Aberrant Promoter Methylation in Chinese Patients with Non-Small Cell Lung Cancer: Patterns in Primary Tumors and Potential Diagnostic Application in Bronchoalevolar Lavage[1]

Eunice C. Chan, Shui Y. Lam, Kenneth W. Tsang, Bing Lam, James C. M. Ho, Kin H. Fu, Wah K. Lam, and Yok L. Kwong[2]

University Department of Medicine, Queen Mary Hospital [E. C. C., K. W. T., B. L., J. C. M. H., W. K. L., Y. L. K.]; Department of Pathology, Kowloon Hospital [S. Y. L.]; and Department of Pathology, Tseung Kwan O Hospital [K. H. F.], Hong Kong

ABSTRACT

This study was aimed at defining patterns of aberrant gene methylation in non-small cell lung cancer (NSCLC) in Chinese patients and its use in detecting cancer cells in bronchoalveolar lavage (BAL).

The methylation-specific PCR (MSP) was used to study methylation of the *p16*, retinoic acid receptor-β (*RAR*β), death-associated protein (*DAP*) kinase, and O^6-methylguanine-DNA-methyltransferase (*MGMT*) genes in 75 NSCLCs [44 adenocarcinomas and 31 squamous cell carcinomas (SCCs)] and 68 BALs from suspected lung cancers.

More females had adenocarcinoma than SCC (11 of 44 *versus* 2 of 31, $P = 0.04$). Aberrant methylation in at least one gene was found in 63 of 75 (84%) NSCLCs. *p16*, *RAR*β, *DAP kinase*, and *MGMT* methylation was similar in adenocarcinoma and SCC. However, females with NSCLC showed more frequent *p16* methylation than males (12 of 13 *versus* 36 of 62, $P = 0.02$), because of more frequent *p16* methylation in female adenocarcinomas (10 of 11 *versus* 17 of 33, $P = 0.02$). This sexual difference was not observed in *RAR*β, *DAP kinase*, and *MGMT*. At 92%, the frequency of *p16* methylation in Chinese female NSCLC is one of the highest known. For BAL, MSP and cytological analysis showed concordant and discordant results in 25 of 68 and 43 of 68 samples. Of 41 MSP+/cytology− cases, 35 were eventually shown to have malignant lung lesions, 4 were at high risk but had no evidence of lung cancer, and 2 were lost to follow-up. There were two MSP−/cytology+ cases.

Frequent gene methylations were seen in Chinese NSCLC patients. More frequent *p16* methylation was seen in female patients. MSP is a useful molecular adjunct for cancer cell detection in BAL samples.

INTRODUCTION

Lung cancer is the most frequent cause of cancer death worldwide (1). Histologically, about 80% are non-small cell and 20% are small cell lung cancers (2). Among the NSCLCs,[3] adenocarcinoma and SCC are the most common histological subtypes. Often grouped together in clinical trials for treatment purposes (3), adenocarcinoma and SCC may have biological differences. Although both subtypes are related to cigarette smoking (4), adenocarcinoma is more prevalent in non-smokers (5). Furthermore, Chinese women in Hong Kong have one of the highest mortality rates of lung cancer in the world (25/100,000 population) with a preponderance of adenocarcinoma, and approximately 80% of these patients have never smoked (6). Therefore, whether there are differences in the pathogenesis and genetic aberrations within the NSCLC remains undefined.

Recently, epigenetic silencing of gene expression by promoter CpG island hypermethylation has been shown to be important in cancer formation (7). DNA methylation may be an alternative mechanism to mutations or deletions in disrupting tumor suppressor gene function. Aberrant gene methylation has also been frequently found in NSCLC. This includes the *p16*, O^6-methylguanine-DNA methyltransferase (*MGMT*), death-associated protein (*DAP*) kinase, retinoic acid receptor-β (*RAR*β), *Ras* association domain family 1A (*RASSF1A*), and adenomatous polyposis coli (*APC*) genes (8–15). Furthermore, aberrant methylation of some of these genes can be found in bronchial epithelial cells in people at high risk of lung cancer (16–20), suggesting that this might be an earlier step in carcinogenesis.

Most studies of aberrant gene methylation in lung cancers have been conducted in the West. Mindful that Western patients may have different etiological and genetic factors as compared with Asian ones, as well as our unusual epidemiological observation of frequent lung cancer in non-smoking women, we investigated retrospectively a series of Chinese NSCLC patients for aberrant promoter methylation with a candidate gene approach, selecting genes that have been reported to show high frequencies of methylation in Western patients: *p16* at 25% (14), *RAR*β at 40% (11, 14), *MGMT* at 25–29% (9, 14), and *DAP kinase* at 19–44% (10, 14). We also studied prospectively the

Received 5/17/02; revised 7/17/02; accepted 7/24/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by the Kadoorie Charitable Foundation.
[2] To whom requests for reprints should be addressed, at University Department of Medicine, Professorial Block, Queen Mary Hospital, Pokfulam Road, Hong Kong. Phone: 852-2-855-4597; Fax: 852-2-974-1165; E-mail: ylkwong@hkucc.hku.hk.

[3] The abbreviations used are: NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma; MGMT, O^6-methylguanine-DNA-methyltransferase; DAP, death-associated protein; RAR, retinoic acid receptor; BAL, bronchoalveolar lavage; MSP, methylation-specific PCR.

Table 1 Primers for MSP

Primer	Sequence (5′–3′)	Annealing temperature	Product size
p16 M_F[a]	TTATTAGAGGGTGGGGCGGATCGC	65°C	150 bp
p16 M_R	GACCCCGAACCGCGACCGTAA		
p16 U_F	TTATTAGAGGGTGGGGTGGATTGT	60°C	151 bp
p16 U_R	CAACCCCAAACCACAACCATAA		
*RAR*β M_F	GGATTGGGATGTCGAGAAC	64°C	93 bp
*RAR*β M_R	TACAAAAAACCTTCCGAATACG		
*RAR*β U_F	AGGATTGGGATGTTGAGAATG	54°C	95 bp
*RAR*β U_R	TTACAAAAAACCTTCCAAATACA		
DAP kinase M_F	GGATAGTCGGATCGAGTTAACGTC	64°C	98 bp
DAP kinase M_R	CCCTCCCAAACGCCGA		
DAP kinase U_F	GGAGGATAGTTGGATTGAGTTAATGTT	64°C	106 bp
DAP kinase U_R	CAAATCCCTCCCAAACACCAA		
MGMT M_F	TTTCGACGTTCGTAGGTTTTCGC	56°C	81 bp
MGMT M_R	GCACTCTTCCGAAAACGAAACG		
MGMT U_F	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	59°C	93 bp
MGMT U_R	AACTCCACACTCTTCCAAAAACAAAACA		

[a] M_F, forward methylated primer; M_R, reverse methylated primer; U_F, forward unmethylated primers; U_R, reverse unmethylated primers.

potential use of gene methylation as a surrogate tumor marker in BAL in patients with suspected lung cancer.

MATERIALS AND METHODS

Primary Lung Cancer Specimens. Primary tumor specimens were obtained from surgically resected lung cancers. Specimens collected prospectively were snap-frozen at −20°C until use. Archival specimens were paraffin embedded. Before DNA extraction, cancer tissues were micro-dissected from surrounding normal tissue on 5-μm-thick sections as described (21).

BAL. BAL samples were obtained during bronchoscopic evaluation of suspected lung cancer, with informed consent. After centrifugation at 3000 *rpm* for 10 min, the cell pellet was harvested and frozen at −20°C until use.

MSP. DNA was extracted from primary tumor/BAL samples with standard phenol-chloroform protocols. For archival paraffin sections, the QIAamp DNA mini kit (Qiagen, Hilden, Germany) was used for DNA extraction. DNA modification was by the bisulphite reaction using the CpGenome DNA Modification Kit (Intergen Co., Purchase, NY), as reported (22). MSP was performed as described (23), with primers for the methylated (M_F and M_R) and unmethylated (U_F and U_R) alleles of *p16*, *RAR*β, *MGMT*, and *DAP kinase* as shown in Table 1. MSP was carried out in a final volume of 50 μl containing 5 μl of bisulphite-modified DNA, 250 μM dNTP (Life Technologies, Inc., Gaithersburg, MD), 1 μM of each primer (Genosys, Cambridgeshire, United Kingdom), 1.5–3 mM $MgCl_2$, 1× PCR gold buffer, and 2 units of AmpliTaq Gold (PE Biosystems, Foster City, CA), in an MJ PTC-200 thermocycler (M. J. Research, Inc., Cambridge, MA) with the following cycling parameters: 95°C for 12 min; 32–40 cycles of 94°C for 1 min, specific annealing temperature (Table 1) for 1 min, 72°C for 1 min; and a final extension step at 72°C for 10 min. Normal and methylated DNA (Intergen) were used to optimize the MSP conditions, and included as normal and positive controls in every experiment.

Specificity and Sensitivity of MSP. All MSPs were performed in duplicate. As controls, amplifications for the unmethylated/methylated alleles on bisulphite-modified normal/methylated DNA and template-blank controls were performed. To test the sensitivity of the MSP, methylated DNA (Intergen) was serially diluted in normal DNA, bisulphite treated, and amplified with primers for the methylated allele. To confirm the specificity of the MSP, PCR products were gel purified and sequenced in both directions with the same primers used for MSP (dRhodamine Terminator Cycle Sequencing Ready Reaction Kit; PE Biosystems), and analyzed by an automated DNA sequencer (ABI Prism 377; PE Biosystems).

RESULTS

Specificity and Sensitivity of MSP. MSP for *p16*, *RAR*β, *DAP kinase*, and *MGMT* showed the expected results for the positive and negative controls in all of the experiments. The PCR products were sequenced and showed the expected changes induced by bisulphite treatment (Fig. 1). MSP had a sensitivity of 10^{-2} for *MGMT*, 10^{-3} for *p16* and *DAP kinase*, and 10^{-4} for *RAR*β (Fig. 1).

Primary Tumors. A total of 44 adenocarcinomas and 31 SCCs were studied (Table 2). There were significantly more female patients with adenocarcinoma than SCC (11 of 44 *versus* 2 of 31, $P = 0.04$, χ^2 test). Methylation of one or more genes occurred in 63 of 75 (84%) of cases. Adenocarcinoma and SCC had similar frequencies of gene methylation (*p16*, 55–68%; *RAR*β, 70–71%; *DAP kinase*, 34–35%; *MGMT*, 14–16%). However, in adenocarcinoma, female patients had a significantly higher frequency of *p16* methylation as compared with males (10 of 11 *versus* 17 of 33, $P = 0.02$, Fisher's exact test). This sex difference in *p16* methylation was not demonstrable in SCC, probably because of the small number of female patients (2 of 2 *versus* 19 of 29, $P > 0.05$). However, when both adenocarcinoma and SCC were considered together, *p16* methylation was still significantly more frequent in female than male patients (12 of 13 *versus* 36 of 62, $P = 0.02$, Fisher's exact test). This sex difference was not found in *RAR*β, *MGMT*, and *DAP kinase* methylation in both histological subtypes (Fig. 1).

BAL: Concordant Results. The results and clinicopathological correlations are shown in Table 3. Concordant results

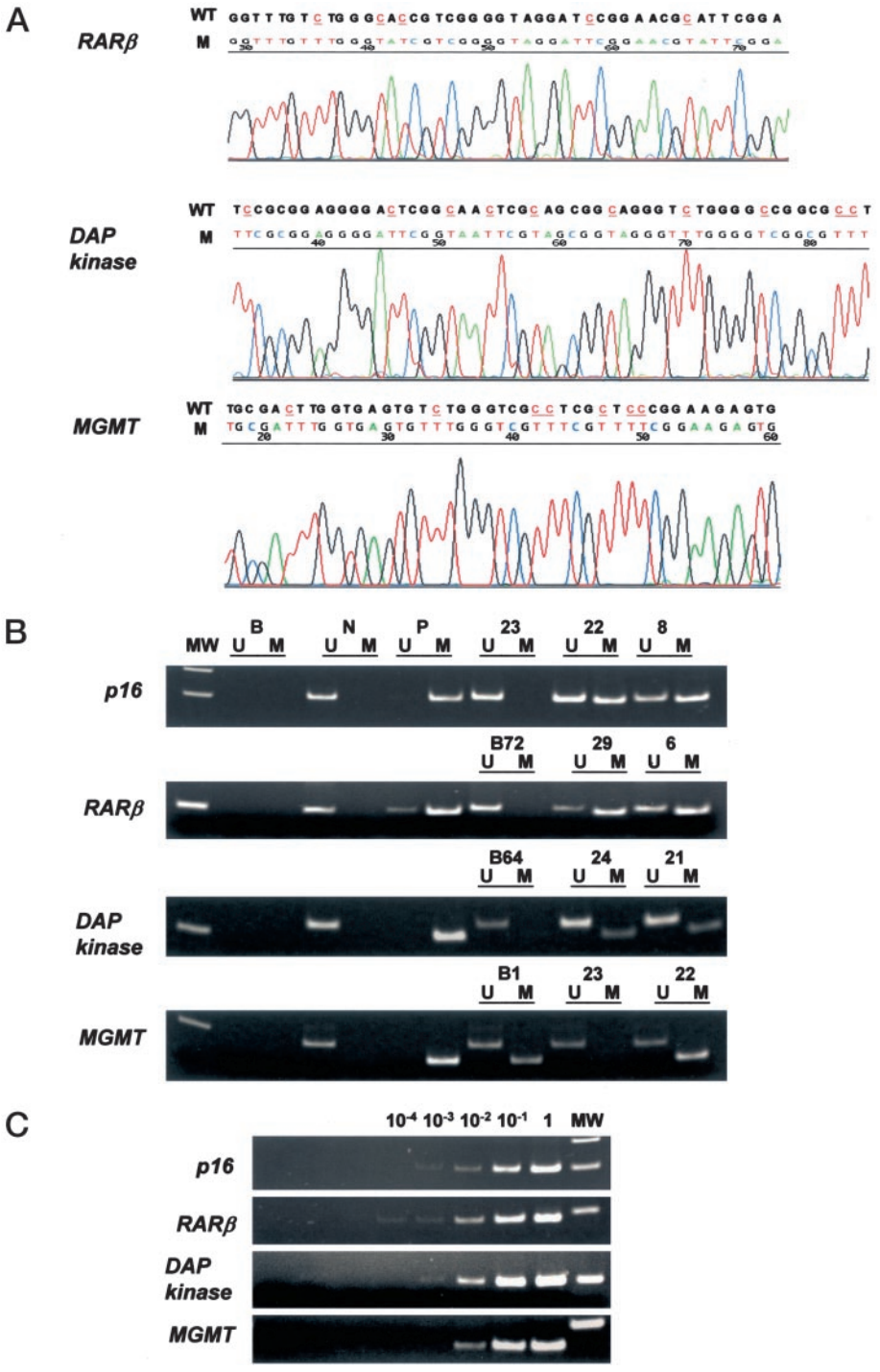

Fig. 1 *A*, sequence of the M-MSP PCR product (*M*) of *RAR*β, *DAP kinase*, and *MGMT* aligned against the wild-type (*WT*) sequences, showing change of C (*underlined red*) to T by bisulphite treatment. C in CpG islands that were methylated were unaffected. The results of *p16* have been published previously (29). *B*, MSP of *p16*, *RAR*β, *DAP kinase*, and *MGMT*. *MW*, molecular weight marker; *B*, reagent blank; *N*, normal control, showing positive amplification for the U-MSP but not the M-MSP; *P*, positive control of universally methylated DNA, showing positive amplification in the M-MSP. Occasional batches of methylated DNA might be incompletely methylated, accounting for a faint band as observed in the *RAR*β *panel*. *p16*, absence of methylation in case 23, but methylation in cases 22 and 8; *RAR*β, absence of methylation in case B72 (*B*, BAL), but methylation in cases 29 and 6; *DAP kinase*, absence of methylation in case B64, but methylation in cases 24 and 21; *MGMT*, absence of methylation in case 23, but methylation in cases B1 and 22. *C*, Sensitivity of MSP, with MGMT at 10^{-2}, *p16* and *DAP kinase* at 10^{-3}, and *RAR*β at 10^{-4}.

were observed in 25 of 68 samples. Fifteen specimens showed malignant cells on cytological analysis, and MSP was positive in at least one gene tested. Ten samples did not show malignant cells on cytological analysis, and the MSP results were correspondingly negative. On follow-up of the 10 cytology-negative and MSP-negative cases, 6 were shown to be infectious in nature. However, four cases were finally found to be NSCLC by further investigations.

BAL: Discordant Results. The results of cytology and MSP were discordant in 43 or 68 samples. Forty-one samples were cytologically negative, but MSP positive in one or more genes. Of these, 25 cases underwent further BAL and transbronchial biopsies and were shown to have malignant diseases, 22 of pulmonary origin and 3 from the large bowel or uterine cervix. Ten cases had evidence of systemic metastases, and the patients declined further histological evaluation. Interestingly, four cases

Table 2 Frequencies of methylation of *p16*, *RAR*β, *DAP kinase*, and *MGMT* in 75 cases of NSCLC

Histologic subtype	Sex	No.	Methylation of gene(s) (% of cases)				
			p16	*RAR*β	*DAP kinase*	*MGMT*	At least one gene
Adenocarcinoma	Male	33	17 (52%)	22 (67%)	10 (30%)	5 (15%)	26 (79%)
	Female	11	10 (91%)	9 (82%)	5 (45%)	2 (18%)	11 (100%)
	Overall	44	24 (55%)	31 (70%)	15 (34%)	7 (16%)	37 (85%)
SCC	Male	29	19 (66%)	21 (72%)	10 (34%)	4 (14%)	24 (83%)
	Female	2	2 (100%)	1 (50%)	1 (50%)	0 (0%)	2 (100%)
	Overall	31	21 (68%)	22 (71%)	11 (35%)	4 (14%)	26 (84%)
Both histologic subtypes	Male	62	36 (58%)	43 (70%)	20 (33%)	9 (15%)	50 (81%)
	Female	13	12 (92%)	10 (77%)	6 (46%)	2 (15%)	13 (100%)
	Overall	75	48 (64%)	53 (71%)	26 (35%)	11 (15%)	63 (84%)

Table 3 Correlation between cytology results and MSP in BAL

Results		*n*	Methylation (%)				Remarks
			p16	*RAR*β	*DAP kinase*	*MGMT*	
Concordant							
Cytology (+)[a]/MSP (+)	Male	10	4 (40%)	9 (80%)	3 (30%)	2 (20%)	Included one patient with small cell lung cancer
	Female	5	1 (20%)	4 (100%)	1 (20%)	0 (0%)	
	Overall	15	5 (33%)	13 (87%)	4 (27%)	2 (13%)	
Cytology (−)/MSP (−)	Male	7					Two cases of pneumonia, two cases of tuberculosis Three cases eventually diagnosed with NSCLC
	Female	3					Two case of pneumonia One case eventually diagnosed with NSCLC
Discordant							
Cytology (−)/MSP (+)	Male	25	7 (28%)	21 (84%)	8 (32%)	2 (8%)	15 cases: later diagnosis of NSCLC (2 days–3 months) 8 cases: clinically malignant with metastases 1 case: metastatic large bowel cancer 1 case: chronic smoker with lung shadow on follow-up[b]
	Female	16	2 (13%)	14 (88%)	2 (13%)	2 (13%)	7 cases: later diagnosis of NSCLS (2 days–1 months) 2 cases: clinically malignant with metastases 2 cases: metastases from colon and cervical cancer 1 case: scleroderma with lung shadow on follow-up[c] 1 case: chronic smoker with lung shadow on follow-up[d] 1 case: pulmonary tuberculosis on follow-up[e] 2 cases: lost to follow-up[f]
	Overall	41	9 (22%)	35 (85%)	10 (24%)	4 (10%)	
Cytology (+)/MSP (−)	Male	2					Both cases were NSCLC
	Female	0					

[a] Cytology (+/−), cancer cells found/not found in BAL.
[b–f] Genes methylated. [b] *RAR*β and *DAP kinase*; [c] *RAR*β; [d] *RAR*β; [e] *RAR*β; [f] 1 case each of *RAR*β and *DAP kinase.*

with positive MSP have not yet had evidence of a malignancy. Two were chronic smokers, one had systemic sclerosis and fibrosing alveolitis, and one had a positive culture for *Mycobacterium tuberculosis* and was receiving treatment. All still have persistent lung shadows at the latest follow-up. Finally, there were two cases where the cytology was positive for malignant cells, but the MSP was negative in all of the four genes tested.

DISCUSSION

In this study, we have observed a preferential increase in female patients with adenocarcinoma as compared with SCC, similar to previous studies in Chinese patients with lung cancer (6). The more frequent occurrence of adenocarcinoma in females, who are non-smokers, suggests that etiological or carcinogenic factors for adenocarcinoma may be different from those of SCC. Previous studies from Hong Kong showed that this could not be explained by different *K-ras* or *p53* mutations (24, 25). In this study, the difference between adenocarcinoma and SCC was also not reflected in the frequencies of methylation of *p16*, *RAR*β, *DAP kinase*, and *MGMT*, because results were comparable in both histological types. However, an interesting observation was that *p16* was methylated in nearly all (92%) of the female patients studied. This sexual difference in *p16* methylation has not been previously observed in Western patients, in whom the risk factor for male and female patients alike is cigarette smoking. This frequency of *p16* methylation was one of the highest known for any gene in any malignancy, suggesting that an inherent predisposition to *p16* methylation might have existed in these Chinese women. Recently, it has been observed that polymorphisms of the *glutathione S-transferase P1* gene (A to G at bp 104) and the *NADPH quinone oxidoreductase* gene (C at bp 909) are associated with increased risks of *p16* methylation in bronchial epithelial cells of subjects

at high risk of lung cancers (19). Hence, whether polymorphisms in genes involved in modulating or repairing DNA, or other genetic predispositions, may explain our observations will need to be investigated further.

Our results also showed that *RAR*β was methylated at a frequency (around 70%) considerably higher than that (around 40%) found in Western patients (11, 14), whereas *DAP kinase* and *MGMT* were methylated at frequencies (35% and 15%) similar to those reported (9, 10, 14). The biological implications of this observation will need to be studied further.

We have also examined the potential application of MSP as a molecular marker for the detection of cancer cells in BAL samples. For MSP to be a useful clinical test, it has to be shown to be more sensitive than conventional cytological analysis. Such a comparison has been performed in 21 samples in one study, where *p16* and *MGMT* methylation was shown to be more sensitive than cytological analysis (12). In another study, molecular markers including aberrant methylation of *p16* and *p53*/*K-ras* mutations were detected at high frequencies in 50 BAL samples (26). However, whether these samples were also cytologically positive was unknown. To our knowledge, our study is the first to systematically compare head-to-head the sensitivity of MSP *versus* conventional cytological analysis in detecting cancer cells in BAL samples. We showed that 15 of 17 (88%) cytologically positive BAL samples showed aberrant methylation of at least one gene in an MSP panel comprising *p16*, *RAR*β, *DAP kinase*, and *MGMT*, a frequency that was similar to that of 84% in primary tumors. More importantly, MSP was positive in 35 cases in which the cytology was negative, but the pulmonary lesions were shown to be malignant by later investigations. In these cases, a positive MSP has most likely identified the presence of neoplastic cells. It would be interesting to investigate the neoplastic lung lesions in these cases, to define whether the patterns of aberrant methylation in the BAL samples matched those of the primary tumors. Unfortunately, the diagnostic materials in these cases were either cytological fluid or small transbronchial biopsies, which were inadequate or not available for further analysis. There were, however, four cases where MSP was positive, with the patients not having evidence yet of a neoplastic lesion. Two were chronic smokers with persistent lung shadows, and it has been shown previously that MSP might be positive in these subjects at high risk of lung cancers (12, 17–20, 26). Another patient had scleroderma, a disease well known to be associated with lung cancer, particularly adenocarcinoma (27, 28). These patients with negative cytology and positive MSP will have to be followed closely for further evidence of a malignancy. Therefore, our results confirm that MSP is a useful and sensitive molecular adjunct for the detection of small numbers of cancer cells in BAL.

Finally, another interesting observation was that in MSP of the BAL the positive rate of *p16* methylation (25%) was lower than that (64%) in primary tumors whereas the frequencies of methylation of *RAR*β (88%), *DAP kinase* (25%), and *MGMT* (11%) were comparable with those in primary tumors (71%, 35%, and 15%, respectively). For the BAL cases, we have not tested the corresponding tumors, because most of them were unresectable. However, it has been shown that the patterns of methylation in bronchial epithelial cells do not necessarily parallel those of the primary tumors (12, 26). Furthermore, BAL presumably contained small numbers of tumor cells. The lower detection rate of *p16* methylation (as compared with, for example, *RAR*β methylation) may be related to the sensitivity of the MSP as well as the respective clone sizes of the neoplastic cells harboring methylation of these genes, which may become significant when a small number of cells are tested. Therefore, prospective comparison of the methylation patterns of the cancer cells in exfoliative cytology against those of the primary tumor will be both important and interesting. Finally, quantitative studies of gene methylation (15) in BAL samples to quantify the amount of neoplastic cells will be needed.

ACKNOWLEDGMENTS

The cytologic evaluation of BAL was performed in the Department of Pathology, University of Hong Kong.

REFERENCES

1. Greenlee, R. T., Hill-Harmon, M. B., Murray, T., and Thun, M. Cancer statistics, 2001. CA Cancer J. Clin., *51:* 15–36, 2001.

2. Martin, J., Ginsberg, R. J., Venkatraman, E. S., Bains, M. S., Downey, R. J., Korst, R. J., Kris, M. G., and Rusch, V. W. Long-term results of combined-modality therapy in resectable non-small-cell lung cancer. J. Clin. Oncol., *20:* 1989–1995, 2002.

3. Schiller, J. H., Harrington, D., Belani, C. P., Langer, C., Sandler, A., Krook, J., Zhu, J., and Johnson, D. H. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N. Engl. J. Med., *346:* 92–98, 2002.

4. Howe, H. L., Wingo, P. A., Thun, M. J., Ries, L. A., Rosenberg, H. M., Feigal, E. G., and Edwards, B. K. Annual report to the nation on the status of cancer (1973 through 1998), featuring cancers with recent increasing trends. J. Natl. Cancer Inst., *93:* 824–842, 2001.

5. Dev, D., Capewell, S., Sankaran, R., Lamb, D., and Sudlow, M. F. Adenocarcinoma of the lung—clinical features and survival. Respir. Med., *90:* 333–337, 1996.

6. Lam, B., Lam, W. K., Lam, C. L., Ooi, G. C., Ho, J. C. M., Wong, M. P., and Tsang, K. W. Adenocarcinoma of the lung in Chinese patients: a revisit and some perspectives from the literature. Postgrad. Med. J., *77:* 708–712, 2001.

7. Baylin, S. B., and Herman, J. G. DNA hypermethylation in tumorigenesis. Trends Genet., *16:* 168–174, 2000.

8. Belinsky, S. A., Nikula, K. J., Palmisano, W. A., Michels, R., Saccomanno, G., Gabrielson, E., Baylin, S. B., and Herman, J. G. Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. Proc. Natl. Acad. Sci. USA, *95:* 11891–11896, 1998.

9. Esteller, M., Hamilton, S. R., Burger, P. C., Baylin, S. B., and Herman, J. G. Inactivation of the 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. Tang, X., Khuri, F. R., Lee, J. J., Kemp, B. L., Liu, D., Hong, W. K., and Mao, L. Hypermethylation of the death-associated protein (DAP) kinase promoter and aggressiveness in stage I non-small-cell lung cancer. J. Natl. Cancer Inst., *92:* 1511–1516, 2000.

11. Virmani, A. K., Rathi, A., Zochbauer-Muller, S., Sacchi, N., Fukuyama, Y., Bryant, D., Maitra, A., Heda, S., Fong, K. M., Thunnissen, F., Minna, J. D., and Gazdar, A. F. Promoter methylation and silencing of the retinoic acid receptor-β gene in lung carcinomas. J. Natl. Cancer Inst., *92:* 1303–1307, 2000.

12. Palmisano, W. A., Divine, K. K., Saccomanno, G., Gilliland, F. D., Baylin, S. B., Herman, J. G., and Belinsky, S. A. Predicting lung cancer by detecting aberrant promoter methylation in sputum. Cancer Res., *60:* 5954–5958, 2000.

13. Burbee, D. G., Forgacs, E., Zochbauer-Muller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S., Sekido, Y., Latif, F., Milchgrub, S., Toyooka, S., Gazdar, A. F., Lerman,

M. I., Zabarovsky, E., White, M., and Minna, J. D. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J. Natl. Cancer Inst., *93:* 691–699, 2001.

14. Zochbauer-Muller, 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.

15. Usadel, H., Brabender, J., Danenberg, K. D., Jeronimo, C., Harden, S., Engles, J., Danenberg, P. V., Yang, S., and Sidransky, D. Quantitative adenomatous polyposis coli promoter methylation analysis in tumor tissue, serum, and plasma DNA of patients with lung cancer. Cancer Res., *62:* 371–375, 2002.

16. Wistuba, I. I., Lam, S., Behrens, C., Virmani, A. K., Fong, K. M., LeRiche, J., Samet, J. M., Srivastava, S., Minna, J. D., and Gazdar, A. F. Molecular damage in the bronchial epithelium of current and former smokers. J. Natl. Cancer Inst., *89:* 1366–1373, 1997.

17. Kersting, M., Friedl, C., Kraus, A., Behn, M., Pankow, W., and Schuermann, M. Differential frequencies of p16(INK4a) promoter hypermethylation, p53 mutation, and K-ras mutation in exfoliative material mark the development of lung cancer in symptomatic chronic smokers. J. Clin. Oncol., *18:* 3221–3229, 2000.

18. Soria, J. C., Rodriguez, M., Liu, D. D., Lee, J. J., Hong, W. K., and Mao, L. Aberrant promoter methylation of multiple genes in bronchial brush samples from former cigarette smokers. Cancer Res., *62:* 351–355, 2002.

19. Gilliland, F. D., Harms, H. J., Crowell, R. E., Li, Y. F., Willink, R., and Belinsky, S. A. Glutathione S-transferase P1 and NADPH quinone oxidoreductase polymorphisms are associated with aberrant promoter methylation of p16(INK4a) and *O*(6)-methylguanine-DNA methyltransferase in sputum. Cancer Res., *62:* 2248–2252, 2002.

20. Belinsky, S. A., Palmisano, W. A., Gilliland, F. D., Crooks, L. A., Divine, K. K., Winters, S. A., Grimes, M. J., Harms, H. J., Tellez, C. S., Smith, T. M., Moots, P. P., Lechner, J. F., Stidley, C. A., and Crowell, R. E. Aberrant promoter methylation in bronchial epithelium and sputum from current and former smokers. Cancer Res., *62:* 2370–2377, 2002.

21. Moskaluk, C. A., and Kern, S. E. Microdissection and polymerase chain reaction amplification of genomic DNA from histological tissue sections. Am. J. Pathol., *150:* 1547–1552, 1997.

22. Siu, L. L., Chan, J. K., Wong, K. F., and Kwong, Y. L. Specific patterns of gene methylation in natural killer cell lymphomas: p73 is consistently involved. Am. J. Pathol., *160:* 59–66, 2002.

23. Herman, J. G., Graff, J. R., Myohanen, 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.

24. Lung, M., Wong, M., Lam, W. K., Lau, K. S., Kwan, S., Fu, K. H., Cheung, H., and Yew, W. W. Incidence of ras oncogene activation in lung carcinomas in Hong Kong. Cancer (Phila.), *70:* 760–763, 1992.

25. Lung, M. L., Wong, M. P., Skaanild, M. T., Fok, C. L., Lam, W. K., and Yew, W. W. p53 gene mutations in non-small cell lung carcinomas in Hong Kong. Chest, *109:* 718–726, 1996.

26. Ahrendt, S. A., Chow, J. T., Xu, L. H., Yang, S. C., Eisenberger, C. F., Esteller, M., Herman, J. G., Wu, L., Decker, P. A., Jen, J., and Sidransky, D. Molecular detection of tumor cells in bronchoalveolar lavage fluid from patients with early stage lung cancer. J. Natl. Cancer Inst., *91:* 332–339, 1999.

27. Rosenthal, A. K., McLaughlin, J. K., Gridley, G., and Nyren, O. Incidence of cancer among patients with systemic sclerosis. Cancer (Phila.), *76:* 910–914, 1995.

28. Yang, Y., Fujita, J., Tokuda, M., Bandoh, S., and Ishida, T. Lung cancer associated with several connective tissue diseases: with a review of literature. Rheumatol. Int., *21:* 106–111, 2001.

29. Chim, C. S., Liang, R., Tam, C. Y., and Kwong, Y. L. Methylation of p15 and p16 genes in acute promyelocytic leukemia: potential diagnostic and prognostic significance. J. Clin. Oncol., *19:* 2033–2040, 2001.