Jpn. J. Cancer Res. **93**, 184–189, February 2002

Inactivation of O^6-Methylguanine-DNA Methyltransferase in Human Lung Adenocarcinoma Relates to High-grade Histology and Worse Prognosis among Smokers

Hiroyuki Hayashi, Takuya Yazawa, Koji Okudela, Jun-ichi Nagai, Takaaki Ito, Masayoshi Kanisawa and Hitoshi Kitamura

Department of Pathology, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004

To evaluate the significance of O^6-methylguanine-DNA methyltransferase (MGMT) activity in the development of human lung adenocarcinoma (AC), we investigated promoter hypermethylation of the *MGMT* gene by methylation-specific PCR, and the expression of MGMT protein by immunohistochemistry in relation to smoking history of the patients. In total, 31 of 87 AC patients (35.5%) showed hypermethylation of the *MGMT* gene, and no significant difference was observed between smokers (37.3%) and non-smokers (33.3%). However, hypermethylation of the *MGMT* gene increased in parallel with lesser differentiation grade of tumors among smokers (well, 16.7%; moderately, 42.1%; poorly, 57.1%; $P=0.022$), although this trend was not observed among non-smokers. Almost all the tumors with promoter hypermethylation of the *MGMT* gene showed consistently negative MGMT staining by immunohistochemistry. When the prognosis of stage-I patients was compared among smokers, it was apparent that the prognosis of patients with inactivated MGMT was worse than that of MGMT-positive patients ($P=0.036$). Such differences in the prognoses were not observed among non-smokers. In conclusion, MGMT inactivation is related to the differentiation grade and the prognosis of lung AC patients among smokers. Although further studies are required, we speculate that smoking may induce hypermethylation, not only of the *MGMT* gene, but also of other important tumor suppressor genes.

Key words: Lung — Adenocarcinoma — Smoking — O^6-Methylguanine-DNA methyltransferase — DNA methylation

O^6-Methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein that protects cells against the carcinogenic and cytotoxic effects of alkylating agents by transferring alkyl groups of guanine from the O^6 position to an internal cysteine residue.[1] Since O^6-methylguanine tends to pair with thymine during replication and results in a guanine-to-adenine mutation,[2] a lack of MGMT expression may have a role in carcinogenesis induced by alkylating agents such as N-nitrosamines. There is substantial evidence that a lack of MGMT expression is a source of guanine-to-adenine mutation.[3–6] Transgenic expression of MGMT has been reported to protect colonic mucosa,[3] lung cells,[4] and thymic lymphocytes[5] from these carcinogenic effects in mice. In humans, a lack of its expression may be related to the development of gliomas,[7] non-small cell lung cancers,[8] and colonic cancers.[6, 9]

The lack of MGMT expression is not commonly due to deletion, mutation, or rearrangement of the *MGMT* gene.[10] Hypermethylation of CpG islands in its promoter region is the most important mechanism.[11]

Undoubtedly, tobacco smoke is a leading cause of lung cancer, especially of squamous cell carcinoma and small cell carcinoma. In the case of adenocarcinoma (AC), however, the carcinogenic effects of smoking are still controversial. Some AC are caused by smoking, while the remainder is unrelated. It has been reported that MGMT activity was significantly higher in smokers than in non-smokers in both the normal and neoplastic lung tissues.[8, 12] This implies that smoking induces MGMT activation in human lungs. Experimental studies have shown that 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) treatment caused a rapid increase in the DNA-methyltransferase (DNA-MTase) activity in A/J mice, which are genetically susceptible to lung carcinogenesis.[13] Since DNA-MTase modulates DNA methylation patterns, this process might result in inactivation of MGMT, as well as other genes, by DNA hypermethylation.

Thus far, MGMT expression has been studied in various human neoplasms, but not in their precursors of any kind. K-*ras* gene mutation is regarded as a relatively early event during the development of lung AC, and occurs in atypical adenomatous hyperplasia (AAH) cells, a precursor of lung AC.[14] In MGMT-transgenic mouse lung tumors, K-*ras* gene mutations were less frequent than in controls, probably due to repair of the injured K-*ras* gene by elevated MGMT activity.[4] It therefore seems important to evaluate

E-mail: hirohaya@med.yokohama-cu.ac.jp

the role of MGMT in the occurrence and prognosis of human lung cancer.

In the present study, we investigated the methylation status of CpG islands in the *MGMT* gene promoter region, as well as its expression, to clarify the role of MGMT activity in the development and prognosis of lung AC, and we compared its significance in smokers and non-smokers.

MATERIALS AND METHODS

Patients and tissues Of the 149 consecutive Japanese patients with lung AC who had been surgically treated between 1987 and 1995 in Kanagawa Cardiovascular and Respiratory Center, frozen tissues were available from 87 patients. They consisted of 38 lesions of well-differentiated AC (W-AC), 31 of moderately differentiated AC (M-AC), and 18 of poorly differentiated AC (P-AC), including 33 bronchioloalveolar carcinoma, 14 papillary types and 40 mixed or solid types. Histological grade and type were determined according to the WHO classification.[15] The mean age of the patients was 65 (range 37–84); 37 were female and 50 were male. Among them, 51 patients had a smoking history, and the others were non-smokers. Methylation-specific PCR (MSP) was performed on the 87 tumors. Immunohistochemistry was performed on all of them as well as on 28 additional stage I-tumors, whose DNA was not available for MSP. Ten cases of AAH were also examined by immunohistochemistry alone. In 10 AAH patients, 5 were smokers, 2 were non-smokers, and in 3 no smoking history was available. In total, 125 patients were studied by MSP and/or immunohistochemistry.

MSP DNA was extracted by the phenol-chloroform method from frozen tissues. Bisulfite pretreatment was performed using a CpGenome DNA Modification Kit (Intergen Co., Purchase, NY) according to the manufacturer's instructions. Control methylated DNA was obtained from CpGenome Universal Methylated DNA (Intergen Co.). Primers were designed to amplify individually unmethylated and methylated alleles. Primer sequences of the promoter region of the *MGMT* gene were 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′ and 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′ for the unmethylated reaction (product size: 93 bp), and 5′-TTTCGACGTTCGTAGGTTTTCGC-3′ and 5′-GCACTCTTCCGAAAACGAAACG-3′ for the methylated reaction (81 bp).[9, 16, 17] Forty PCR cycles (94°C for 30 s, 60°C for 1 min, and 72°C for 1 min) were performed. Each PCR product was loaded onto nondenaturing 12% polyacrylamide gels, and visualized by Silver Stain Plus Kit (Bio-Rad Lab., Hercules, CA) according to the manufacturer's instructions.

Immunohistochemistry For immunohistochemistry, we used mouse primary monoclonal antibody against MGMT (MT3.1; Chemicon International, Inc., Temecula, CA) at a 1:100 dilution. Four micrometer-thick formalin-fixed paraffin-embedded tissue samples were deparaffinized, and microwave pretreatment was performed for 15 min at 95°C. The sections were incubated overnight at 4°C with the primary antibody and then processed with a streptavidin-biotin-peroxidase detection kit (Histofine, Nichirei, Tokyo), in which diaminobenzidine was used as the chromogen. The MGMT expression was evaluated by scoring the staining intensity (0 = negative; 1 = weak; 2 = intermediate; 3 = strong) and percentage of positive cells of the entire lesion (0 = 0%; 1 = <25%; 2 = 26–50%; 3 = >50%) according to the method of Mattern *et al*.[8] A score >2 corresponds to a positive immunohistochemical result.

Statistical analysis Associations between the MGMT status and the clinicopathological parameters were determined by Fisher's exact test. *P* values of less than 0.05 were considered significant.

Survival analysis To eliminate the effect of stage, we analyzed stage-I cases[18] used in MSP studies and 28 additional stage-I cases, whose frozen tissues were not available. Of the 149 consecutive lung AC patients, 51 stage-I patients who had undergone complete tumor resection with mediastinal lymph node dissection were analyzed. The patients consisted of 26 females and 25 males whose mean age was 64 (range 42–81). Twenty-five of them were smokers. Those who died within 3 months after surgery, those who died of causes other than lung cancer within 5 years, and those who were followed for less than 5 years (for the surviving patients) were excluded from the survival analysis. Kaplan-Meier survival analysis with the log-rank test was used to evaluate the relationship between MGMT expression and survival distribution.

RESULTS

Methylation status of MGMT By MSP, a methylated allele of the *MGMT* gene was observed in 37.3% of smokers and 33.3% of non-smokers (Fig. 1 and Table I). All cases, including cases with a methylated allele, also showed the unmethylated allele, possibly due to contamination with normal tissues during the process of DNA extraction. However, partial methylation or allele-specific methylation can not be ruled out. By immunohistochemistry, positive nuclear immunoreactivity of MGMT was observed. Most of the positive cases showed clonal patterns (labeling index >50%) with strong intensity, although others were focally positive with moderate intensity. Some scattered reactivity with weak intensity was observed in some of the negative cases. Tumors with a methylated allele showed negative immunostaining, and *vice versa*, except for 2 cases (Fig. 2, a and b). These exceptional tumors were positive for MGMT immunohistochemistry, but the methylated allele was observed by MSP. Normal bronchial epithelium and some interstitial

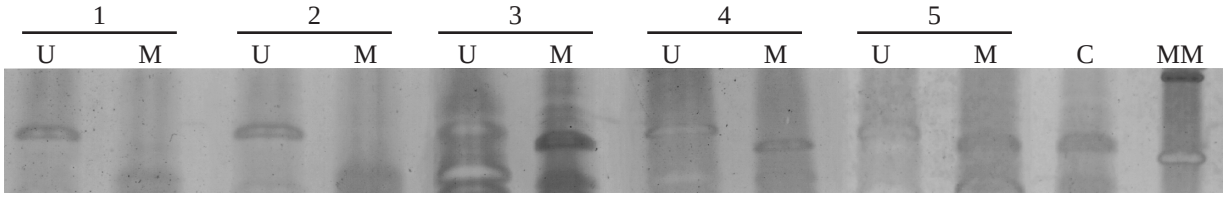

Fig. 1. The result of MSP. Only unmethylated allele (U, 93 bp) was observed in cases #1 and #2, whereas methylated allele (M, 81 bp) was also observed in cases #3, #4, and #5. #1 and #3 were smokers. C: positive control for M allele. MM: marker (162 and 79 bp).

Table I. Promoter Hypermethylation of the *MGMT* Gene by MSP and the Result of Immunohistochemistry

	n	MET		IHC	
		(+)	(%)	(−)	(%)
Age					
≤65	44	20	(45.5)	18	(40.9)
>65	43	11	(25.6)	11	(25.6)
Gender					
Male	50	18	(36.0)	17	(34.0)
Female	37	13	(35.1)	12	(32.4)
Stage					
I	47	16	(34.0)	14	(29.8)
II	14	4	(28.6)	4	(28.6)
III	24	10	(41.7)	10	(41.7)
IV	2	1	(50.0)	1	(50.0)
Smoking and histology					
Smokers	51	19	(37.3)	19	(37.3)
W-AC	18	3	(16.7)[a)]	3	(16.7)
M-AC	19	8	(42.1)	8	(42.1)
P-AC	14	8	(57.1)[a)]	8	(57.1)
Non-smokers	36	12	(33.3)	10	(27.8)
W-AC	20	7	(35.0)	5	(25.0)
M-AC	12	4	(33.3)	4	(33.3)
P-AC	4	1	(25.0)	1	(25.0)
Total	87	31	(35.6)	29	(33.3)

MET, methylation; IHC, immunohistochemistry.
a) $P=0.022$.

lymphocytes were positive for MGMT and thus they served as internal positive controls.

Methylation status was evaluated according to the differentiation of tumors (Table I). Among smokers, promoter hypermethylation of the *MGMT* gene tended to increase in parallel with differentiation grade, and its inactivation was significantly lower in W-AC than in P-AC ($P=0.022$). Such a trend was not observed in non-smokers. All ten cases of AAH showed positive staining for MGMT (Fig. 2c). No relationships were observed among histological subtypes or cell types.

Survival analysis Among stage-I cases, 11 tumors (44.0%) of smokers and 8 tumors (30.8%) of non-smokers were MGMT-negative by immunohistochemistry. Log-rank test revealed that the MGMT-negative patients had a worse prognosis than those without inactivation in stage-I smokers (Fig. 3; $P=0.036$). Analyses made upon non-smokers ($P=0.98$) or on both groups mixed ($P=0.19$) revealed no significant differences. Histological grade ($P=0.51$), age ($P=0.79$), and gender ($P=0.17$) were not significant factors.

DISCUSSION

Loss of MGMT expression has been reported to have a significant role in carcinogenesis in various organs.[7)] In

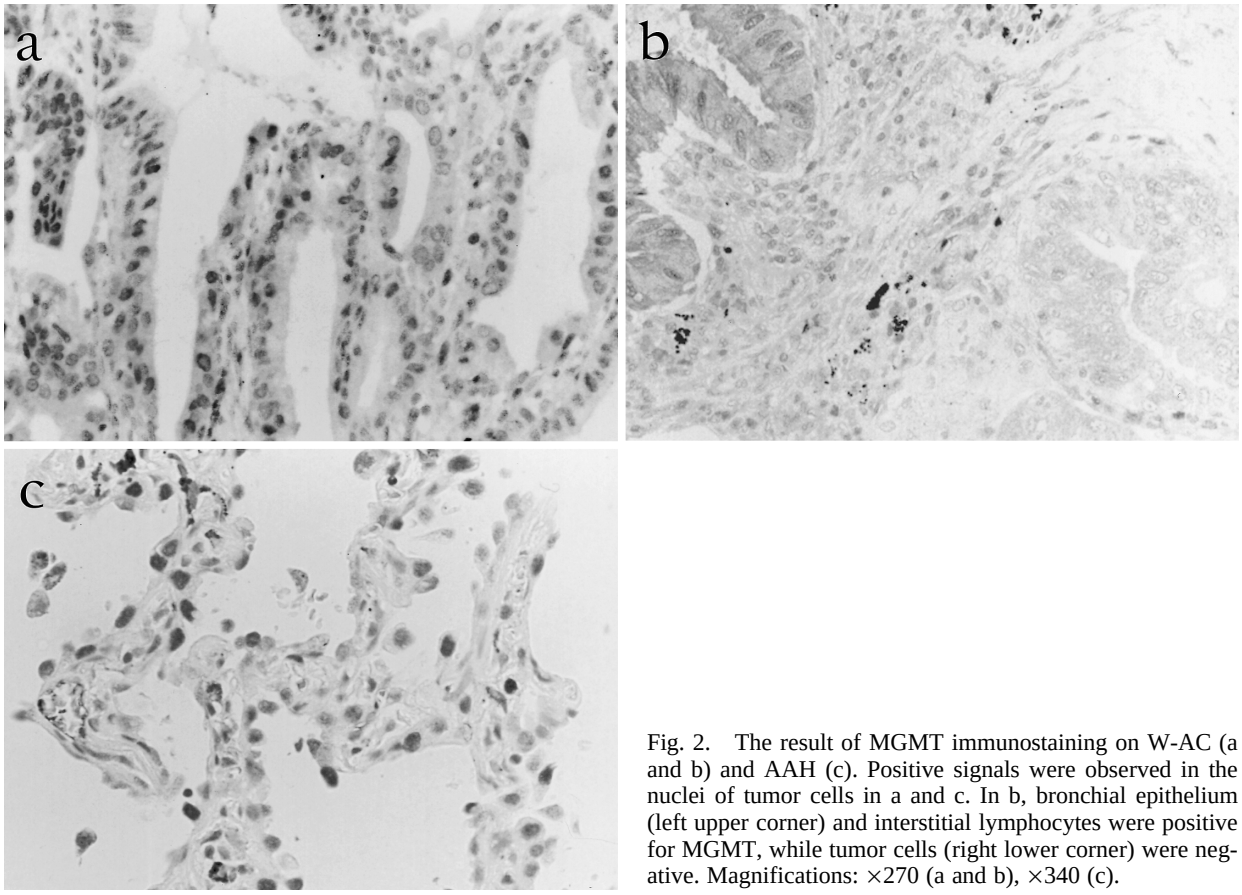

Fig. 2. The result of MGMT immunostaining on W-AC (a and b) and AAH (c). Positive signals were observed in the nuclei of tumor cells in a and c. In b, bronchial epithelium (left upper corner) and interstitial lymphocytes were positive for MGMT, while tumor cells (right lower corner) were negative. Magnifications: ×270 (a and b), ×340 (c).

lung carcinoma, 25–29% of non-small cell lung carcinoma[8, 9] and 37% of AC have been reported to show loss of MGMT expression.[8] Our results reconfirmed that loss of its expression is frequently associated with carcinogenesis in lung cancer, including AC.

Mattern *et al.* and Drin *et al.* have reported that MGMT activity and expression tend to be higher in tumors (especially in squamous cell carcinoma) and normal tissues of smokers.[8, 12] In this study, such an association between smoking history and MGMT expression was not found. This discrepancy may be due to differences in the histological subtypes of lung cancer. Our results suggested that there is little or no difference between smokers and nonsmokers as regards lack of MGMT expression in lung AC. Spontaneous methylation or other mechanisms might be more important.

We analyzed the results in relation to the differentiation grade of AC. In the smokers group, promoter hypermethylation of the *MGMT* gene was more frequent in P-AC. Furthermore, patients with MGMT-negative tumors had a worse prognosis than those with MGMT-positive tumors. Since MGMT is a DNA repair protein, loss of MGMT

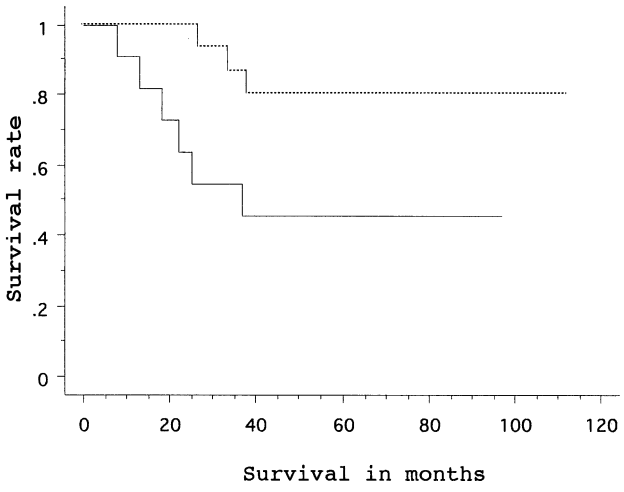

Fig. 3. Kaplan-Meier survival curves of stage-I AC patients of the smoking group. MGMT expression status is indicated as positive (dotted line) or negative (solid line). The MGMT-negative group had a worse prognosis than the MGMT-positive group (P=0.036).

expression seems to be associated with the progression of AC in smokers. It is speculated that smokers whose tumors are MGMT-negative may be more susceptible to the effects of carcinogenic agents, such as tobacco-specific nitrosamines, than those with MGMT-positive tumors.

An alternative explanation is that DNA-MTase activity increases in parallel with tumor progression, although the basic mechanisms are unknown.[19, 20] During this process, many tumor suppressor genes, including MGMT, might be involved by hypermethylation, resulting in the inactivation of their functions. Thus, the observations presented here may represent a coincidental inactivation of MGMT and other tumor suppressor genes, such as *p16INK4*, which is more frequently methylated in smokers than in nonsmokers.[21]

Our results in AAH demonstrated that loss of MGMT expression is not an early event during carcinogenesis of lung AC. To our knowledge, this is the first report of MGMT expression in precancerous lesions of the lung.

In conclusion, the inactivation of MGMT is associated with lung AC development. Although the frequency of inactivation of MGMT was similar in smokers and nonsmokers, its effects on progression were observed in smokers only. The inactivation of MGMT might also reflect a poor prognosis. It might be valuable to study the inactivation of MGMT in smoking-related cancers, including precancerous lesions, to clarify the carcinogenic processes of these cancers.

ACKNOWLEDGMENTS

We thank M. Ikeda, H. Mitsui, and T. Suzuki for their excellent technical assistance. We also thank Dr. Nobuo Ogawa (Pulmonary Surgery, Kanagawa Cardiovascular and Respiratory Center) who kindly offered the lung cancer cases. This work was supported by grants from the Smoking Research Foundation, the Japanese Ministry of Education, Culture, Sports, Science and Technology and the Yokohama Foundation for Advancement of Medical Science, Japan.

(Received August 22, 2001/Revised October 30, 2001/Accepted December 4, 2001)

REFERENCES

1) Pegg, A. E. Mammalian O^6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res.*, **50**, 6119–6129 (1990).
2) Beland, F. A. and Poirier, M. C. DNA adducts and carcinogenesis. *In* "The Pathobiology of Neoplasia," ed. A. E. Sirica, pp. 57–80 (1989). Plenum Press, New York.
3) Zaidi, N. H., Pretlow, T. P., O'Riordan, M. A., Dumenco, L. L., Allay, E. and Gerson, S. L. Transgenic expression of human MGMT protects against azoxymethane-induced aberrant crypt foci and G to A mutations in the K-ras oncogene of mouse colon. *Carcinogenesis*, **16**, 451–456 (1995).
4) Liu, L., Qin, X. and Gerson, S. L. Reduced lung tumorigenesis in human methylguanine DNA-methyltransferase transgenic mice achieved by expression of transgene within the target cell. *Carcinogenesis*, **20**, 279–284 (1999).
5) Allay, E., Veigl, M. and Gerson, S. L. Mice over-expressing human O^6-alkylguanine-DNA alkyltransferase selectively reduce O^6 methylguanine mediated carcinogenic mutations to threshold levels after N-methyl-N-nitrosourea. *Oncogene*, **18**, 3783–3787 (1999).
6) Esteller, M., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M. A., Watkins, D. N., Issa, J.-P. J., Sidransky, D., Baylin, S. B. and Herman, J. G. Inactivation of the DNA repair gene O^6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. *Cancer Res.*, **60**, 2368–2371 (2000).
7) Rolhion, C., Penault-Llorca, F., Kemeny, J.-L., Kwiatkowski, F., Lemaire, J.-J., Chollet, P., Finat-Duclos, F. and Verrelle, P. O^6-Methylguanine-DNA methyltransferase gene (MGMT) expression in human glioblastomas in relation to patient characteristics and p53 accumulation. *Int. J. Cancer (Pred. Oncol.)*, **416**, 416–420 (1999).
8) Mattern, J., Koomägi, R. and Volm, M. Smoking-related increase of O^6-methylguanine-DNA methyltransferase expression in human lung carcinomas. *Carcinogenesis*, **19**, 1247–1250 (1998).
9) Esteller, M., Hamilton, S. R., Burger, P. C., Baylin, S. B. and Herman, J. G. Inactivation of DNA repair gene O^6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res.*, **59**, 793–797 (1999).
10) Fornace, A. J., Jr., Papathanasiou, M. A., Hollander, M. C. and Yarosh, D. B. Expression of the O^6-methylguanine-DNA methyltransferase gene MGMT in MER^+ and MER^- human tumor cells. *Cancer Res.*, **50**, 7908–7911 (1990).
11) Qian, X. C. and Brent, T. P. Methylation hot spots in the 5′ flanking region denote silencing of the O^6-methylguanine-DNA methyltransferase gene. *Cancer Res.*, **57**, 3672–3677 (1997).
12) Drin, I., Schoket, B., Kostic, S. and Vincze, I. Smoking-related increase in O^6-alkylguanine-DNA alkyltransferase activity in human lung tissue. *Carcinogenesis*, **15**, 1535–1539 (1994).
13) Belinsky, S. A., Nikula, K. J., Baylin, S. B. and Issa, J.-P. J. Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer. *Proc. Natl. Acad. Sci. USA*, **93**, 4045–4050 (1996).
14) Westra, W. H., Baas, I. O., Hruban, R. H., Askin, F. B., Wilson, K., Offerhaus, G. J. A. and Slebos, R. J. C. K-ras oncogene activation in atypical adenomatous hyperplasia of

the human lung. *Cancer Res.*, **56**, 2224–2228 (1996).
15) WHO. Histological classification of lung and pleural tumours. *In* "Histological Typing of Lung and Pleural Tumours," pp. 21–66 (1999). Springer-Verlag, Berlin and Heidelberg.
16) Harris, L. C., Potter, P. M., Tano, K., Shiota, S., Mitra, S. and Brent, T. P. Characterization of the promoter region of the human O^6-methylguanine-DNA methyltransferase gene. *Nucleic Acids Res.*, **19**, 6163–6167 (1991).
17) Herman, J. G., Graff, J. R., Myöhänen, S., Nelkin, B. D. and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA*, **93**, 9821–9826 (1996).
18) Mountain, C. F. Revisions in the international system for staging lung cancer. *Chest*, **111**, 1710–1717 (1997).
19) Kautiainen, T. L. and Jones, P. A. DNA methyltransferase levels in tumorigenic and nontumorigenic cells in culture. *J. Biol. Chem.*, **261**, 1594–1598 (1986).
20) Laird, P. W., Jackson-Grusby, L., Fazeli, A., Dickinson, S. L., Jung, W. E., Li, E., Weinberg, R. A. and Jaenisch, R. Suppression of intestinal neoplasia by DNA hypomethylation. *Cell*, **81**, 197–205 (1995).
21) Zöchbauer-Müller, S., Fong, K. M., Virmani, A. K., Geradts, J., Gazdar, A. F. and Minna, J. D. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. *Cancer Res.*, **61**, 249–255 (2001).