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Aberrant DNA methylation profiles of non-small cell lung cancers in a Korean population

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Summary We performed this study to investigate the aberrant methylation profile of the cancer-related genes in Korean non-small cell lung cancer (NSCLC) that previously exhibited high frequencies of methylation in Western populations. The aberrant promoter methylation of eight genes (*GSTP1*, *p16*, *FHIT*, *APC*, *RASSF1A*, *hMLH1*, *hMSH2*, *AGT*) was determined by MSP in 99 surgically resected NSCLCs and their corresponding nonmalignant lung tissues. Methylation in the tumor samples was detected at 15% for *GSTP1*, 22% for *p16*, 34% for *FHIT1*, 48% for *APC*, 40% for *RASSF1A*, 18% for *hMLH1*, 8% for *hMSH2* and 21% for *AGT*, whereas it occurred at lower frequencies in the corresponding nonmalignant lung tissues, particularly in the *p16* (1%) and *RASSF1A* (1%) genes. These results suggest that the methylation profiles of NSCLCs in a Korean population are similar to those in Western populations.
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1. Introduction

The aberrant methylation of CpG islands in the promoter region of genes has been associated with the transcriptional inactivation of tumor suppressor genes in human cancers. This epigenetic process is considered to be the major gene silencing mechanism for tumor suppressor genes along with genetic alterations such as mutations and deletions [1,2]. Several known and putative genes have been found to be frequently inactivated by promoter methylation in lung cancers. These are implicated in the different roles of lung carcinogenesis including carcinogen detoxification [glutathione *S-transferase P1* (*GSTP1*)], tumor suppression [*p16*, fragile histidine triad (*FHIT*), adenomatous polyposis coli (*APC*) and Ras association domain family 1A (*RASSF1A*)] and DNA repair [*hMLH1*, *hMSH2*, O^6-alkyllguanine-DNA-alkyltransferase (*AGT*)] [3—9].

Although methylated gene profiles have been widely studied in lung cancer, the majority of the previous studies were performed on Western populations [9—14]. The epidemiologic characteristics of lung cancer in East Asian countries are remarkably different from those of Western countries. For example, lung adenocarcinoma in women in East Asian countries frequently arises in never-smokers [15,16]. In addition, several recent studies have shown that mutations in the kinase domain of the *epidermal growth factor receptor* gene are more frequent in never-smokers, females and East Asian populations, whereas *KRAS* mutations are more frequent in smokers, males and Western populations [17,18]. These observations, coupled with the differences in the epidemiological characteristics of lung cancer, suggest that the genetic and environmental factors leading to lung cancer in East Asian populations may be different from those of Western populations [17,18]. Therefore, it is possible that methylated gene profiles in lung cancer from East Asian populations may be different from those from Western countries. In this study, we analyzed the promoter methylation of eight genes (*GSTP1*, *p16*, *FHIT*, *APC*, *RASSF1A*, *hMLH1*, *hMSH2*, *AGT*) in Korean non-small cell lung cancers (NSCLCs) that have been reported to be frequently methylated in lung cancers.

2. Materials and methods

2.1. Patients and tissue samples

Tumor and corresponding nonmalignant lung tissue specimens were obtained from 99 Korean NSCLC patients who underwent curative resection at the Kyungpook National University Hospital (Daegu, Korea) from January 2002 to June 2004. None of these patients received chemotherapy and radiotherapy before the surgery. Informed consent was obtained from each patient before the surgery. This study was approved by the institutional review board of the Kyungpook National University Hospital. Eighty males (81%) and 19 females (19%) with mean age of 63 ± 8.4 (range: 41—82) were included in this study. There were 20 never-smokers and 79 ever-smokers (current- or former-smokers) with mean pack-years of 39 ± 17.2. There were 61 cases of squamous cell carcinomas and 38 adenocarcinomas. The pathologic stages were as follows: 56 were at stage I, 18 were at stage II and 25 were at stage III.

All of the tumor and macroscopically normal lung tissue samples were obtained at the time of surgery, and were rapidly frozen in liquid nitrogen and stored at $-80^{\circ}C$ until genomic DNA preparation. Only tumors with greater than 80% of the tumor component were sent for DNA extraction and methylation analysis. The macroscopically normal lung tissues were confirmed to be normal by hematoxylin-eosin staining.

Of the 99 cases in this study, 78 (43 out of 61 squamous cell carcinomas and 35 out of 38 adenocarcinomas) had previously been examined for *EGFR* (exon 18—21), *ERBB2* (exon 19 and 20) and *KRAS* (exon 1) mutations in another study [19]. Of these 78 cases, *EGFR* and *ERBB2* mutations were found only in the adenocarcinomas (8 of the 35 cases, 22.9% and 1 of the 35 cases, 2.9%, respectively). The *EGFR* or *ERBB2* mutations in the adenocarcinomas were significantly more common in women than in men (7 of 15 cases, 46.7% versus 2 of 20 cases, 10.0%; $P = 0.02$), and in never-smokers than in ever-smokers (7 of 13 cases, 53.8% versus 2 of 22 cases, 9.1%; $P = 0.006$). The *KRAS* mutation was found in 6 out of the 78 cases (7.7% overall; 2 of 43 squamous cell carcinomas, 4.7% and 4 of 35 adenocarcinomas, 11.4%). All 6 cases with the *KRAS* mutation were ever-smoker men.

2.2. Methylation analysis

The aberrant promoter methylation of the eight genes was determined using methylation-specific PCR (MSP) with primers specific for the methylated and unmethylated alleles of each gene after treatment of the genomic DNA with sodium bisulfite [20]. The primer sequences and annealing temperatures are summarized in Table 1. CpGenome™ Universal methylated and unmethylated DNA (Chemicon, Temecula, CA, USA) were used as positive controls for the methylated and unmethylated genes, respectively. The PCR products (10 μl) were analyzed on a 2% agarose gel, stained with ethidium bromide and visualized under UV light. Each MSP was repeated at least once to confirm the results.

2.3. Statistical analysis

The relationship between the methylation and the clinicopathological characteristics was analyzed using a χ^2 test or Fisher's exact test for categorical variables. A logistic regression test was conducted to estimate the relationship between methylation and the covariates of age, gender, exposure to tobacco smoke and histology. The methylation index (MI), a reflection of the methylation status of all of the genes tested, is defined as the number of genes methylated divided by the number of genes analyzed. The MIs of the different groups were compared using analysis of variance (ANCOVA), adjusted with covariates.

3. Results

We used MSP assay to determine the frequency of methylation of the *GSTP1*, *p16*, *FHIT*, *APC*, *RASSF1A*, *hMLH1*, *hMSH2*, *AGT* genes in 99 resected NSCLCs and their corresponding

Table 1 Primers and annealing temperatures for methylation-specific PCR (MSP)

Gene	Forward primer	Reverse primer	*T* (°C)	Product size (bp)
GSTP1	M: 5′-TTCGGGGTGTAGCGGTCGTC-3′	M: 5′-GCCCCAATACTAAATCACGACG-3′	58	91
	U: 5′-GATGTTTGGGGTGTAGTGTTGTT-3′	U: 5′-CCACCCCAATACTAAATCACAACA-3′	58	97
p16	M: 5′-TTATTAGAGGGTGGGGCGGATCGC-3′	M: 5′-GACCCCGAACCGCGACCGTAA-3′	58	150
	U: 5′-TTATTAGAGGGTGGGGTGGATTGT-3′	U: 5′-CAACCCCAAACCACAACCATAA-3′	58	151
FHIT	M: 5′-TAGGCGGCGTTTCGGTTTCGC-3′	M: 5′-CACCGCCCCGTAAACGACGCC-3′	66	167
	U: 5′-TAGGTGGTGTTTTGGTTTTGT-3′	U: 5′-CACCACCCCATAAACAACACC-3′	66	167
APC	M: TATTGCGGAGTGCGGGTC-3′	M: 5′-TCGACGAACTCCCGACGA-3′	58	98
	U: 5′-GTGTTTTATTGTGGAGTGTGGGTT-3′	U: 5′-CCAATCAACAAACTCCCAACAA-3′	58	108
RASSF1A	M: 5′-GTGTTAACGCGTTGCGTATC-3′	M: 5′-AACCCGCGAACTAAAAACGA-3′	60	94
	U: 5′-TTTGGTTGGAGTGTGTTAATGTG-3′	U: 5′-CAAACCCCACAAACTAAAAACAA-3′	60	108
hMLH1	M: 5′-ACGTAGACGTTTTATTAGGGTCG-3′	M: 5′-CCTCATCGTAACTACCCGCG-3′	58	115
	U: 5′-TTTTGATGTAGATGTTTTATTAGGGTTGT-3′	U: 5′-ACCACCTCATAACTACCCACA-3′	56	124
hMSH2	M: 5′-TCGTGGTCGGACGTCGTTC-3′	M: 5′-CAACGTCTCCTTCGACTACACCG-3′	56	136
	U: 5′-GGTTGTTGTGGTTGGATGTTGTTT-3′	U:5′-CAACTACAACATCTCCTTCAACTACACCA-3′	56	136
AGT	M: 5′-TTTCGACGTTCGTAGGTTTTCGC-3′	M: 5′-GCACTCTTCCGAAAACGAAACG-3′	58	81
	U: 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′	U: 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′	58	93

T, annealing temperature; M, methylated and U, unmethylated.

nonmalignant tissues. Representative examples of the MSP assay are illustrated in Fig. 1. The methylation frequencies of the eight genes in the 99 primary tumors were as follows: 15% for *GSTP1*, 22% for *p16*, 34% for *FHIT1*, 48% for *APC*, 40% for *RASSF1A*, 18% for *hMLH1*, 8% for *hMSH2* and 21% for *AGT* (Table 2). At least one of the eight genes was methylated in 84% of the tumors; only one gene was methylated in 23% of the tumors, two in 24% of the tumors, three in 17% of the tumors, four in 14% of the tumors, five in 2% of the tumors and six in 3% of the tumors; giving MIs of 0 in 16 tumors, 0.125 in 23 tumors, 0.250 in 24 tumors, 0.375 in 17 tumors, 0.500 in 14 tumors, 0.625 in 2 tumors and 0.750 in 3 tumors, respectively. Methylation of *GSTP1*, *p16*, *RASSF1A*, *hMSH2* and *AGT* in the corresponding nonmalignant lung tissues was detected at low frequencies (5, 1, 1, 3 and 4%, respectively), but it was seen at a frequencies of >10% for the *FHIT* (17%), *APC* (33%) and *hMLH1* (12%) genes.

The methylation of *p16* was more frequent in the older patients than in the younger patients (29% versus 9%; $P < 0.05$); however, there were no age-related differences

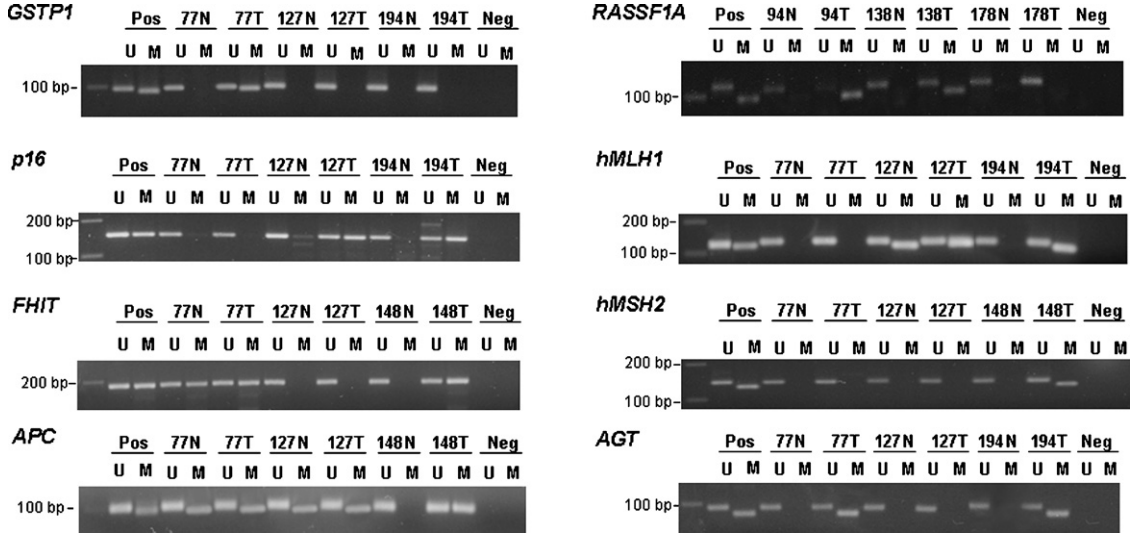

Fig. 1 Methylation analysis of eight genes in primary non-small cell lung cancers and their corresponding nonmalignant lung tissues by MSP. The gene studied is given at the left of each panel. Pos, positive control; N, nonmalignant lung tissue; T, tumor; Neg, negative control; Lane U, amplified product with primers recognizing unmethylated sequence and Lane M, amplified product with primers recognizing methylated sequence.

Table 2 Methylation frequency and methylation index (MI) by clinicopathologic characteristics of non-small cell lung cancer patients

	Methylation positive (%) (number of methylation positive/number of total cases)								
	GSTP1	*p16*	*FHIT*	*APC*	*RASSF1A*	*hMLH1*	*hMSH2*	*AGT*	MI (mean ± S.D.)[a]
All subjects ($n = 99$)	15 (15/99)	22 (22/99)	34 (34/99)	48 (48/99)	40 (40/99)	18 (18/99)	8 (8/99)	21 (21/99)	0.26 ± 0.19
Age (years)									
<60 ($n = 34$)	9 (3/34)	9 (3/34)	35 (12/34)	50 (17/34)	29 (10/34)	18 (6/34)	6 (2/34)	18 (6/34)	0.22 ± 0.17
≥60 ($n = 65$)	18 (12/65)	29 (19/65)*	34 (22/65)	48 (31/65)	46 (30/65)	18 (12/65)	9 (6/65)	23 (15/65)	0.28 ± 0.19
Gender									
Male ($n = 80$)	15 (12/80)	25 (20/80)	38 (30/80)	49 (39/80)	44 (35/80)	18 (14/80)	10 (7/80)	24 (19/99)	0.28 ± 0.19*
Female ($n = 19$)	16 (3/19)	11 (2/19)	21 (4/19)	47 (9/19)	26 (5/19)	21 (4/19)	5 (1/19)	11 (2/19)	0.20 ± 0.13
Smoking status									
Never ($n = 20$)	15 (3/20)	20 (4/20)	20 (4/20)	40 (8/20)	30 (6/20)	15 (3/20)	5 (1/20)	15 (3/20)	0.20 ± 0.13
Ever ($n = 79$)	15 (12/79)	23 (18/79)	38 (30/79)	51 (40/79)	43 (34/79)	19 (15/79)	9 (7/79)	23 (18/79)	0.28 ± 0.20*
Histologic types									
Squamous cell ca. ($n = 61$)	10 (6/61)	21 (13/61)	38 (23/61)	46 (28/61)	36 (22/61)	18 (11/61)	5 (3/61)	25 (15/61)	0.25 ± 0.19
Adenoca ($n = 38$)	24 (9/38)*	24 (9/38)	29 (11/38)	53 (20/38)	47 (18/38)*	18 (7/38)	13 (5/38)	16 (6/38)	0.28 ± 0.18
Adenoca[b]									
EGFR mutation (−) ($n = 26$)	23 (6/26)	27 (7/26)	31 (8/26)	54 (14/26)	46 (12/26)	19 (5/26)	12 (3/26)	15 (4/26)	0.28 ± 0.18
EGFR mutation (+) ($n = 9$)	22 (2/9)	22 (2/9)	33 (3/9)	44 (4/9)	57 (5/9)	11 (1/9)	11 (1/9)	22 (2/9)	0.28 ± 0.21

* $P < 0.05$.

[a] The MIs of the different groups were compared using analysis of covariance (ANCOVA).

[b] Adenocarcinoma cases examined for *EGFR*, *ERBB2* and *KRAS* mutations. Mutation (+); cases with *EGFR* ($n = 8$) or *ERBB2* ($n = 1$) mutation.

seen in the methylation rates of the other seven genes or in the MI value of the eight genes studied (Table 2). The methylation rate of each gene did not significantly differ between the males and females, or between the never- and ever-smokers, but the MI value was significantly higher in males than in females, and was significantly higher in ever-smokers than in the never-smokers (0.28 ± 0.19 versus 0.20 ± 0.13; $P < 0.05$ and 0.28 ± 0.20 versus 0.20 ± 0.13; $P < 0.05$, respectively). The methylation rates of *GSTP1* and *RASSF1A* were significantly higher in cases of adenocarcinoma than in cases of squamous cell carcinoma (24% versus 10% and 47% versus 36%, respectively), whereas the methylation rates of the other six genes and the MI value of the eight genes did not differ significantly between squamous cell carcinomas and adenocarcinomas. Aberrant methylation in lung tumors showed no significant correlation with tumor stage.

The methylation profiles were next compared according to the *EGFR* mutation status in adenocarcinomas. Of the 38 adenocarcinomas, 35 had previously been examined for *EGFR*, *ERBB2* and *KRAS* mutations in another study [19]. There was no significant difference in the rates of methylation or the MI value of the eight genes examined according to the *EGFR* mutation status in the adenocarcinomas (Table 2).

4. Discussion

The methylation rates of *GSTP1*, *p16*, *FHIT*, *APC*, *RASSF1A*, *hMLH1*, *hMSH2*, *AGT* in Korean NSCLCs were comparable to those in previous studies, where methylation was detected at 7—10% for *GSTP1*, 23—44% for *p16*, 36—37% for *FHIT*, 0—55% for *APC*, 15—45% for *RASSF1A*, 0—67% for *hMLH1*, 29—34% for *hMSH2* and 10—55% for *AGT* [5,7—14,21—23].

Interestingly, Toyooka et al. [24] investigated the geography-related differences in the methylation profiles of NSCLC. The methylation rates of *p16*, *APC* and *RASSF1A* in their study did not differ between cases in the USA and Australia and cases in Japan and Taiwan, whereas the methylation rates of *GSTP1* and *AGT* were significantly higher in the American and Australian cases (15% for *GSTP1* and *AGT*) than in those from Japan and Taiwan (1% for *GSTP1* and *AGT*). In addition, Yanagawa et al. [22] also reported that the methylation of *GSTP1* was detected at a low frequency in Japanese NSCLCs (1 of 75 cases). In contrast to the Japanese studies [22,24], we found that the *GSTP1* and *AGT* genes were frequently methylated in Korean NSCLCs, which was similar to the findings of the Caucasian studies. Although the reason for these differences in the methylation rates of *GSTP1* and *AGT1* from Japanese and Korean studies is unclear, several factors may account for these differences. These differences might depend on the MSP assay sensitivity and the position of the CpG sites examined [14,25]. However, the conventional MSP with the same primers was used to determine the methylation status of the *GSTP1* and *AGT* promoters in the Japanese studies and the present study. Therefore, it is unlikely that any differences could be attributable to MSP procedure. Several studies have reported that gene-specific methylation varied according to the type of carcinogen exposure [26—28]. Based on these observations, it is possible that the differences in the methylation rates of *GSTP1* and *AGT* from Japanese and Korean NSCLCs may be related to the geographical or cultural differences in carcinogen exposures, including cigarette smoking, dietary factors, occupational and environmental chemical exposure [26—29]. The discrepant results might also have been due to genetic differences of the study populations. A recent study reported that subjects carrying functional polymorphic variants of *GSTP1* had a higher risk of aberrant promoter methylation of *AGT* [30]. This suggests that functional variants in the genes involved in the folate metabolism, DNA methylation, carcinogen metabolism and the repair of methylation may play an important role in the susceptibility to methylation [29]. In addition, there is increasing evidence of significantly different genetic polymorphisms between Japanese and Koreans. This suggests that genetic differences between Japanese and Koreans can affect the susceptibility to gene-specific methylation. However, it is possible that these differences are attributable to chance as a result of the relatively small number of the study subjects examined. Therefore, further study with more subjects will be needed.

Although there is no direct evidence that smoking induces aberrant promoter methylation, several studies have reported that the methylation of tumor-related genes was associated with tobacco smoking [10,21,24,31]. The MI value in the ever-smokers, in the present study, was significantly higher than that of never-smokers although the methylation rate of each gene was not significantly different between the ever-smokers and never-smokers, which is consistent with the findings of the previous studies.

5. Conclusion

We determined the frequency of methylation of the *GSTP1*, *p16*, *FHIT*, *APC*, *RASSF1A*, *hMLH1*, *hMSH2*, *AGT* genes in Korean NSCLCs and examined their relationships to the clinicopathologic features of lung cancers. In this study, we found high methylation rates in Korean NSCLC patients with methylation profiles very similar to those previously reported in Western populations.

Conflict of interest

None.

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