or tissues and/or regional lymph nodes; Distant: a tumor that has spread to parts of the body remote from the primary tumor by direct extension or by metastasis; Unstaged: information is not sufficient to assign stage. The relative five year survival rates (white bars) are based on follow-up of patients from 1983–1996 and represent the likelihood that a patient will not die from the cancer within five years. Note that for both SCLC and NSCLC the largest group is the Distant category, and that survival of this group is 10–50-fold lower than that of Localized cancers

DNA methylation patterns are altered in cancer cells

In mammalian cells, DNA methylation occurs at the carbon-5 position of cytosine, in palindromic CpG dinucleotides (recently reviewed by Costello and Plass, 2001; Robertson, 2001; Bird, 2002). DNA methylation is essential for proper mammalian development and plays a role in transcriptional repression, modulation of chromatin structure, X-chromosome inactivation, genomic imprinting, and suppression of repetitive and parasitic DNA sequences. CpG dinucleotides are less common than expected in the genome, because methylated cytosine is converted to thymidine upon spontaneous deamination and such a thymidine is repaired much less efficiently than uracil, the deamination product of unmethylated cytosine. Despite this CpG depletion, the mammalian genome contains CpG islands, CpG-rich areas of 0.2 to 1 kilobase in length. CpG islands are frequently found in the promoter regions of genes, and are protected from deamination-mediated CpG depletion because they are usually largely unmethylated in normal cells.

For more than fifteen years, it has been recognized that the methylation patterns of tumor cells are significantly altered compared to those of normal cells (reviewed by Laird, 1997; Jones and Laird, 1999; Baylin *et al*., 2001; Robertson, 2001; Rountree *et al*., 2001). In cancer cells, a genome-wide hypomethylation is observed, which could contribute to carcinogenesis through oncogene activation, retrotransposon activation and chromosome instability. At the same time, local hypermethylation of certain CpG islands is seen. CpG island hypermethylation is associated with gene silencing; the methylated residues are thought to recruit methyl-binding proteins (e.g. MeCP2) and associated factors, such as histone deacetylases, leading to chromatin remodeling and transcriptional shut-down (reviewed by Wade, 2001). Thus, hypermethylation of CpG islands in the promoter regions of growth controlling genes (cell cycle regulators, DNA repair enzymes, and other potential tumor suppressor genes), can contribute importantly to cancer development and/or progression. The identities of the hypermethylated regions can vary between cancers from different organs (Costello *et al*., 2000); certain genes such as CDKN2A ($p16^{INK4A}$) show promoter hypermethylation in almost all cancers, while others show high frequencies of methylation only in very specific tumor types. For instance, glutathione S-transferase π (GSTP1) is methylated in a high fraction of liver cancers and in about 30% of breast cancers, but shows little or no methylation in other types of cancers (Esteller *et al*., 2001).

The study of DNA methylation changes in cancer and their effects has become a topic of intense investigation, as evidenced by the many reviews (e.g. Baylin *et al*., 2001; Costello and Plass, 2001; Roberts *et al*., 1991) and research publications devoted to the topic. DNA methylation changes can not only yield insight in to the complex molecular pathways leading to cancer, but also promise to provide a highly sensitive and specific diagnostic tool. Below, we review our current knowledge of DNA methylation changes in lung cancer, and discuss its implications for patient screening and diagnosis.

DNA hypomethylation in lung cancer

Early studies showed genome-wide and gene-specific hypomethylation (of the human growth hormone, γ-globin, and HRAS genes) in lung tumors compared to the normal tissue counterparts (Feinberg and Vogel-

stein, 1983a,b; Wain et al., 1986). Hypomethylation has been proposed to participate in tumorigenesis by several possible mechanisms (reviewed by Costello and Plass, 2001; Robertson, 2001). Originally, the most important effect of hypomethylation was thought to be the transcriptional activation of oncogenes. However, there has been little evidence to support a role of hypomethylation in cancer by this mechanism. One possible example might be the activation of MAGE genes in NSCLC (Jang et al., 2001). MAGE genes, whose function is unknown, are normally only expressed in the testis, placenta, and skin during wound healing, but are activated in many different cancers. Expression of these genes has been observed in 70–85% of NSCLC tumors, where it was significantly correlated with loss of methylation, detected in 75–80% of tumors. Until the function of the MAGE genes is elucidated, it remains unclear whether their expression can be classified as an example of oncogene activation.

Another possible mechanism by which hypomethylation could contribute to cancer development is through the activation of parasitic genetic elements, such as retroviruses and retrotransposons. Activation of these elements could lead to transcriptional activation of adjacent genes by existing or newly transposed elements, or to the disruption of tumor suppressor genes following transposition. To our knowledge there are no reports to support a role of hypomethylation in lung cancer by this mechanism.

Chromosomal instability resultant from hypomethylation could also potentially affect cancer development (reviewed by Costello and Plass, 2001). This is based on the observation of deletions and translocations of hypomethylated regions of chromosome 1 and 16 in breast adenocarcinomas, ovarian epithelial and other cancers. One study of NSCLC showed that samples exhibiting loss of one of the HRAS alleles were twice as likely to show methylation of the remaining HRAS gene (Vachtenheim et al., 1994), indicating that hypomethylation of the 3′ region of HRAS may stimulate gene loss. Loss of the wild type HRAS gene has been implicated in cancer development in mice (Bremner and Balmain, 1990). In humans, the loss of heterozygosity (LOH) in NSCLC tumors could extend beyond HRAS to other genes that map along chromosome 11p, a chromosomal region that is prone to loss in a variety of tumor types.

Although the biological consequences of hypomethylation in cancer remain largely unknown, consistently observed hypomethylation could be useful as a biological marker for cancer (Jang et al., 2001; Woodson et al., 2001). However, documented cases of methylation loss have thus far been rare.

DNA hypermethylation in lung cancer: a summary of findings

While reports of hypomethylation in lung cancer have been very limited, a flood of studies showing hypermethylation in lung cancer has emerged in the last few years. This is not unexpected, given the large number of studies demonstrating abnormal promoter CpG island methylation of many genes in a variety of neoplasms (Baylin et al., 2001; Costello and Plass, 2001; Robertson, 2001). In lung cancer, the methylation status of over 40 genes has been assessed in tumors, cell lines, serum, and/or sputum. Table 1 summarizes methylation analyses of lung tumors and cell lines to date. Due to differences between the design of the various studies, the materials used, and the techniques applied, the results for each gene listed may not be directly comparable (Siegmund and Laird, 2002). Heterogeneity in the samples or inherent differences between the kinds of samples used (e.g. primary tumors vs cell lines or different lung cancer histologies) may affect the percentage methylated samples observed. Some of the techniques used to assess hypermethylation yield qualitative data (such as Methylation Specific PCR or MSP), resulting in yes/no answers with regard to the presence of methylation, while others generate quantitative data, such as the real-time PCR-based MethyLight assay (Eads et al., 2000; Trinh et al., 2001). The various methods also differ in the number of CpG dinucleotides for which methylation is determined during the analysis. This varies from minimally one, e.g. when using methylation-sensitive restriction enzymes, to ten or more, when using MethyLight or bisulfite genomic sequencing. Bisulfite treatment, which converts unmethylated Cs to Us but does not affect methylated Cs (Olek et al., 1996), is used to incorporate the methylation information in the DNA sequence. The location of the sampled CpGs may also affect outcome, since the methylation patterns within a given CpG island may not be uniform (Zheng et al., 2000). Despite these differences, it is reassuring that overall the results obtained for individual loci agree reasonably well (Table 1). While some variability exists, the various studies consistently identify certain genes as showing no detectable or low methylation, while others are methylated in a substantial percentage of lung cancers. For example, CDKN2B/p15^{INK4B}, ARF/p14 and GSTP1 are not or infrequently methylated in lung cancers. In contrast, multiple reports indicate high percentages of lung tumors showing methylation of APC, CDKN2A/p16^{INK4A}, and RARB (Table 1).

In many cases, cell lines have also been studied. They provide a virtually limitless supply of DNA as well as the possibility for mechanistic studies of the effect of hypermethylation, including measuring gene expression following drug-mediated DNA methylation reversal. How well does the data from tumors agree with that obtained from cell lines? While global analyses of DNA methylation suggest that cell lines are consistently more heavily methylated than primary tissues (Flatau et al., 1983; Smiraglia et al., 2001), the source of this difference is presently unclear. For many loci listed in Table 1, the percentage of cell lines positive for methylation was in the same range as that of positive tumors (e.g. APC, BCL2, CDH1, CDH13, CDKN2A,

Table 1 Hypermethylation of promoter CpG islands in human lung cancer

Genes[a]	Alternative gene name	*Samples* Fraction methylated	Percentage methylated	Histological type[b]	Sample type[c]	Technique used[d]	Reference
AR	Androgen receptor	19/47, 20/44	40, 45	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
ARF	p14	9/107, 0/126, 4/62[e]	8, 0, 6[e]	NSCLC	TU	MSP	Esteller *et al*., 2001; Kim *et al*., 2001b; Zochbauer-Muller *et al*., 2001b
		0/46, 0/43	0, 0	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
APC	Adenomatous polyposis coli	86/91	95	NSCLC	TU	ML	Brabender *et al*., 2001
		22/48, 0/17[e]	46, 0[e]	NSCLC	TU	MSP	Esteller *et al*., 2001; Virmani *et al*., 2001
		95/99	96	NSCLC/other	TU	ML	Usadel *et al*., 2002
		34/58, 13/50	59, 26	NSCLC, SCLC	CL	MSP	Virmani *et al*., 2001
		34/46, 25/43	74, 58	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
BCL2	B-cell CLL/lymphoma-2	28/120, 0/14	23, 0	NSCLC, SCLC	TU	SO	Nagatake *et al*., 1996
		6/11	55	NSCLC	CL	SO	Nagatake *et al*., 1996
BRCA1	breast cancer 1	1/22[e]	4[e]	NSCLC	TU	MSP	Esteller *et al*., 2001
CALCA	calcitonin	8/20, 16/18	40, 89	NSCLC, SCLC	TU, CL	SO	Baylin *et al*., 1986
		35/46, 37/43	76, 86	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
CDH1	E-cadherin	19/107	18	NSCLC	TU	MSP	Zochbauer-Muller *et al*., 2001b
		13/46, 9/43	28, 21	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
CDH13	H-cadherin	18/42	43	NSCLC	TU	MSP	Toyooka *et al*., 2001a
		9/20, 5/7	45, 71	SCLC	TU/CL	MSP	Sato *et al*., 1998
		15/30, 6/30	50, 20	NSCLC, SCLC	CL	MSP	Toyooka *et al*., 2001a
CDKN2A	cyclin-dependent kinase inhibitor 2A, p16INK4A	14/33, 10/29, 9/22, 27/107, 51/185, 8/38, 12/47, 19/50, 9/21, 18/68, 28/89[e], 11/18, 8/10	42, 34, 41, 25, 28, 21, 26, 38, 43, 26, 31[e], 61, 80	NSCLC	TU	MSP	Esteller *et al*., 1999b; Kashiwabara *et al*., 1998; Ng *et al*., 2002; Kim *et al*., 2001b; Zochbauer-Muller *et al*., 2001b; Ahrendt *et al*., 1999; Sanchez-Cespedes *et al*., 1999, 2001; Seike *et al*., 2000; Gorgoulis *et al*., 1998; Belinsky *et al*., 1998; Esteller *et al*., 2001; Palmisano *et al*., 2000
		3/4	75	NSCLC	TU	MSP-ISH	Nuovo *et al*., 1999
		6/19	32	NSCLC	TU	MSP	Hou *et al*., 1999
		21/101, 8/15	21, 53	NSCLC	TU	SO	Herman *et al*., 1996; Tanaka *et al*., 1998
		0/5, 0/6	0, 0	SCLC	TU	SO	Herman *et al*., 1996; Merlo *et al*., 1995
		1/6	17	SCLC	TU	MSP	Seike *et al*., 2000
		14/33	42	NSCLC	TU	MSP	Ng *et al*., 2002
		3/8, 2/3	38, 67	NSCLC, SCLC	CL	SO	Zhu *et al*., 2001
		8/24, 7/11, 7/9, 6/32	33, 64, 78, 19	NSCLC	CL	SO	Herman *et al*., 1996; Otterson *et al*., 1995; Hamada *et al*., 1998; Merlo *et al*., 1995
		3/4, 1/10, 1/10	75, 10, 10	SCLC	CL	SO	Herman *et al*., 1996; Otterson *et al*., 1995; Merlo *et al*., 1995
		22/46, 16/43	48, 37	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
CDKN2B	cyclin-dependent kinase inhibitor 2B, p15	0/27[e], 0/21	0[e], 0	NSCLC	TU	MSP	Esteller *et al*., 2001; Seike *et al*., 2000
		0/15, 0/6	0, 0	NSCLC, SCLC	TU	SO	Herman *et al*., 1996
		1/11, 1/10	9, 10	NSCLC, SCLC	CL	SO	Herman *et al*., 1996
		1/20	5	NSCLC	CL	MSP	Hanada *et al*., 1998
		6/46, 8/43	13, 19	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002;
CDKN2D	cyclin-dependent kinase inhibitor 2D, p19	0/3, 0/8	0	NSCLC/SCLC	CL	MSP	Zhu *et al*., 2001
CTNNB1	β-catenin	0/47, 0/42	0, 0	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
DAPK	death-associated protein kinase	59/135, 47/185	44, 25	NSCLC	TU	ML	Kim *et al*., 2001a; Tang *et al*., 2000
		20/107, 5/22	19, 23	NSCLC	TU	MSP	Esteller *et al*., 1999b; Zochbauer-Muller *et al*., 2001b
		10/64[e]	16[e]	NSCLC	TU	MSP	Esteller *et al*., 2001

Continued

Table 1 (*Continued*)

Genes[a]	Alternative gene name	Samples: Fraction methylated	Percentage methylated	Histological type[b]	Sample type[c]	Technique used[d]	Reference
EDN1	endothelin 1	14/20	70	NSCLC	TU	SO	Takai *et al*., 2001
EGFR	epidermal growth factor receptor	7/7, 2/3	100, 67	NSCLC, SCLC	CL	SO	Gamou *et al*., 1988
ESR1	estrogen receptor α	11/46, 4/11	24, 36	NSCLC, SCLC	TU, CL	SO	Issa *et al*., 1996
		34/46, 24/43	74, 56	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
ESR2	estrogen receptor β	29/47, 23/44	62, 52	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
FHIT	fragile histidine triad	40/107	37	NSCLC	TU	MSP	Zochbauer-Muller *et al*., 2001a
		16/25, 14/22	64, 64	NSCLC, SCLC	CL	MSP	Zochbauer-Muller *et al*., 2001a
GDF10	growth/differentiation factor (BMP3B)	5/6	83	NSCLC	CL	RLGS/SO	Dai *et al*., 2001
GSTP1	glutathione S-transferase π	2/21[e]	9[e]	NSCLC	TU	MSP	Esteller *et al*., 2001
		7/107, 2/22	7, 9	NSCLC	TU	MSP	Esteller *et al*., 1999b; Zochbauer-Muller *et al*., 2001b
		19/47, 26/44	40, 59	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
HIC1	hypermethylated in cancer	17/51	33	NSCLC	TU	SO	Eguchi *et al*., 1997
		46/47, 44/44	98, 100	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
HOXB	homeobox B cluster	3/4[f]	75[f]	SCLC	TU	MSP	Flagiello *et al*., 1996
HTR1B	5-hydroxytryptamine receptor 1B	14/20, 2/2	70, 100	NSCLC	TU, CL	SO	Takai *et al*., 2001
MGMT	0^6-methylguanine-DNA methyltransferase	27/92, 22/107, 6/22, 6/10	29, 21, 27, 60	NSCLC	TU	MSP	Esteller *et al*., 1999a; Palmisano *et al*., 2000; Wolf *et al*., 2001; Zochbauer-Muller *et al*., 2001b
		18/83[e]	21[e]	NSCLC	TU	MSP	Esteller *et al*., 2001
		10/41, 10/34	24/29	NSCLC, SCLC	TU	MSP	Esteller *et al*., 1999a
		38/47, 40/44	81, 91	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
		8/47[g], 10/44[g]	17[g], 23[g]	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
MLH1	*Mut* L homolog	0/20[e]	0[e]	NSCLC	TU	MSP	Esteller *et al*., 2001
		2/46, 1/43	4, 2	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
MTHER	5-,10-methylene-tetra-hydrofolate reductase	46/47, 42/42	98, 100	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
MYOD1	myogenic factor 3	30/46, 18/43	65, 42	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
PGR	progesterone receptor	38/47, 37/42	81, 88	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
PTGS2	prostaglandin G/H synthase, cyclooxygenase-2	36/46, 41/43	78, 95	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
PTHRP[h]	parathyroid hormone related protein	6/7	85; 0 at Cpg island[h]	NSCLC	TU	BGS	Ganderton *et al*., 1995
RARB	retinoic acid receptor β	21/49, 13/21	43, 62	NSCLC, SCLC	TU	MSP	Virmani *et al*., 2000
		43/107	40	NSCLC	TU	MSP	Zochbauer-Muller *et al*., 2001b
		31/78, 50/66	40, 76	NSCLC, SCLC	CL	MSP	Virmani *et al*., 2000
RASSF1A	RAS effector homologue	32/107	30	NSCLC	TU	MSP	Burbee *et al*., 2001
		22/58, 14/41	38, 34	NSCLC	TU	BGS	Agathanggelou *et al*., 2001; Dammann *et al*., 2000
		2/2, 21/29	100, 72	SCLC	TU	BGS	Agathanggelou *et al*., 2001
		16/16	100	SCLC	CL	BGS	Dammann *et al*., 2000
		17/27, 47/47	63, 100	NSCLC, SCLC	CL	MSP	Burbee *et al*., 2001
		4/11, 18/25	36, 72	NSCLC, SCLC	CL	BGS	Agathanggelou *et al*., 2001

Continued

Table 1 (*Continued*)

Genes[a]	Alternative gene name	Samples: Fraction methylated	Percentage methylated	Histological type[b]	Sample type[c]	Technique used[d]	Reference
RB1	retinoblastoma 1	0/14	0	NE/SCLC	TU	SO	Gouyer *et al*., 1998
		0/120, 0/14	0, 0	NSCLC, SCLC	TU	SO	Nagatake *et al*., 1996
		0/47, 0/43	0, 0	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
RIZI	retinoblastoma protein-interacting zinc finger	2/7, 1, 1	29, 100	NSCLC, SCLC	CL	BGS/SO	Du *et al*., 2001
S100A2	nuclear calcium-binding protein	8/9	89	NSCLC	CL	MSP	Feng *et al*., 2001
SRBC	serum deprivation response factor, c-raf-1	11/14	79	NSCLC	TU	BGS	Xu *et al*., 2001
TGFBR2	transforming growth factor	3/25, 0.21	12, 0	NSCLC, SCLC	CL	MSP, SO	Hougaard *et al*., 1999; Osada *et al*., 2001
	β receptor II	0/47, 3/42	0, 7	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
THBS1	thrombospondin	1/47, 2/44	2, 5	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
TIMP3	tissue inhibitor of	28/107	26	NSCLC	TU	MSP	Zochbauer-Muller *et al*., 2001b
	metalloproteinase 3	4/21[e], 6/12	19[e], 50	NSCLC	TU, CL	MSP	Bachman *et al*., 1999; Esteller *et al*., 2001
		14/47, 22/44	30, 50	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002
TP73	tumor protein 73	0/22[e]	0[e]	NSCLC	TU	MSP	Esteller *et al*., 2001
TSLC1	tumor suppressor in lung cancer 1	21/54, 6/12	39, 50	NSCLC	TU, CL	BGS	Kuramochi *et al*., 2001
TYMS	thymidylate synthetase	0/47, 1/44	0, 2	NSCLC, SCLC	CL	ML	Virmani *et al*., 2002

[a]Human Genome Organization (HUGO) designation used when available. Genes for which silencing has been demonstrated are indicated in **bold**; [b]Small cell lung carcinoma (SCLC) or non-small cell lung carcinoma (NSCLC); [c]Sample type denoted as tumor (TU) or cell line (CL); [d]Techniques used for methylation analysis: Methylation Specific PCR (MSP), Southern blot (SO), MethyLight (ML), Bisulfite Genomic Sequencing (BGS), Restriction Landmark Genomic Sequencing (RLGS), and Methylation Specific PCR In Situ Hybridization (MSP-ISH); [e]Tumor type was not specified by authors, however NSCLC tumors are the most widely used and SCLC tumors are rare so it is likely that the tumor samples are NSCLC; [f]3/4 tumors were methylated for more than 5 HOX genes, and 1/4 tumors was methylated for 1 HOX gene; [g]The region analysed for methylation was at the first exon/intron boundary instead of the promoter region; [h]Methylation was found at a CpG cluster upstream of the CpG island at the promoter of the PTHRP gene; no methylation was found in the promoter

RARB, RASSF1A, and TIMP3), suggesting that local methylation changes in tumors may be generally maintained in cell lines. A recent analysis of bladder cancers and their corresponding cell lines over time supports this observation (Markl *et al*., 2001). These results indicate that cell lines might be viable tools to prescreen large sets of potential new DNA methylation markers, an exciting possibility (Virmani *et al*., 2002). Further comparisons of tumors and cell lines will be required to determine whether this is true.

DNA hypermethylation in lung cancer: biological implications

The genes thus far found to be hypermethylated in over 30% of lung tumors (based on at least two independent studies) are: APC, CDKN2A, CHD13, RARB, and RASSF1A (Table 1). Each of these five genes has been demonstrated to be transcriptionally silenced in cell lines/tissues showing methylation (Table 1). Re-expression of these genes was seen in lung cancer cell lines following treatment with the methylation inhibitor 5-aza-2′ deoxycytidine, further supporting the notion that methylation caused their inactivation (Brabender *et al*., 2001; Dammann *et al*., 2000; Merlo *et al*., 1995; Otterson *et al*., 1995; Toyooka *et al*., 2001a; Virmani *et al*., 2000; Zhu *et al*., 2001). These commonly methylated genes are potential tumor suppressors, involved in the regulation of cell cycle, adhesion, apoptosis, and signal transduction. Their silencing argues for DNA methylation as a common mechanism for gene inactivation in lung cancer. The observation of methylation of multiple other genes, albeit less well documented, suggests that many more genes are inactivated by this mechanism. However, caution should be exercised, since methylation does not guarantee gene silencing (Baylin and Herman, 2001). Irrespective of whether or not they lead to gene silencing, consistently occurring methylation events are of great interest due to their potential applicability as epigenetic lung cancer markers.

While much can be learned from which genes are methylated in lung cancer, information can also be gleaned from the (sometimes unexpected) absence of hypermethylation. A number of genes shown to be methylated in other cancers (ARF, CDKN2B, CTTNB1, MLH1, RB1) have exhibited little or no methylation in lung cancer. For example, no methylation of the RB1 gene was detected in lung cancer cell

Table 2 Detection of promoter hypermethylation in remote media (blood/exfoliative material) from lung cancer patients and high-risk subjects

Genes	*Remoted medium source*	*Fraction of tumors showing methylation*	*Fraction of remote medium samples positive for methylation and relationship to matched tumors*	*Subsequent tumor development in patients with positive remote medium samples*	*Reference*
CDKN2A	Sputum collected from smokers before knowledge of medical history	ND	8/33 sputum samples	3/8 subjects diagnosed with lung cancer at time of sputum collection (2 NSCLC and 1 SCLC/NSCLC), 1/8 developed SCLC one year later, 4/8 remain cancer-free	Belinsky *et al*., 1998
	Sputum collected from high risk cancer-free patients exposed to tobacco and/or radon	0/3	18/123 sputum samples	so far no subjects positive for CDNK2A methylation have developed lung cancer; 3 lung cancer cases developed in cases with negative sputum	Palmisano *et al*., 2000
	Sputum collected from chronic smokers with no cancer	3/3	7/25 sputum, bronchoalveolar lavage or brushing samples	3/25 subjects developed cancer (2 lung, 1 esophagus), all 3 positive for methylation	Kersting *et al*., 2000
	Sputum collected 5–35 months prior to SCC diagnosis	10/11	10/10 sputum samples from methylated tumors, 1/11 from unmethylated tumors	only sputum samples from patients who developed SCC were analysed	Palmisano *et al*., 2000
	Sputum from SCC patients collected at time of diagnosis	8/10	8/8 sputum samples from methylated tumors, 0/2 from unmethylated tumors	NA	Palmisano *et al*., 2000
	Sputum collected from lung cancer patients	4/5	26/51 sputum, bronchoalveolar lavage or brushing samples	NA	Kersting *et al*., 2000
	Serum collected from lung cancer patients	9/22	3/9 serum samples from methylated tumors, 0/13 from unmethylated tumors	NA	Esteller *et al*., 1999b
	Bronchoalveolar lavage from 50 patients with resectable (stage I–IIIa) lung cancer	19/50	12/19 bronchoalveolar lavage samples from methylated tumors, 0/31 from unmethylated tumors	NA	Ahrendt *et al*., 1999
MGMT	Sputum collected from high risk cancer free patients exposed to tobacco and/or radon	2/3	31/123 sputum samples	so far 2 subjects positive for MGMT methylation have developed lung cancer, both tumors are for positive for methylation; 1 methylation negative lung cancer developed from a sputum negative subject	Palmisano *et al*., 2000
	Sputum collected 5–35 months prior to SCC diagnosis	9/11	7/11 sputum samples from methylated tumors, 0/2 from unmethylated tumors	NA	Palmisano *et al*., 2000
	Sputum from SCC patients collected at time of diagnosis	6/10	4/6 sputum samples from methylated tumors, 1/4 from unmethylated tumors	NA	Palmisano *et al*., 2000
	Serum collected from lung cancer patients	6/22	4/6 serum samples from methylated tumors, 0/16 from unmethylated tumors	NA	Esteller *et al*., 1999b
DAPK	Serum collected from lung cancer patients	5/22	4/5 serum samples from methylated tumors, 0/17 from unmethylated tumors	NA	Esteller *et al*., 1999b
GSTP1	Seurm collected from lung cancer patients	2/22	1/2 serum samples from methylated tumors, 0/20 from unmethylated tumors	NA	Esteller *et al*., 1999b
APC	Serum/plasma collected from lung cancer patients at time of surgery	95/96	42/89 available serum/plasma samples; 0/1 from the unmethylated tumor; 0/50 from cancer-free controls	NA	Usadel *et al*., 2002

ND: not done, NA: not applicable. Highlighted areas indicate that sputum samples were taken from subjects for which presence of cancer was unknown

lines (Gouyer *et al*., 1998; Nagatake *et al*., 1996; Virmani *et al*., 2002) even though RB1 is thought to be inactivated in virtually all SCLC (Viallet and Minna, 1990), and RB1 is inactivated by methylation in a significant proportion of retinoblastoma tumors (Stirzaker *et al*., 1997). Similarly, the cell cycle regulator genes ARF and CDKN2B show very little methylation in lung cancer (Esteller *et al*., 2001; Zochbauer-Muller

et al., 2001b; Herman *et al*., 1996; Virmani *et al*., 2002), but have been shown to be inactivated in lung cancer by other means, such as deletions (Herman *et al*., 1996; Sanchez-Cespedes *et al*., 1999). The basis for the differences in mechanisms of gene silencing/loss in cancers arising in different organs is intriguing. It is likely related to the timing of the inactivation of individual genes (whether they occur early or late in a particular kind of cancer) and the mechanisms for gene inactivation available in the cell at those times. For example, the previous inactivation of repair enzymes by methylation may promote mutation as a common mechanism for subsequent inactivation events. The type of carcinogenic stimuli to which different kinds of tissues are exposed is also likely to influence the mechanism and sequence of gene inactivation. For the moment, data about the timing of hypermethylation events in lung cancer is limited and sometimes contradictory.

One of the genes that is thought to be prone to hypermethylation early during lung cancer development is cell cycle regulator CDKN2A. CDKN2A is the gene that has been studied most in DNA methylation analyses of lung cancer (Table 1). The strongest evidence supporting early methylation of CDKN2A is the observation that methylation of this gene can precede clinical diagnosis of lung cancer. Two studies independently report the detection of CDKN2A in sputum of individuals with no detectable cancer (Kersting *et al*., 2000; Palmisano *et al*., 2000). In one of these studies, CDKN2A methylation was evident in two sputum samples which had been collected from subjects almost three years prior to diagnosis (Palmisano *et al*., 2000). Very early methylation of CDKN2A is also supported by an analysis of four SCC samples using methylation-specific PCR *in situ* hybridization (MSP-ISH), which allows the methylation status of a gene to be studied in individual cells (Nuovo *et al*., 1999). MSP-ISH showed that methylation of CDKN2A first becomes visible in dysplastic cells that are the earliest progenitors of SCC.

Studies similar to those described above for CDKN2A will be very useful to determine the timing of hypermethylation of other genes. To date, such studies have been limited to a handful of genes (Table 2). In combination with the use of animal models, in which methylation changes are studied in animals exposed to carcinogens from tobacco smoke (Belinsky, 1998; Belinsky *et al*., 1998; Swafford *et al*., 1997), this should eventually yield an epigenetic roadmap describing the contribution of hypermethylation in the molecular pathway to lung cancer.

Sensitivity and specificity of DNA methylation markers for lung cancer detection

An early diagnosis of lung cancer is not easily made (reviewed by Black, 1999). Small lesions detected by radiography or other imaging methods must be sampled in order to be histologically or cytologically examined, but tumor tissue is frequently difficult to obtain. Resections are invasive procedures, carried out only when they clearly benefit the patient (usually limited to NSCLC stages I, II and IIIA). Fine needle aspirates (FNA) may be used to obtain tumor material for diagnosis, but yields may be low and the reliability of the obtained sample is highly dependent on the ability to accurately locate the biopsy area. Exfoliative material (present in sputum, bronchoalveolar lavage, and bronchial brushings) offers diagnostic possibilities, but the sensitivity of current cytological tests is low. Diagnostic tools that would provide high specificity and sensitivity would clearly be of enormous benefit to patients, particularly if the specimens could be obtained by non-invasive means. DNA-based diagnostics allow signal amplification by PCR, and thus could provide an improved sensitivity. Tumor DNA can be found in exfoliative material (Ahrendt *et al*., 1999; Belinsky *et al*., 1998; Kersting *et al*., 2000; Palmisano *et al*., 2000) and in the blood of a substantial fraction of cancer patients (Esteller *et al*., 1999b; Leon *et al*., 1977; Usadel *et al*., 2002). Recent reports of DNA methylation analyses carried out with serum, plasma, sputum and bronchial lavages or brushings (henceforth referred to collectively as ‘remote media’) are therefore of great interest (Table 2).

Of primary concern for the utility of DNA methylation markers for lung cancer diagnosis is the sensitivity and specificity of the marker. Sensitivity is the ability to identify a cancer case, and is mathematically defined as the ratio of correctly identified positive samples (‘true positives’) over all existing cancer-positive patients (‘true positives’ plus ‘false-negatives’). Optimal sensitivity is achieved when the number of false negatives approaches zero. Specificity is the ability to correctly identify cancer-free subjects, and is mathematically defined as the ratio between correctly identified non-cancer cases (‘true negatives’) and all existing cancer-free subjects (‘true negatives’ plus ‘false-positives’). Specificity is high when the number of false positives is low (Fleiss, 1981).

How sensitive and specific can DNA methylation analysis be when applied to lung cancer diagnosis? The sensitivity depends foremost on the prevalence of the methylation change in lung cancer. If hypermethylation of the tested gene is absent in a substantial percentage of tumors, the sensitivity will be poor. This appears to be so in all cases but those of APC and RASSF1A (Table 1, and white sections, Table 2). Low prevalence problems can be addressed by using panels of genes instead of individual ones (Kersting *et al*., 2000; Virmani *et al*., 2002). Signatures derived from multiple genes can provide unprecedented pattern recognition, as evidenced by the recent observation that expression profiles could be used to predict clinical outcome of breast cancer patients (van’t Veer *et al*., 2002). This appears to hold true for methylation patterns as well; methylation patterns differ between cancers from different organs (Costello *et al*., 2000; Esteller *et al*., 2001) and even between different cancer histologies from the same organ (Toyooka *et al*., 2001b; Virmani *et*

al., 2002). The applicability of DNA methylation analysis to the discrimination between different histological subtypes of lung cancer is strongly supported by the observation that methylation patterns of SCLC cell lines differ from those of NSCLC lines. In a study of almost 100 NSCLC and SCLC cell lines, the methylation analysis of a mere 23 loci yielded seven genes exhibiting statistically significant differences in methylation levels between the two groups (Virmani *et al*., 2002). The subsequent application of a hierarchical clustering algorithm using data from the seven genes allowed the distinction between SCLC and NSCLC cell lines with a specificity and sensitivity of 78% (Virmani *et al*., 2002). While clinically applicable markers require better sensitivity and specificity, it should be emphasized that this study was the first of its kind, and that the panel of markers tested was very modest, in particular when compared with the size of panels routinely used for expression analyses (thousands). Thus, it is highly likely that with the expansion of the collection of informative markers, the ability of methylation panels to discriminate between different histological subtypes of lung cancer will improve substantially. Such panels would be extremely valuable at several different levels. In their most simple application, they could be used to analyse samples derived from lung cancer tissue (obtained by resection, biopsy or FNA). They would serve as diagnostic aids for the pathologist, helping establish the correct histology of the cancer. The ability to discriminate between NSCLC and SCLC is of high clinical relevance due to the important differences in treatment and survival of patients with these cancers. As more knowledge is gained about clinical correlations with methylation patterns, methylation signatures may also provide information about response to therapy and prognosis within a given histological group.

If the cancer-specific methylation signature is detectable in remote media, its presence could be used to monitor the patient's response to therapy; loss of markers from lung cancer patient sera following resection, and return of markers following recurrence has been reported (Usadel *et al*., 2002). Whether methylation analysis of remote media could be used as a diagnostic tool for lung cancer would depend on the frequency with which methylation signatures can be detected. This will depend on the level of 'shedding' of tumor DNA into the sampled tissue or fluid, and will likely vary dependent on tumor location and stage. Poor representation in the remote medium will negatively affect sensitivity. The presence of tumor DNA in patient blood is generally estimated to occur in less than half of cancer cases, thereby strongly reducing the sensitivity of serum analyses (e.g. see APC, Table 2 (Usadel *et al*., 2002)). In contrast, detection can approach 100% when sputum of SCC patients is analysed (Palmisano *et al*., 2000). This is encouraging, although it remains to be seen whether it will also hold true for other histological subtypes of lung cancer that arise more peripherally in the lung.

If methylation signatures obtained from remote media are to be used as diagnostic tools, a high specificity is crucial. How frequently a given methylation signature is seen in patients who do not develop cancer is an important question to address, since false positive results are highly undesirable due to the psychological and clinical consequences. False positives could be caused by environmental factors such as exposure to smoke, lung disease, and age of the subject, which could affect methylation patterns even in subjects that never develop cancer (Belinsky *et al*., 1998). A number of studies have been done to determine the effect of smoking on DNA methylation (Belinsky, 1998; Eguchi *et al*., 1997; Hou *et al*., 1999; Kersting *et al*., 2000; Kim *et al*., 2001b; Swafford *et al*., 1997). An increase in cytosine DNA-methyltransferase activity in alveolar type II cells was reported in mice treated with the tobacco-specific carcinogen NKK (Belinsky, 1998), and hypermethylation of CDKN2A has been seen in rats exposed to NKK, tobacco smoke, radioisotopes and/or X-rays (Belinsky *et al*., 1998; Swafford *et al*., 1997). Contradictory results have been obtained from studies of the relationship between smoking and DNA methylation in human lung tumors. Several analyses of hypermethylation of CDKN2A, DAPK, GSTP1, and APC showed no correlation between methylation at these loci and the smoking history of subjects (Brabender *et al*., 2001; Kim *et al*., 2001a; Sanchez-Cespedes *et al*., 2001). However, other studies suggest that such a link does exist: an analysis of 185 primary NSCLC cases showed significant associations between CDKN2A methylation and pack years smoked as well as duration of smoking (Kim *et al*., 2001b). Another study showed a close association between a higher deposition of black dust matter in the lung and hypermethylation of CDKN2A (Hou *et al*., 1999). In an analysis of eight symptomatic chronic smokers with no detectable cancer but evidence of CDKN2A methylation in sputum, three subjects later developed malignancies (two lung cancers and one esophageal cancer) and all three showed methylation of CDKN2A in the tumors (Kersting *et al*., 2000). Methylation of the HIC1 locus in paired tumor and non-tumor lung tissue from 51 NSCLC patients was also shown to be associated with smoking status, when DNA of tumor as well as non-tumor tissue was examined (Eguchi *et al*., 1997). Thus, it appears that exposure to smoke can affect methylation patterns. Age of the tested subjects could also influence the observed methylation patterns, since methylation has been seen to increase with age (Issa, 2000). Further studies will be required to more clearly define the role of smoking and age in the development of methylation patterns, and determine the specificity of methylation markers obtained from smokers and non-smokers of different ages.

Related to the issue of specificity is the question of similarities in methylation patterns found in tumors from different organs. Some genes which show high levels of methylation in lung cancer, such as APC (Brabender *et al*., 2001; Usadel *et al*., 2002; Virmani *et al*., 2001), are also highly methylated in other cancers (in the case of APC, cancers of the digestive tract (Esteller *et al*., 2001). Thus, a patient with a positive

APC methylation signal in serum might have lung cancer, colon cancer, colon cancer metastasized to the lung, or any other neoplasm in which APC methylation is common. While the source of the methylation signature and other clinical characteristics of a patient may help pinpoint the location of a malignancy, ideally, specific methylation signatures should be identified to distinguish between neoplasms from different organs. With the sampling of sufficiently large panels of methylation markers, this should be feasible (Esteller *et al*., 2001; Virmani *et al*., 2002).

Screening high risk groups for lung cancer using DNA methylation signatures

Whether screening of high risk groups such as long-term smokers for lung cancer is feasible or even desirable has often been debated (reviewed by Black, 1999; Ellis and Gleeson, 2001; Marcus, 2001). Previous trials of chest radiography and sputum cytology did not improve survival. It has also been argued that screening may lead to overdiagnosis and the administration of invasive treatments to subjects who might never have required treatment under normal circumstances. The development of highly specific and sensitive methylation analyses would remove these objections, provided adequate non-invasive means of obtaining these signatures could be developed. The sensitivity and specificity of sputum cytology and chest radiography are very low compared to what could potentially be achieved with a highly informative panel of methylation markers. In addition, studies exploring correlations between clinical parameters (survival, risk of relapse, response to treatment, etc.) and methylation signatures could be carried out, and with the screening of sufficient markers, will likely generate powerful prognostic indicators. Therefore, next to identifying patients with cancer, methylation signatures could also provide information on which treatment is indicated. One of the biggest challenges will be the improvement of non-invasive procedures for obtaining these signatures. The technical optimization of assays such as MethyLight, and the combination of sputum and serum analyses might increase sensitivity. The most immediate challenge, however, is to identify a panel of markers with the highest sensitivity and specificity.

How would such a panel be developed? One method for doing so is to use a candidate gene approach in which promoter CpG islands, selected based on knowledge about the corresponding genes (their putative function, their inactivation/methylation in certain cancers, etc.) are tested (Virmani *et al*., 2002). Alternatively, a global approach can be applied, in which thousands of CpG islands are tested simultaneously (Costello *et al*., 2000). The gene BMP3B/GDF10 was identified as a possible new methylation marker for lung cancer from a screen of over 1000 CpG islands in NSCLC cell lines (Dai *et al*., 2001). The power of methylation analyses to generate specific signatures is formidable, considering that the genome contains an estimated 45 000 CpG islands, each of which could be methylated or not (resulting in $2^{45\,000}$ possible distinct methylation patterns). While this complexity may not entirely approach that which may be obtained using gene expression profiles, technically methylation analysis has several advantages. The first is that the variables studied (CpG island methylation) are largely negative in normal tissues, so that positive methylation will usually be informative. In contrast, in expression studies, genes may be transcribed at different levels in a variety of tissues or under a variety of normal conditions that are not necessarily related to cancer. The second advantage is that methylation analyses require DNA, instead of the more labile RNA, and can be carried out on many different kinds of samples (including paraffin-embedded fixed material) using a high through-put technology (Eads *et al*., 2000).

Harnessing the power of methylation signatures will be a challenge, requiring the identification of possible markers, the testing of these markers through case–control association studies and prospective trials (Sullivan Pepe *et al*., 2001), and the development of statistical tools to analyse complex methylation data (Siegmund and Laird, 2002). However, the investment will be well worth the effort, as it promises to yield one of the most powerful diagnostic tools yet.

Acknowledgements
The authors are grateful for the generous support provided by Mr and Mrs Eri and Mary Lou Metler and the Lung Cancer Methylation Fund, and for critical comments on the manuscript by members of the Laird-Offringa laboratory.

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