3. Centrifuge the tube and rinse the pellet with 70% ethanol. Dry the pellet to a degree that it is dry but still retains water (*see* **Note 11**). Dissolve the pellet in 4 μL of 3 × EE solution (*see* **Note 12**).
4. Denaturation and re-annealing are successively performed. Place one drop of mineral oil onto the 4 μL 3 × EE solution in tube C. Denature the DNA mixture by heating at 96°C for 10 min in a thermal cycler. Add 1 μL of 5 M NaCl, paying attention so that the tube is "not" cooled during the handling (*see* **Note 13**), and keep the tube at 67°C for 16–24 h for re-annealing.

3.3. Selective Amplification

1. The denatured and re-annealed mixture is diluted by adding 45 μL of 1 M NaCl preheated at 67°C. To prevent evaporation, 1 M NaCl should be prepared in a large volume (400–500 μL). Great care should be taken that the mixture does not cool down.
2. Prepare a PCR solution (387 μL for now, but final 400 μL) that contains 1 × PCR III buffer, 300 μM dNTP, 1 M betaine, and 15 U of Taq polymerase; keep the solution at 72°C.
3. To the PCR solution, add 5 μL of the diluted mixture (step 1) at 72°C avoiding cool down of the mixture (*see* **Note 14**). This step is necessary to fill in 3′ ends of re-annealed products.
4. Add 8 μL of JHpa24 primer (50 μM) to the PCR solution at the first 95°C step, and perform 10 cycles of PCR (95°C for 1 min and 70°C for 3 min). After 10 cycles of PCR, keep the PCR solution at 72°C. Do not cool it down.
5. Take the tubes out of the thermal cycler and add immediately 40 μL of 10 × mung bean buffer to the PCR tube (*see* **Note 15**). Then add 10 μL of mung bean nuclease (10 U/μL). Keep the tube at 30°C for 30 min.
6. Extract the solution twice with phenol, twice with chloroform, and precipitate it with ethanol using NH_4OAc (*see* **Note 16**). Dissolve the pellet in 30 μL of TE.
7. Prepare a PCR solution that contains 1 × PCR III buffer, 300 μM dNTPs, 15 U Taq polymerase, 1 M betaine, and 3 μL of the above solution (step 6) (*see* **Note 17**). Heat the tube to 95°C, and then add 8 μL of JHpa24 primer (*see* **Note 18**). Perform 20 cycles of PCR (95°C for 1 min and 70°C for 3 min).
8. Check the degree of PCR amplification by running 10 μL of the PCR solution in a 2% NuSieve agarose gel (**Fig. 10.6**). If the amplification is not sufficient, add 2–10 cycles of PCR.
9. Extract the PCR solution twice with phenol, twice with chloroform, and precipitate it with ethanol using NH_4OAc. Dissolve the pellet in 50 μL of TE, and quantify the solution. This is the product of the first cycle of competitive hybridization (C1).

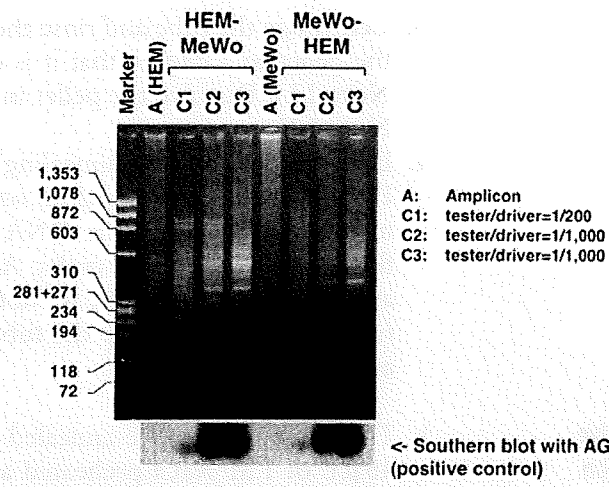

Fig. 10.6. A typical course of MS-RDA. Amplicons were prepared from a normal human embryonic melanocyte (HEM) and a melanoma cell line (MeWo). Two series of MS-RDA were performed using HEM as the tester (HEM – MeWo) and MeWo as the tester (MeWo – HEM). In C1, some differentially methylated fragments seem to be enriched. In C2 and C3, differentially methylated fragments became clearly visible by ethidium bromide staining of the entire DNA. Appropriate enrichment of differentially methylated fragments was confirmed by adding an unmethylated and methylated DNA fragment (AG8) to the tester and driver, respectively, at a concentration of one copy per haploid genome, and by observing its amplification by Southern blot analysis.

3.4. The Second Cycle

1. Digest 5–20 μg of C1 with 50–200 U of *Msp*I to switch the JHpa adaptor to the NHpa adaptor.
2. In a similar manner to the adaptor switch from RHpa to JHpa (step 8 of **Section 3.1** amplicon preparation), remove the digested JHpa adaptor, and confirm that the removal was complete.
3. To the C1 whose adaptor was removed (C1/MspI/E1), ligate the NHpa adaptor in a 30 μL solution that contains 500 pmol of the NHpa adaptor, 200 ng of the C1/MspI/E1, 1 × T4 ligase buffer, and 800 U of T4 ligase. Keep the solution at 16°C overnight.
4. Add 70 μL of TE to the ligation solution, and extract the solution once with phenol. Mix 40 ng (20 μL of the diluted solution) of the diluted ligation solution with 40 μg of driver amplicon (E1 and E2). Extract the mixture once with phenol, twice with chloroform, and precipitate it with ethanol using NaOAc.
5. Perform competitive hybridization and selective amplification as described for C1 in steps 3 and 4 of **Section 3.2** (competitive hybridization) and steps 1–9 of **Section 3.3** (selective amplification), except that the NHpa24 primer is used (*see* **Note 19**). This will produce C2. For C2, 20 cycles are usually enough for the second PCR as described in step 7

of **Section 3.3** (selective amplification). Confirm that selective amplification took place by running C2 in a 2% NuSieve agarose (**Fig. 10.6**).

3.5. The Third Cycle (OPTIONAL)

1. If selective amplification is not satisfactory in C2, a third cycle could be performed. In this case, switch the adaptor from NHpa back to JHpa, and perform competitive hybridization and selective amplification as described above (*see* **Note 20**).

3.6. Analysis of the MS-RDA Product

1. Clone the product of the last selective amplification (C2 or C3) into pGEM-T Easy vectors (*see* **Note 21**). After transformation into competent cells, pick up 96 clones and sequence them (*see* **Note 22**, *see also* **Chapter 14**). The cloned fragments have the final adaptor on their ends, and their sequence could be searched in genome databases.

4. Notes

1. This step is the only step that detects the DNA methylation status of the various genomic regions in the tester and driver. Therefore, the digestion by a methylation-sensitive restriction enzyme must be complete. Complete digestion can be confirmed by electrophoresing an aliquot of the digestion solution in a 0.9% agarose gel and observing the smear pattern. Also, some DNA samples are contaminated with RNA, and, if so, RNA must be eliminated during the digestion by RNase treatment. This will allow an appropriate amount of genomic DNA to be used for the following steps to prepare an amplicon.
2. Once an adaptor is prepared, it can be stored at −20°C for years.
3. The ligation time can be shortened down to 4 h. Since ATP is necessary for the ligation reaction, 10 × T4 ligase buffer should be handled on ice.
4. Betaine is known to facilitate amplification of G+C-rich sequences *(14)*.
5. These can be prepared at room temperature, while some of the later steps need careful temperature control. The ligation solution can be used without any purification.
6. To prevent nonspecific amplification, Taq polymerase should be added at a high temperature. A hot-start using an engineered-Taq polymerase, such as AmpliTaq Gold® (Applied Biosystems), is not suitable because the 3′ ends of the ligation products must be filled in at the initial incubation step at 72°C (**Fig. 10.2**).

7. For the following steps, a sufficient quantity of amplicon should be prepared. However, if too many cycles of PCR are performed, the amplification bias among DNA fragments will become significant. Therefore, the number of PCR cycles should be kept to a minimum within the range that yields a sufficient quantity of amplicon.
8. Typically, the driver amplicon yields 200–400 μg of amplified DNA.
9. Since 40 μg of driver amplicon without the adaptor are necessary for a competitive hybridization, 80–120 μg of the driver amplicon are necessary for a complete MS-RDA procedure. Use of E2 is sometimes inevitable to secure sufficient amounts of driver amplicon without the adaptor. However, for the tester, only E1 should be used because any residual adaptor can be ligated again and impairs the efficiency of competitive hybridization and selective amplification.
10. Since the efficiency of competitive hybridization and re-annealing is critical for the success of the experiment, the mixture is purified completely.
11. As with usual ethanol precipitation, the pellet should be dried to an appropriate degree. If it is too dry, its dissolution will become very difficult. If it contains much ethanol, the volume becomes larger and the efficiency of re-annealing will be impaired.
12. Ethanol precipitation is typically performed in a 1.5 mL Eppendorf tube, and the next step will be performed in a 0.5 mL Eppendorf tube. To dissolve the pellet of 40 μg of DNA (in tube A) completely:
    (a) take two 0.5 mL tubes (B, C),
    (b) put 5 μL of 3 × EE in tube B,
    (c) move 2 μL of 3 × EE from tube B to the pellet in tube A,
    (d) vortex tube A for more than 1 min paying attention that the solution is on the pellet, and spin it down (the solution is very sticky),
    (e) move the 2(+α) μL of the solution in tube A to tube C,
    (f) add fresh 2 μL of 3 × EE from tube B to tube A without changing the tip,
    (g) vortex tube A for more than 1 min, and spin it down,
    (h) move the 2 μL in tube A to tube C without changing the tip, and
    (i) vortex tube C, and spin it down.
13. The 5 M NaCl can be preheated, but care should be taken to avoid evaporation. Wear gloves to avoid burns while handling a hot tube. Cooling down of the tube accelerates non-specific annealing, and will impair the efficiency of RDA.

14. The remaining solution (45 μL) from step 1 is usually kept at 67°C until the success of step 8 is confirmed. If the pellet is lost at step 6, it can be started over from this point.
15. While adding the mung bean buffer to the PCR solution, its temperature goes down to 30–40°C. Do not cool it down too much.
16. The pellet is very tiny, and the highest care must be taken that it is not lost. This step is one of the most difficult steps in the entire RDA procedure.
17. At this step, for the first time, all the DNA molecules in the tube are completely double stranded because all the single-stranded DNA molecules were digested by mung bean nuclease. Therefore, taking care of the temperature is not necessary during preparation of the PCR solution.
18. Only for this PCR, hot-start PCR can be used, if addition of a primer at 95°C is troublesome. The entire solution can be prepared at room temperature using an engineered-Taq polymerase, such as AmpliTaq Gold®.
19. The product of the first 10 cycles of PCR of the second competitive hybridization is usually much more abundant than the one in the first competitive hybridization, and the risk of loss is lower.
20. An addition of a fourth cycle does not improve the experiment. If selective amplification is not obtained in the third cycle, the quality of the initial samples, selection of the initial samples, and technical errors should be considered.
21. Any TA cloning vector is fine.
22. The number of clones sequenced is dependent upon the diversity of the final product. If redundant clones are observed by sequencing, sequencing can be suspended at that time point. If only nonredundant clones are observed, more clones should be sequenced.

Acknowledgements

The protocol is improved through the support of a Grant-in-Aid for the Third-Term Comprehensive Cancer Control Strategy from the Ministry of Health, Labour and Welfare, Japan.

References

1. Ushijima, T., Morimura, K., Hosoya, Y., et al. (1997) Establishment of methylation-sensitive representational difference analysis and isolation of hypo- and hypermethylated genomic fragments in mouse liver tumors. *Proc Natl Acad Sci USA* 94, 2284–2289.
2. Kaneda, A., Takai, D., Kaminishi, M., et al. (2003) Methylation-sensitive representational

difference analysis and its application to cancer research. *Ann NY Acad Sci* 983, 131–141.
3. Ushijima, T. (2005) Detection and interpretation of altered methylation patterns in cancer cells. *Nat Rev Cancer* 5, 223–231.
4. Takai, D., Yagi, Y., Wakazono, K., et al. (2001) Silencing of *HTR1B* and reduced expression of *EDN1* in human lung cancers, revealed by methylation-sensitive representational difference analysis. *Oncogene* 20, 7505–7513.
5. Kaneda, A., Kaminishi, M., Nakanishi, Y., et al. (2002) Reduced expression of the *insulin-induced protein 1* and *p41 Arp2/3 complex* genes in human gastric cancers. *Int J Cancer* 100, 57–62.
6. Miyamoto, K., Asada, K., Fukutomi, T., et al. (2003) Methylation-associated silencing of heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase-2 (3-OST-2) in human breast, colon, lung and pancreatic cancers. *Oncogene* 22, 274–280.
7. Miyamoto, K., Fukutomi, T., Akashi-Tanaka, S., et al. (2005) Identification of 20 genes aberrantly methylated in human breast cancers. *Int J Cancer* 116, 407–414.
8. Hagihara, A., Miyamoto, K., Furuta, J., et al. (2004) Identification of 27 5′ CpG islands aberrantly methylated and 13 genes silenced in human pancreatic cancers. *Oncogene* 23, 8705–8710.
9. Abe, M., Ohira, M., Kaneda, A., et al. (2005) CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. *Cancer Res* 65, 828–834.
10. Furuta, J., Nobeyama, Y., Umebayashi, Y., et al. (2006) Silencing of peroxiredoxin 2 and aberrant methylation of 33 CpG islands in putative promoter regions in human malignant melanomas. *Cancer Res* 66, 6080–6086.
11. Kaneda, A., Wakazono, K., Tsukamoto, T., et al. (2004) *Lysyl oxidase* is a tumor suppressor gene inactivated by methylation and loss of heterozygosity in human gastric cancers. *Cancer Res* 64, 6410–6415.
12. Abe, M., Westermann, F., Nakagawara, A., et al. (2007) Marked and independent prognostic significance of the CpG island methylator phenotype in neuroblastomas. *Cancer Lett* 247, 253–258.
13. Lisitsyn, N., Lisitsyn, N., Wigler, M. (1993) Cloning the differences between two complex genomes. *Science* 259, 946–951.
14. Henke, W., Herdel; K., Jung, K., et al. (1997) Betaine improves the PCR amplification of GC-rich DNA sequences. *Nucleic Acids Res* 25, 3957–3958.

The Presence of Aberrant DNA Methylation in Noncancerous Esophageal Mucosae in Association With Smoking History

A Target for Risk Diagnosis and Prevention of Esophageal Cancers

Daiji Oka, MD[1,2]; Satoshi Yamashita, PhD[1]; Tadashi Tomioka, BTech[1]; Yukihiro Nakanishi, MD, PhD[3]; Hoichi Kato, MD, PhD[4]; Michio Kaminishi, MD, PhD[2]; and Toshikazu Ushijima, MD, PhD[1]

BACKGROUND: Esophageal squamous cell carcinomas (ESCCs) tend to have multiple primary lesions, and it is believed that they arise from background mucosae with accumulation of genetic/epigenetic alterations. In this study, the objective was to elucidate the effects of smoking and drinking on the accumulation of epigenetic alterations in background mucosae. **METHODS:** Genes that are silenced in human ESCCs were searched for by treating 3 ESCC cell lines with the demethylating agent, 5-aza-2′-deoxycytidine and performing oligonucleotide microarrays. Methylation levels were analyzed by quantitative methylation-specific polymerase chain reaction analysis of 60 ESCCs and their corresponding background mucosae. **RESULTS:** Forty-seven genes were identified as methylation-silenced in at least 1 of the 3 ESCC cell lines, and 14 of those genes (claudin 6 [*CLDN6*]; G protein-coupled receptor 158 [*GPR158*]; homeobox A9 [*HOXA9*]; metallothionein 1M [*MT1M*]; neurofilament, heavy polypeptide 200 kDa [*NEFH*]; plakophilin 1 [*PKP1*]; protein phosphatase 1, regulatory [inhibitor] subunit 14A [*PPP1R14A*]; pyrin domain and caspase recruitment domain containing [*PYCARD*]; R-spondin family, member 4 [*RSPO4*]; testis-specific protein, Y-encoded–like 5 [*TSPYL5*]; ubiquitin carboxyl-terminal esterase L1 [*UCHL1*]; zinc-finger protein 42 homolog [*ZFP42*]; zinc-finger protein interacting with K protein 1 homolog [*ZIK1*]; and zinc-finger and SCAN domain containing 18 [*ZSCAN18*]) were used as markers. In the background mucosae, methylation levels of 5 genes (*HOXA9*, *MT1M*, *NEFH*, *RSPO4*, and *UCHL1*) had significant correlations with smoking duration ($\rho = .268$; $P = .044$; $\rho = .405$; $P = .002$; $\rho = .285$; $P = .032$; $\rho = .300$; $P = .024$; and $\rho = .437$; $P = .001$, respectively). In contrast, an inverse correlation between *PYCARD* methylation levels and alcohol intake was observed ($\rho = -.334$, $P = .025$) among individuals with the inactive aldehyde dehydrogenase 2 (*ALDH2*) genotype. **CONCLUSIONS:** The current results suggested that ESCCs developed from an epigenetic field for cancerization, which was induced by exposure to carcinogenic factors, such as tobacco smoking. The epigenetic field defect will be a novel target for risk diagnosis and prevention of ESCCs. **Cancer 2009;115:3412–26.**

KEY WORDS: epigenetics, DNA methylation, esophageal cancer, tobacco smoking, alcohol drinking.

Corresponding author: Toshikazu Ushijima, MD, PhD, Carcinogenesis Division, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan; Fax: (011) 81-3-5565-1753; tushijim@ncc.go.jp

[1]Carcinogenesis Division, National Cancer Center Research Institute, Tokyo, Japan; [2]Department of Gastrointestinal Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [3]Pathology Division, National Cancer Center Research Institute, Tokyo, Japan; [4]Esophageal Surgery Division, National Cancer Center Hospital, Tokyo, Japan

Received: August 26, 2008; **Revised:** December 9, 2008; **Accepted:** January 7, 2009

Published online: May 26, 2009 © 2009 American Cancer Society

DOI: 10.1002/cncr.24394, www.interscience.wiley.com

Esophageal cancer is 1 of the most lethal cancers and imposes reduced quality of life even in patients who receive curative treatment.[1] Squamous cell carcinoma (SCC) and adenocarcinoma are 2 major histologic types of esophageal cancer, and SCC is the predominant histologic type in Asian countries.[2] Most patients with esophageal SCC (ESCC) have a history of chronic smoking and/or heavy drinking, and these are established risk factors for ESCC.[1] With regard to the interactions between smoking and drinking, controversial reports are available; however, to our knowledge, to date, the combined risk for ESCC has not been clarified.[3-5]

Patients with ESCC often have multiple primary lesions,[6] and the frequency of multiple occurrence reaches up to 30%.[7,8] In addition, dysplastic lesions frequently are observed in the background mucosae surrounding ESCCs.[9] The incidence of multiple occurrences of ESCC and/or dysplastic lesions reportedly is high in heavy smokers and heavy drinkers.[7,10,11] It is believed that both tobacco smoking and alcohol drinking can induce genetic/epigenetic alterations in esophageal mucosal cells and that genetic/epigenetic alterations already have accumulated in the normal-appearing esophageal mucosae of individuals who have a long history of smoking and drinking, forming a "field for cancerization."[12]

DNA methylation of a CpG island (CGI) in a promoter region causes silencing of its downstream gene and is known as a major epigenetic mechanism for inactivation of tumor-suppressor genes.[13] In ESCCs, various tumor-suppressor genes, such as adenomatous polyposis coli (*APC*), cadherin 1 (*CDH1*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), retinoic acid receptor β (*RARβ*), Ras association domain family member 1-isoform A (*RASSF1A*), and ubiquitin carboxyl-terminal esterase L1 (*UCHL1*), reportedly are methylated.[14,15] In contrast to the deep involvement of aberrant DNA methylation in ESCCs, its inducers in the esophagus have been poorly clarified, except for induction methylation of the fragile histidine triad gene *FHIT* by nicotine, a tobacco component, in human esophageal epithelial cell lines.[16] Tobacco smoking reportedly induced methylation of several genes in bronchial and oral epithelium,[17–20] but no information was available in esophageal epithelial cells.

In the current study, our objective was to clarify the effects of tobacco smoking and alcohol drinking on the induction of DNA methylation in esophageal mucosae. The mucosae are not clonal, and the degree of methylation is correlated with the power of methylation induction. Because different genes have different susceptibility to methylation induction,[21] first, we screened genes that were silenced in ESCCs by treating ESCC cell lines with a demethylating agent and analyzing the genes that were up-regulated. Then, we quantified the methylation levels of the "marker" genes and 4 tumor-suppressor genes in ESCCs and in noncancerous esophageal mucosae by using quantitative methylation-specific polymerase chain reaction (PCR) (MSP) analysis.

MATERIALS AND METHODS

Cell Lines and 5-Aza-2′-deoxycitidine Treatment

The ESCC cell lines KYSE30, KYSE220, and KYSE270, which were derived from well differentiated, moderately differentiated, and well differentiated ESCCs, respectively,[22] were purchased from Health Science Research Resources Bank (Osaka, Japan). For 5-aza-2′-deoxycitidine (5-Aza-dC) treatment, 2×10^5 KYSE30 cells per 10-cm dish, 5×10^5 KYSE220 cells per 10-cm dish, and 4×10^5 KYSE270 cells per 10-cm dish were seeded on Day 0 and exposed to freshly prepared 5-Aza-dC (Sigma, St. Louis, MO) for 24 hours on Days 1 and 3. The concentration of 5-Aza-dC was 1 μM for KYSE30 and KYSE220 cells and 0.3 μM for KYSE270. After each treatment, the cells were placed in fresh medium and harvested on Day 4. Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan) and purified using an RNeasy Mini kit (Qiagen, Valencia, Calif). DNA methyltransferase 1 depletion was confirmed by Western blot analysis of the whole cell extract.[23]

Oligonucleotide Microarray Analysis and Database Search

Oligonucleotide microarray analysis was performed using the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, Calif) with 47,400 probe sets from 38,500 genes and GeneChip Operating Software. The signal intensities were normalized so that the average

of all genes on a GeneChip was 500. Database searches were performed at a GenBank website, and we searched for CGIs by using a modified criteria of Takai and Jones[24]: 1) CpG score ≥0.60, 2) guanine (G) and cytosine (C) content ≥50%, and 3) length ≥500 base pairs.

Patients and Tissue Samples

Sixty primary ESCC specimens and their paired noncancerous background mucosae were collected from patients who underwent esophagectomy and who were diagnosed histologically with invasive SCC at the National Cancer Center Hospital, Tokyo, Japan (51 men and 9 women; average age, 63.3 years [range, 41-83 years]). Informed consent was obtained from all patients. Background mucosae were resected from areas stained by iodine that were considered histologically normal.[25] All samples were stored in RNAlater (Applied Biosystems, Foster City, Calif) at −80°C until the extraction of genomic DNA. Genomic DNA was extracted by using the phenol-chloroform method.

History of tobacco smoking and alcohol drinking was obtained from 57 patients and 55 patients, respectively, in interviews with the patients by medical staff. Mean daily alcohol intake was calculated, converting a cup of sake (180 mL), a cup of shochu (180 mL), a single finger of whisky (30 mL), a bottle of wine (750 mL), and a bottle of beer (633 mL) into 27 g, 45 g, 12 g, 105 g, and 25 g of alcohol, respectively.

Aldehyde Dehydrogenase Genotyping

Aldehyde dehydrogenase 2 (*ALDH2*) genotyping of each patient was performed on genomic DNA extracted from background mucosae by PCR-restriction fragment length polymorphism.[26] Exon 12 of the *ALDH2* gene was amplified with forward primer (5′-CAAATTACAGGGTCAACTG CT-3′) and reverse primer (5′-CCACACTCACAGTTTTC TCTT-3′). The PCR products were digested with *Eam*1104I (*Ksp*632I) (Fermentas International Inc., Burlington, Ontario, Canada) and electrophoresed on ethidium bromide-stained 2% NuSieve agarose gels.

Bisulfite Modification and Semiquantitative Methylation-specific PCR

DNA was digested by *Bam*HI, and 1 μg of the digested DNA was denatured in 0.3 N NaOH at 37°C for 15 minutes. The samples underwent 15 cycles of 30-second denaturation at 95°C and a 15-minute incubation at 50°C in 3.1 N sodium bisulfite (pH 5.0) and 0.5 mM hydroquinone. The samples were desalted with Zymo-Spin IC Columns (Zymo Research, Orange, Calif), desulfonated in 0.3 N NaOH, and dissolved in 40 μL to 160 μL of Tris-ethylene diamine tetraacetic acid buffer.

MSP was performed with a primer set specific to the methylated (M) or unmethylated (U) sequence using 12.5 ng of the sodium bisulfite-treated DNA and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, Calif). The primers used are listed in Table 1. DNA methylated with *Sss*I methylase (New England Biolabs, Beverly, Mass) and DNA amplified twice by a GenomiPhi DNA amplification kit (GE Healthcare Bio-Sciences, Buckinghamshire, England) were used as fully methylated and unmethylated control DNA, respectively. For semiquantitative MSP, "10%-methylated DNA" was prepared by mixing 10% of methylated DNA and 90% of fully unmethylated control DNA after bisulfite modification.

Quantitative Methylation-specific PCR and Calculation of Methylation Levels

Real-time MSP was performed using 50 ng of the sodium bisulfite-treated DNA, SYBR Green I (BioWhittaker Molecular Applications, Rockland, Md), and an iCycler Thermal Cycler (Bio-Rad Laboratories). Methylated and unmethylated standard DNA was prepared by cloning PCR products from fully methylated and unmethylated control DNA, respectively, into the pGEM-T Easy vector (Promega, Madison, Wis) or by purifying PCR products using the Wizard SV Gel and PCR clean-up system (Promega). The numbers of methylated and unmethylated molecules in a sample were determined by comparing its amplification with that of methylated and unmethylated standard DNA, respectively, that contained known numbers of molecules (10 to 10^6 molecules).

On the basis of the numbers of methylated and unmethylated molecules for a genomic region in a sample, methylation levels were calculated as the fraction of methylated molecules in the total number of DNA molecules (the number of methylated molecules + the number of

Table 1. Primers for Qualitative or Quantitative Methylation-specific Polymerase Chain Reaction

Gene Symbol	Status	Primer Sequence		Annealing Temperature, °C		
		Forward	Reverse	Qualitative	Quantitative	No. of Cycles*
CLDN6	M	ATAAGTTTGGGATTCGTAC	ATCTTAAAAAAACGATAACG	52	54	35
	U	TTGATAAGTTTGGGATTTGTAT	CAAAAATCTTAAAAAAACAATAACA	52	54	35
GPR158	M	GTAATTTTTAGTCGGTTTTTC	GAATAACTAAACTACCGTCG	56	52	35
	U	TGTAATTTTTAGTTGGTTTTTTGT	CCAAATAACTAAACTACCATCA	56	58	35
HOXA9	M	TCGGATTATTAATAGCGTGC	ATCACCTAATAAATTACCGACG	60	58	33
	U	TAATAGTGTGTGGAGTGATTTATGT	CAATCACCTAATAAATTACCAACA	60	60	33
MT1M	M	GTATAGTTTTTTTCGCGTAAATTC	AACCCAACATAAATACCGAACG	59	55	32
	U	TTATTTGGTGTATAGTTTTTTTTGT	TAAACCCAACATAAATACCAAACA	56	52	38
NEFH	M	TTAAGGGGTTGGATTCGGTC	CGAAACGAAACGAAAAACACTACG	61	58	35
	U	GTTAAGGGGTTGGATTTGGTT	CCAAAACAAAACAAAAAACACTACA	61	61	35
PKP1	M	TTTTGTTTTTAAGAGCGTTGGTTTC	ACCCACTCCACCGAACCG	58	62	35
	U	TTTTGTTTTTAAGAGTGTTGGTTTT	CACTCCACCAAACCACACA	58	61	34
PPP1R14A	M	ATTTCGGTTCGGGAGTTTTC	TCGACTTAAAACACGCAATCG	58	60	35
	U	GGAGTTTTTGATGTAGGGATTT	TCAACTTAAAACACACAATCATACA	58	57	35
PYCARD	M	CGGGGAATCGCGGAGGTTTC	AATAAAACCCGAAAAAAAACCG	55	57	35
	U	GGTTTGGGGAATTGTGGAGGTTTT	ATCACACCCTCCAACTAACCTACA	55	60	33
RSPO4	M	CGTTAGGGTAGTGTTTCGGTTTC	TACTATAAACGCGCCGAACG	57	60	35
	U	TTTTTTTTTGTTAGGGTAGTGTTTT	ATAAACACACCAAACACATCCA	57	58	35
TSPYL5	M	GGGTCGTTTTTTGCGTAGTC	GTCACGAACGTACAACTATACCG	62	60	35
	U	GGTTGTTTTTTGTGTAGTTGTAGT	CATCACAAACATACAACTATACCA	62	62	35
UCHL1	M	TCGTATTTATTTGGTCGCGATC	CTATAAAACGCCGACCAAACG	62	64	35
	U	GGTTTGTATTTATTTGGTTGTGATT	CAACTATAAAACACCAACCAAACA	60	61	33
ZFP42	M	GCGTCGTTTAGGTGTTAGGC	AAAACGTAACCGACCCCG	62	57	35
	U	GTGTTTTAGGGTGGGGTTGGTTAT	AAACCCACCCTCCAAACTAACACA	62	63	35
ZIK1	M	GTTTGAGGTGACGTTGGGC	GACCCTTTTCTCAACGCGA	64	62	35
	U	TTTGAGGTGATGTTGGGTG	AACAACCCTTTTCTCAACACA	60	59	35
ZSCAN18	M	TTTTTTGTTCGTTTCGGTGC	GATAACGACCGACAAACTACG	59	62	35
	U	TGTTATGGTTTTTTTGTTTGTTTT	CTACACACTAAACCTCACCACA	59	60	35
CDH1	M	TAGGTTTTAGTGAGTTATCGGC	AAACGAAACTAACGACCCG		59	
	U	ATTTTAGGTTAGAGGGTTATTGTG	ATAAACCCCAAAAACACCA		59	
CDKN2A	M	TTGGTAGTTAGGAAGGTTGTATCGC	TCCCTACTCCCAACCGCG		62	
	U	GGTAGTTAGGAAGGTTGTATTGT	TCCCTACTCCCAACCACA		61	
MLH1	M	CGTTAAGTATTTTTTTCGTTTTGC	TCCGCTCTTCCTATTAATTCG		59	
	U	AGTGTTAAGTATTTTTTTTGTTTTGT	CTATCCACTCTTCCTATTAATTCA		56	
RASSF1A	M	GTCGTCGTTGTGGTCGTTC	AACCCGAAAACGAAACTAAACG		62	
	U	TGTTGTTGTTGTGGTTGTTT	AAAACAAAACTAAACACACTCTCA		62	

CLDN6 indicates claudin 6; M, specific to methylated DNA; A, adenine; T, thymine; G, guanine; C, cytosine; U, specific to unmethylated DNA; *GPR158*, G protein-coupled receptor 158; *HOXA9*, homeobox A9; *MT1M*, metallothionein 1M; *NEFH*, neurofilament, heavy polypeptide 200 kDa; *PKP1*, plakophilin 1; *PPP1R14A*, protein phosphatase 1, regulatory (inhibitor) subunit 14A; *PYCARD*, pyrin domain and caspase recruitment domain containing; *RSPO4*, R-spondin family, member 4; *TSPYL5*, testis-specific protein, Y-encoded–like 5; *UCHL1*, ubiquitin carboxyl-terminal esterase L1; *ZFP42*, zinc-finger protein 42 homolog; *ZIK1*, zinc-finger protein interacting with K protein 1 homolog; *ZSCAN18*, zinc-finger and SCAN domain containing 18; *CDH1*, cadherin 1; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *MLH1*, MutL homolog 1; *RASSF1A*, Ras association domain family member 1, isoform A.
*The number of polymerase chain reaction (PCR) cycles for qualitative methylation-specific PCR.

unmethylated molecules). For each gene, the deviation value of a methylation level in the background mucosa of a case was calculated.

Statistical Analysis

Correlations between methylation levels in background mucosae and risk factors of patients were analyzed by Spearman rank-order correlation coefficients (ρ). Methylation levels in groups with different clinicopathologic characters were compared using the Kruskal-Wallis test and the Mann-Whitney *U* test. Correlations between methylation frequencies in ESCCs and methylation levels in background mucosae (mean deviation values) were analyzed using Pearson correlation coefficients. The statistical calculations were conducted with SPSS software (13.0J; SPSS Inc., Chicago, Ill).

RESULTS

Genes Up-regulated by 5-Aza-deoxycitidine Treatment and Their Methylation Analysis

Three ESCC cell lines (KYSE30, KYSE220, and KYSE270) were treated with 5-Aza-dC, and changes in gene expression were analyzed by oligonucleotide microarrays. We searched for genes that 1) were up-regulated above a threshold (8-fold, 16-fold, or 32-fold), 2) had signal intensities of $\leq$100 before the treatment and >100 after the treatment, 3) were not located on chromosome X, and 4) had CGIs 5′ upstream of their putative transcription start sites. The higher threshold we adopted, the fewer genes were up-regulated. Because the objective of the screening was to isolate marker genes for exposure to tobacco smoking and alcohol drinking, we adopted a cutoff value of 16-fold so that a manageable number of candidate genes (72 candidate genes in total) would be obtained.

The methylation status of the CGIs at the putative transcription start sites of the 72 genes was analyzed by MSP in the KYSE30, KYSE220, and KYSE270 cell lines. Forty-seven genes were confirmed as completely methylated at least in 1 of the 3 cell lines and were considered to be methylation silenced. Then, their methylation status was analyzed in 6 primary ESCCs and their background mucosae by using semiquantitative MSP. Thirty-nine of those genes were methylated in at least 1 primary ESCC, but 15 genes were methylated too heavily in the background mucosae ($\geq$10% in all 6 samples). Therefore, the remaining 24 genes were considered the most informative.

Methylation Quantification in ESCCs and Background Mucosae

Among the 24 genes, primers for quantitative MSP were designed successfully for 14 genes: claudin 6 (*CLDN6*); G protein-coupled receptor 158 (*GPR158*); homeobox A9 (*HOXA9*); metallothionein 1M (*MT1M*); neurofilament, heavy polypeptide 200 kDa (*NEFH*); plakophilin 1 (*PKP1*); protein phosphatase 1, regulatory (inhibitor) subunit 14A (*PPP1R14A*); pyrin domain and caspase recruitment domain containing (*PYCARD*); R-spondin family, member 4 (*RSPO4*); testis-specific protein, Y-encoded–like 5 (*TSPYL5*); *UCHL1*; zinc-finger protein 42 homolog (*ZFP42*); zinc-finger protein interacting with K protein 1 homolog (*ZIK1*); and zinc-finger and SCAN domain containing 18 (*ZSCAN18*). Methylation levels of these 14 genes and of 4 tumor-suppressor genes (*CDH1*, *CDKN2A*, mutL homolog 1 [*MLH1*], and *RASSF1A*), 3 of which reportedly are silenced in ESCCs,[27–29] were analyzed in 60 ESCCs and their paired noncancerous background mucosae. Similar to results from an examination of gastric cancers and their background mucosae,[30] distributions of methylation levels revealed different patterns between ESCCs and their background mucosae (Fig. 1).

In the background mucosae, different genes had different methylation distribution from the viewpoints of the fraction of methylation-positive samples and their absolute methylation levels. The first group of genes (*CLDN6*, *CDKN2A*, *MLH1*, and *RASSF1A*) had no methylation. The second group of genes (*PYCARD*, *RSPO4*, *TSPYL5*, *ZIK1*, and *ZSCAN18*) was methylated in a small number of samples, and the levels were low ($\leq$3%). The third group of genes (*UCHL1* only) was methylated also in only a small number of samples, but the methylation level was high in some samples, reaching up to 20%. The fourth group of genes (*GPR158*, *HOXA9*, *MT1M*, *NEFH*, *PKP1*, *PPP1R14A*, *ZFP42*, and *CDH1*) was methylated in a large number of samples, and the methylation levels revealed unimodal distribution with various highest values ranging from 6.8% (*MT1M*) to 25.9% (*ZFP42*).

In the 60 ESCCs, 12 marker genes (*CLDN6*, *GPR158*, *HOXA9*, *MT1M*, *NEFH*, *PPP1R14A*, *RSPO4*, *TSPYL5*, *UCHL1*, *ZIK1*, *ZFP42*, and *ZSCAN18*) and 2 tumor-suppressor genes (*CDH1* and *RASSF1A*) were methylated in 11 to 49 ESCCs and in 3 to 4 ESCCs, respectively, with a cutoff threshold of 6%.[30,31] Two marker genes (*PKP1* and *PYCARD*) and 2 tumor-suppressor genes (*CDKN2A* and *MLH1*) were not methylated. The distribution of methylation levels in methylation-positive ESCCs was much broader than the levels in background mucosae. Also, 11 of 12 marker genes (*CLDN6*, *GPR158*, *HOXA9*, *MT1M*, *NEFH*, *PPP1R14A*, *RSPO4*, *TSPYL5*, *UCHL1*, *ZIK1*, and *ZSCAN18*) and 2 tumor-suppressor genes (*CDH1* and *RASSF1A*) had large numbers of methylation-negative samples at the same time.

Correlations Between Methylation Levels in the Background Mucosae and Exposure Levels to ESCC Risk Factors

Next, we examined correlations between methylation levels in the background mucosae and risk factors for ESCCs; age, smoking duration, and mean daily alcohol intake (Table 2). From the initial 14 genes, *CLDN6*, which did

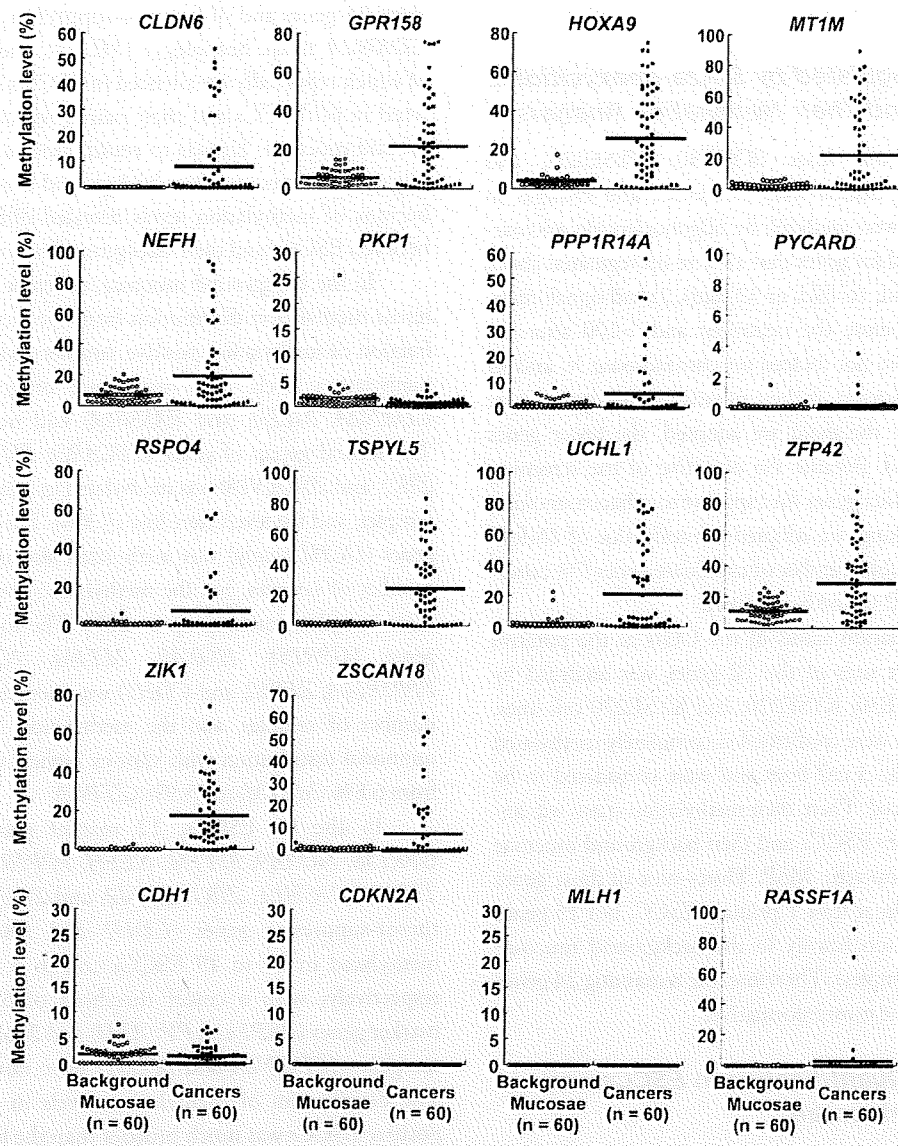

FIGURE 1. Methylation levels of the 14 selected genes and 4 tumor suppressor genes in esophageal squamous cell carcinomas (ESCCs) and their background mucosae are shown. Distinct distributions of methylation levels were observed in the background mucosae and in ESCCs. Mean methylation levels of individual groups are indicated by horizontal lines. *CLDN6* indicates claudin 6; *GPR158*, G protein-coupled receptor 158; *HOXA9*, homeobox A9; *MT1M*, metallothionein 1M; *NEFH*, neurofilament, heavy polypeptide 200 kDa; *PKP1*, plakophilin 1; *PPP1R14A*, protein phosphatase 1, regulatory (inhibitor) subunit 14A; *PYCARD*, pyrin domain (PYD) and caspase recruitment domain (CARD) containing; *RSPO4*, R-spondin family, member 4; *TSPYL5*, testis-specific protein, Y-encoded-like 5; *UCHL1*, ubiquitin carboxyl-terminal esterase L1; *ZFP42*, zinc-finger protein 42 homolog; *ZIK1*, zinc-finger protein interacting with K protein 1 homolog; *ZSCAN18*, zinc-finger and SCAN domain containing 18; *CDH1*, cadherin 1; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *MLH1*, MutL homolog 1; *RASSF1A*, Ras association domain family member 1, isoform A.

not have methylation in the background mucosae, was excluded. With age, a significant correlation was observed only for *TSPYL5* ($\rho = .273$; $P = .035$). It is interesting to note that, with smoking duration ($n = 57$), significant correlations were observed for 5 genes; *HOXA9* ($\rho = .268$; $P = .044$), *MT1M* ($\rho = .405$; $P = .002$), *NEFH* ($\rho = .285$; $P = .032$), *RSPO4* ($\rho = .300$; $P = .024$), and *UCHL1* ($\rho = .437$; $P = .001$). Scatter diagrams are shown in Figure 2, and the mean methylation levels in 3 tertile groups are shown in Figure 3.

Table 2. Correlations (Shown as *P* Values) Between Tobacco Use or Alcohol Use and Methylation Levels in Background Mucosae of the 13 Selected Genes

Variable	*GPR158*	*HOXA9*	*MT1M*	*NEFH*	*PKP1*	*PPP1R-14A*	*PYCARD*	*RSPO4*	*TSPYL5*	*UCHL1*	*ZFP42*	*ZIK1*	*ZSCAN18*
Age													
ρ	.177	.218	.035	.225	.077	.246	.216	.100	.273	−.079	.200	.061	.049
P	.176	.094	.789	.084	.560	.058	.097	.449	.035*	.551	.126	.642	.707
Smoking duration													
ρ	.155	.268	.405	.285	.158	.130	−.118	.300	.215	.437	.181	.027	.108
P	.248	.044*	.002*	.032*	.242	.335	.383	.024*	.109	.001*	.177	.841	.424
Alcohol consumption													
ρ	−.154	−.078	.071	−.139	−.072	−.168	−.249	−.089	−.214	.224	−.249	−.024	−.251
P	.263	.571	.605	.312	.600	.219	.067	.517	.116	.100	.067	.861	.064
Inactive ALDH2†													
ρ	.021	−.036	.228	−.039	−.034	.030	−.334	-.058	−.111	.259	−.142	.009	−.202
P	.890	.812	.132	.798	.822	.843	.025*	.705	.469	.085	.353	.952	.184

GPR158 indicates; G protein-coupled receptor 158; *HOXA9*, homeobox A9; *MT1M*, metallothionein 1M; *NEFH*, neurofilament, heavy polypeptide 200 kDa; *PKP1*, plakophilin 1; *PPP1R14A*, protein phosphatase 1, regulatory (inhibitor) subunit 14A; *PYCARD*, pyrin domain and caspase recruitment domain containing; *RSPO4*, R-spondin family, member 4; *TSPYL5*, testis-specific protein, Y-encoded–like 5; *UCHL1*, ubiquitin carboxyl-terminal esterase L1; *ZFP42*, zinc-finger protein 42 homolog; *ZIK1*, zinc-finger protein interacting with K protein 1 homolog; *ZSCAN18*, zinc-finger and SCAN domain containing 18; ALDH2, aldehyde dehydrogenase 2.

* $P < .05$.

† The $ALDH2^1/ALDH2^2$ heterozygote (n=45) and the $ALDH2^2/ALDH2^2$ homozygote (n=0).

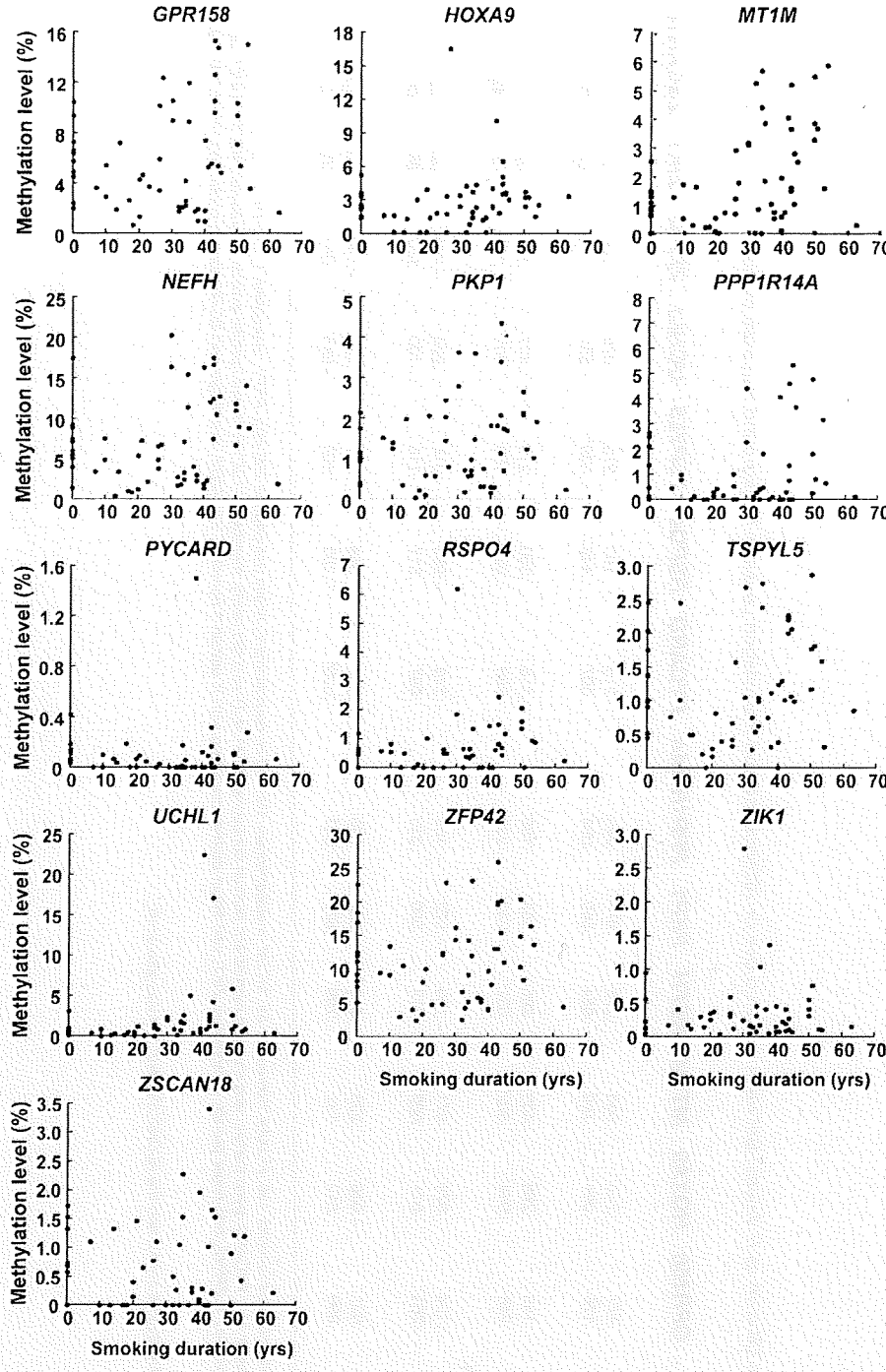

FIGURE 2. Correlations between smoking duration and methylation levels of the 13 selected genes in the background mucosae are illustrated. A positive correlation was observed for homeobox A9 (*HOXA9*) ($P = .044$), metallothionein 1M (*MT1M*) ($P = .002$), neurofilament, heavy polypeptide 200 kDa (*NEFH*) ($P = .032$), R-spondin family, member 4 (*RSPO4*) ($P = .024$), and ubiquitin carboxyl-terminal esterase L1 (*UCHL1*) ($P = .001$) methylation levels. *GPR158* indicates G protein-coupled receptor 158; *PKP1*, plakophilin 1; *PPP1R14A*, protein phosphatase 1, regulatory (inhibitor) subunit 14A; *PYCARD*, pyrin domain (PYD) and caspase recruitment domain (CARD) containing; *TSPYL5*, testis-specific protein, Y-encoded-like 5; *ZFP42*, zinc-finger protein 42 homolog; *ZIK1*, zinc-finger protein interacting with K protein 1 homolog; *ZSCAN18*, zinc-finger and SCAN domain containing 18.

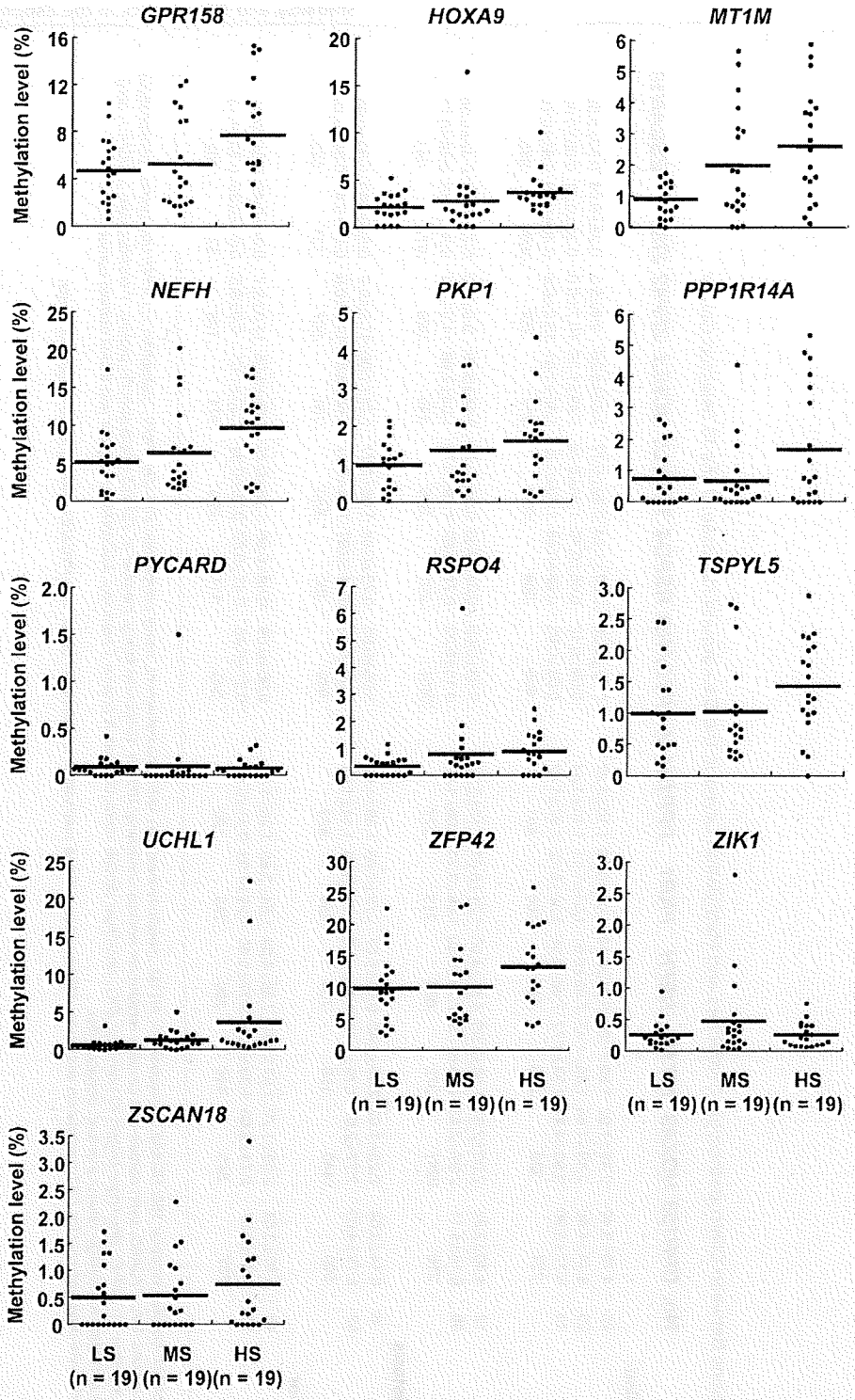

FIGURE 3. Methylation levels of the 13 selected genes in tertiles of smoking duration are shown light smokers (LS) (no or short smoking duration; <21 years); middle smokers (MS) (smoking duration <40 years but ≥21 years); and heavy smokers (HS) (smoking duration ≥40 years). Significantly increasing trends of methylation levels were observed for homeobox A9 (*HOXA9*), metallothionein 1M (*MT1M*), neurofilament heavy polypeptide 200 kDa (*NEFH*), R-spondin family member 4 (*RSPO4*), and ubiquitin carboxyl-terminal esterase L1 (*UCHL1*). *GPR158* indicates G protein-coupled receptor 158; *PKP1*, plakophilin 1; *PPP1R14A*, protein phosphatase 1, regulatory (inhibitor) subunit 14A; *PYCARD*, pyrin domain (PYD) and caspase recruitment domain (CARD) containing; *TSPYL5*, testis-specific protein, Y-encoded-like 5; *ZFP42*, zinc-finger protein 42 homolog; *ZIK1*, zinc-finger protein interacting with K protein 1 homolog; *ZSCAN18*, zinc-finger and SCAN domain containing 18.

Table 3. Associations Between Clinicopathologic Characters and Methylation Levels in the Background Mucosae of the 13 Selected Genes*

Variable	No.	*GPR158*	*HOXA9*	*MT1M*	*NEFH*	*PKP1*	*PPP1R14A-*	*PYCARD*	*RSPO4*	*TSPYL5*	*UCHL1*	*ZFP42*	*ZIK1*	*ZSCAN18*
Tumor differentiation														
Poor	25	4.79±3.42	2.45±1.92	1.80±1.99	6.21±5.16	1.06±0.89	1.10±1.87	0.12±0.30	0.62±1.24	0.99±0.77	2.22±5.36	9.8±5.5	0.28±0.54	0.53±0.58
Moderate	20	6.44±4.40	2.49±1.66	1.65±1.47	7.99±5.61	1.55±1.11	1.10±1.44	0.08±0.12	0.68±0.70	1.18±0.76	1.49±1.38	10.7±6.0	0.36±0.32	0.63±0.93
Well	13	6.72±3.90	3.63±4.24	2.34±1.72	7.46±4.56	3.32±6.72	1.07±1.42	0.03±0.03	0.62±0.58	1.39±0.82	1.45±1.51	13.2±6.4	0.40±0.25	0.65±0.75
P		.224	.328	.340	.410	.160	.441	.605	.568	.339	.262	.362	.006†	.948
Depth of tumor														
T1/T2	12	4.40±2.82	2.00±2.81	1.56±1.90	4.81±4.05	1.21±0.87	1.13±2.26	0.02±0.03	0.34±0.38	0.96±0.75	2.44±6.30	9.5±6.0	0.27±0.18	0.32±0.56
T3/T4	46	6.16±4.11	2.97±2.43	1.95±1.73	7.70±5.30	1.88±3.70	1.08±1.42	0.10±0.23	0.72±1.02	1.19±0.79	1.63±2.66	11.2±5.9	0.35±0.46	0.66±0.77
P		.216	.024†	.372	.076	.715	.277	.046†	.201	.296	.246	.328	.977	.081
Lymph node metastasis														
Negative	8	6.14±3.04	2.23±1.02	2.40±1.42	8.00±5.67	1.72±1.08	1.39±1.90	0.05±0.05	0.73±0.64	1.06±0.77	1.24±1.00	11.9±5.9	0.51±0.46	0.80±0.93
Positive	52	5.79±4.00	2.77±2.67	1.79±1.78	7.02±5.06	1.73±3.49	1.14±1.64	0.09±0.22	0.64±0.97	1.16±0.77	1.88±3.84	10.9±6.0	0.30±0.40	0.55±0.70
P		.500	.446	.158	.704	.254	.429	.946	.480	.863	.761	.557	.131	.522
Multiplicity of tumor														
Solitary	48	5.99±3.84	2.59±1.79	1.97±1.82	7.38±5.04	1.84±3.60	1.26±1.71	0.06±0.09	0.68±0.96	1.18±0.75	1.89±3.95	11.3±5.7	0.34±0.43	0.52±0.62
Multiple	10	4.86±4.37	2.74±2.65	1.38±1.35	5.77±5.85	1.22±1.33	0.31±0.44	0.21±0.46	0.45±0.82	0.98±0.94	1.33±1.61	9.0±6.9	0.31±0.33	0.95±1.13
P		.237	.845	.446	.206	.249	.126	.345	.111	.229	.571	.138	.571	.202

GPR158 indicates; G protein-coupled receptor 158; *HOXA9*, homeobox A9; *MT1M*, metallothionein 1M; *NEFH*, neurofilament, heavy polypeptide 200 kDa; *PKP1*, plakophilin 1; *PPP1R14A*, protein phosphatase 1, regulatory (inhibitor) subunit 14A; *PYCARD*, pyrin domain and caspase recruitment domain containing; *RSPO4*, R-spondin family, member 4; *TSPYL5*, testis-specific protein, Y-encoded–like 5; *UCHL1*, ubiquitin carboxyl-terminal esterase L1; *ZFP42*, zinc-finger protein 42 homolog; *ZIK1*, zinc-finger protein interacting with K protein 1 homolog; *ZSCAN18*, zinc-finger and SCAN domain containing 18.

*Methylation levels are described as average values±standard deviation (%).

†Significant associations were observed only for *ZIK1* methylation and tumor differentiation, *HOXA9* methylation and depth of tumor, and *PYCARD* methylation and depth of tumor.

With alcohol intake (n = 55), in contrast, no correlation was observed in the patients overall. When 55 patients were classified into those who had the active ALDH2 type (*ALDH2*^{1}/*ALDH2*^{1} homozygote; 10 patients) and those who had the inactive ALDH2 type (*ALDH2*^{1}/*ALDH2*^{2} heterozygote; 45 patients), a significant inverse correlation was present for *PYCARD* ($\rho = -0.334$; $P = .025$) among patients who had the inactive ALDH2 type. No positive correlation was observed with any genes in either group.

We also examined associations between methylation levels in the background mucosae and clinicopathologic characters, including tumor differentiation, depth of tumor, positive lymph node metastasis, and multiplicity of tumors (Table 3). Associations were observed only in 3 analyses, *ZIK1* methylation and tumor differentiation, *HOXA9* methylation and depth of tumor, and *PYCARD* methylation and depth of tumor, and the other 49 analyses were negative.

Relation Between Methylation in the Background Mucosae and in ESCCs

Methylation in the background mucosae reflects methylation events in numerous stem/progenitor cells, and its degree can be assessed by methylation levels. To incorporate methylation levels of multiple genes analyzed for a sample into 1 value, we calculated deviation values for the genes, and their average was used. In contrast, methylation in cancer tissue, if it occurred in cancer precursor cells, theoretically is present in all cancer cells in a sample. To assess the degree of methylation in such cancer precursor cells, we obtained the number of methylated genes in an ESCC. Eleven genes were used for the analysis, because *CLDN6* was not methylated at all in the background mucosae, and *PKP1* and *PYCARD* were not methylated in any of the ESCCs (methylation levels, ≤6%). No significant correlation between the methylation levels in the background mucosae and the methylation frequencies in the ESCCs (n = 60) was observed (r = .212; $P = .104$) (Fig. 4).

DISCUSSION

In this study, we demonstrated that duration of tobacco smoking is correlated significantly with DNA methylation levels of promoter CGIs of *HOXA9*, *MT1M*, *NEFH*, *RSPO4*, and *UCHL1* in esophageal mucosae. This strongly indicates that chronic tobacco smoking induces aberrant DNA methylation of multiple genes in esophageal mucosae and that a predisposed field for ESCCs is formed (epigenetic field defect or epigenetic field for cancerization). Ishii et al demonstrated in a qualitative analysis of 14 genes that methylation was more frequent in the background mucosae from patients with ESCC than in mucosae from healthy volunteers,[32] indicating that the presence of aberrant methylation in esophageal mucosae is associated with ESCC development. The degree of aberrant methylation in gastric mucosae is correlated with gastric cancer risk,[21,33] and the presence of aberrant methylation in noncancerous tissues also is associated with the risk of liver cancer,[34] colon cancer,[35] breast cancer,[36] and renal cancer.[37] Therefore, it is highly possible that the degree of aberrant methylation is correlated with the risk of ESCC.

The current study clearly indicated a correlation between the quantity of aberrant DNA methylation and smoking duration, although an association between methylation incidence and smoking (or alcohol intake) was not observed in a previous study.[32] This "discrepancy" most likely occurred because our quantitative analysis was able to detect differences even among methylation-positive individuals and also because we screened and selected genes with methylation levels that were correlated with tobacco smoking. Different genes have different susceptibility to methylation induction by specific methylation-inducing agents,[21,38] partly because genes with low transcription have high susceptibility to methylation induction.[39] In bronchial epithelia, an association between tobacco smoking and the methylation of some genes has been reported by qualitative studies.[17–19] A more extensive search for genes that are methylated in association with smoking duration may lead to the isolation of more marker genes.

The mechanism(s) with which tobacco smoking induces aberrant DNA methylation is important. Generally, as an inducer of aberrant DNA methylation, chronic inflammation is considered important.[39] In gastric mucosae, *Helicobacter pylori* infection induces aberrant methylation, possibly through the induction of chronic inflammation.[21] In colonic mucosae from patients with ulcerative colitis, it is known that aberrant methylation is

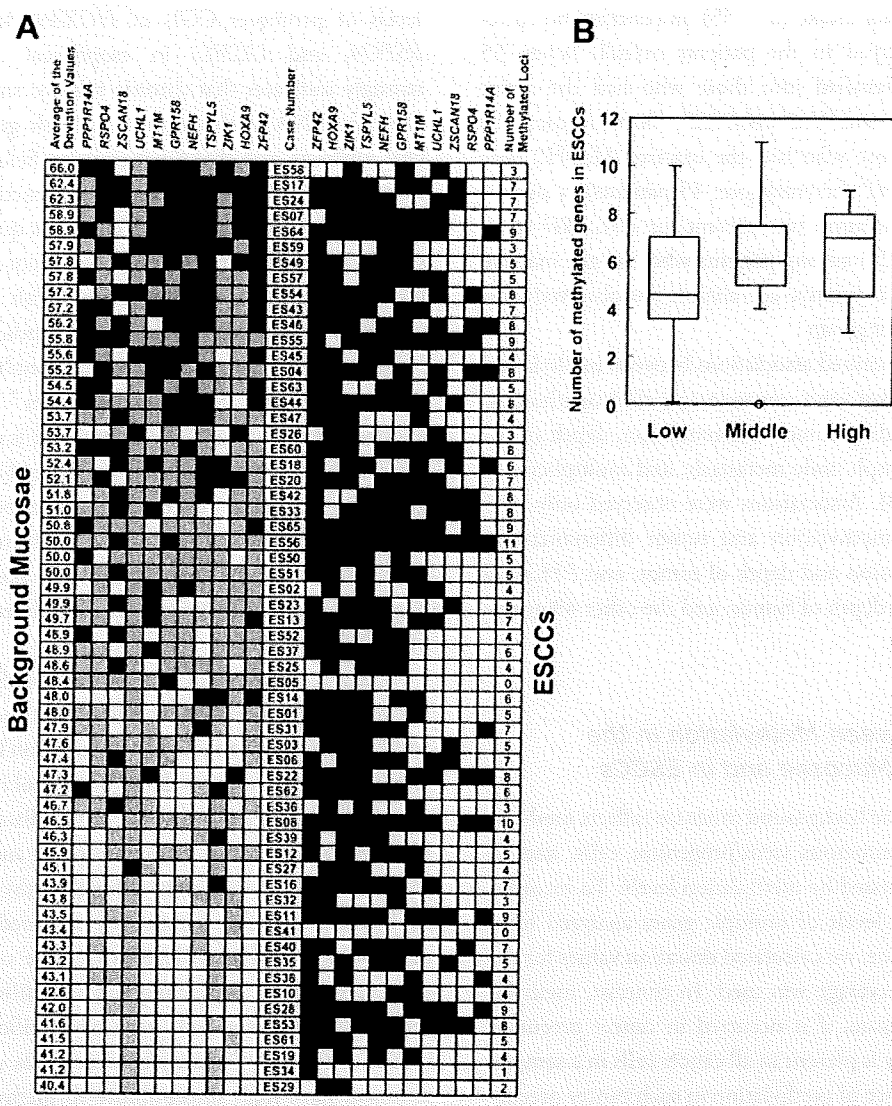

FIGURE 4. Correlations between methylation levels in the background mucosae and frequent methylation in esophageal squamous cell carcinomas (ESCCs) are illustrated. (A) Color coded representation of deviation values in the background mucosae and presence of methylation in ESCCs. The analysis was performed for 11 marker genes with methylation in the background mucosae and methylation-positive ESCCs, and the 11 genes were aligned in the order of frequency of methylation in ESCCs. The 60 samples were aligned in the order of high average of the deviation values. No trend toward a decreasing frequency of methylation in ESCCs was observed. *PPP1R14A* indicates protein phosphatase 1, regulatory (inhibitor) subunit 14A; *RSPO4*, R-spondin family, member 4; *ZSCAN18*, zinc-finger and SCAN domain containing 18; *UCHL1*, ubiquitin carboxyl-terminal esterase L1; *MT1M*, metallothionein 1M; *GPR158*, G protein-coupled receptor 158; *NEFH*, neurofilament, heavy polypeptide 200 kDa; *TSPYL5*, testis-specific protein, Y-encoded-like 5; *ZIK1*, zinc-finger protein interacting with K protein 1 homolog; *ZFP42*, zinc-finger protein 42 homolog; *HOXA9*, homeobox A9. (B) Box graph representation of the number of methylated genes in tertiles of the methylation levels in the background mucosae. The tertiles were obtained by the average of the deviation values in A. No difference in the number of methylated genes was observed.

present.[40] There is little information whether or not tobacco smoking induces chronic inflammation in esophageal mucosae. In bronchial epithelia, it is known that tobacco smoking induces chronic inflammation,[41] and tobacco ingredients that were swallowed induced similar inflammation in esophageal mucosae. It was reported recently that bladder cancers in smokers tended to have methylation of the runt-related transcription factor 3 gene *RUNX3*,[42] and systemic effects of tobacco ingredients also may be possible.

Alcohol drinking, in contrast with smoking, did not induce methylation. Rather, *PYCARD* methylation levels in the background esophageal mucosae decreased significantly with alcohol consumption in patients with who had the inactive ALDH2 genotype. It has been reported that chronic alcohol consumption induces global hypomethylation in rats,[43,44] and it has been suggested that alcohol has an epigenetic action different from tobacco smoking, although both are risk factors for ESCC. In addition, it is suggested that the methylation profile associated with tobacco smoking is different from that associated with alcohol drinking. Once a methylation profile specific to tobacco smoking or alcohol drinking is established, it can be used as a methylation fingerprint to assess past exposure to these factors for clinicopathologic analysis and epidemiology. The use of DNA methylation profiles as fingerprints of exposure to carcinogenic factors is expected to become an important field.[45,46]

To assess the extent of tobacco smoking and alcohol drinking, we used duration (not the amount of intake) and mean intake (not duration), respectively. This was because previous reports suggested that ESCC risk depends mainly on smoking duration rather than mean tobacco intake and on mean alcohol intake rather than drinking duration.[3,5]

Methylation levels in cancers were useful for estimating the roles of genes in ESCC development. *PKP1* and *PYCARD* methylation levels in ESCCs were <6%, and it was unlikely that their methylation took place before monoclonal growth of cancer cells. Similarly, *CDH1* methylation levels in ESCC were <7.1% in all ESCCs, suggesting that *CDH1* methylation was unlikely to be involved in early stages of ESCC development. This finding was in accordance with a previous report that *CDH1* methylation was involved in metastatic progression.[47] The *CDKN2A* and *MLH1* tumor-suppressor genes were not methylated in ESCCs or in their background mucosae. In contrast, the *RASSF1A* tumor-suppressor gene had methylation levels >30% in 2 ESCCs and zero in most ESCCs. This suggested that *RASSF1A* silencing may be involved in the early stages of ESCC development, but the incidence was low. Aberrant methylation of *CDH1*, *CDKN2A*, and *RASSF1A* in ESCCs was reported in 14 of 20 ESCCs, 17 of 34 ESCCs, and 25 of 48 ESCCs, respectively, by qualitative MSP.[27–29] It is known that qualitative MSP tends to overestimate methylation frequencies,[48] and the incidences reported here were considered reasonable. Some genes, such as *NEFH* and *ZFP42* had methylation levels of almost 100% in some ESCCs. Because cancer tissues contained not only cancer cells but also stromal cells, these genes should have been methylated in both cancer cells and stromal cells. Recent studies demonstrated that cancer stromal cells have distinct epigenetic changes,[49] and some of these genes may be involved in such changes.

There was no correlation between methylation levels in the background mucosae and methylation frequencies in ESCCs, as in our previous study on gastric cancers and their background mucosae.[30] This suggests that methylation levels in the background mucosae do not necessarily reflect methylation levels in cancer precursor cells. In conclusion, we have demonstrated that chronic tobacco smoking is associated with the accumulation of aberrant methylation of multiple genes in esophageal mucosae.

Conflict of Interest Disclosures

Supported by Grants-in-aid for the Third-term Comprehensive Cancer Control Strategy and for Pioneering Basic Research from the Ministry of Health, Labor and Welfare, Japan.

Dr. Oka is the recipient of a Research Resident Fellowships from the Foundation for Promotion of Cancer Research.

References

1. Enzinger PC, Mayer RJ. Esophageal cancer. *N Engl J Med.* 2003;349:2241-2252.
2. Vizcaino AP, Moreno V, Lambert R, Parkin DM. Time trends incidence of both major histologic types of esophageal carcinomas in selected countries, 1973-1995. *Int J Cancer.* 2002;99:860-868.
3. Castellsague X, Munoz N, De Stefani E, et al. Independent and joint effects of tobacco smoking and alcohol drinking on the risk of esophageal cancer in men and women. *Int J Cancer.* 1999;82:657-664.
4. Lee CH, Wu DC, Lee JM, et al. Carcinogenetic impact of alcohol intake on squamous cell carcinoma risk of the oesophagus in relation to tobacco smoking. *Eur J Cancer.* 2007; 43:1188-1199.
5. Launoy G, Milan CH, Faivre J, Pienkowski P, Milan CI, Gignoux M. Alcohol, tobacco and oesophageal cancer: effects of the duration of consumption, mean intake and current and former consumption. *Br J Cancer.* 1997;75: 1389-1396.

6. Kuwano H, Ohno S, Matsuda H, Mori M, Sugimachi K. Serial histologic evaluation of multiple primary squamous cell carcinomas of the esophagus. *Cancer.* 1988;61:1635-1638.
7. Kanamoto A, Yamaguchi H, Nakanishi Y, Tachimori Y, Kato H, Watanabe H. Clinicopathological study of multiple superficial oesophageal carcinoma. *Br J Surg.* 2000;87:1712-1715.
8. Pesko P, Rakic S, Milicevic M, Bulajic P, Gerzic Z. Prevalence and clinicopathologic features of multiple squamous cell carcinoma of the esophagus. *Cancer.* 1994;73:2687-2690.
9. Saeki H, Kimura Y, Ito S, Miyazaki M, Ohga T. Biologic and clinical significance of squamous epithelial dysplasia of the esophagus. *Surgery.* 2002;131(1 suppl):S22-S27.
10. Nakanishi Y, Ochiai A, Yoshimura K, et al. The clinicopathologic significance of small areas unstained by Lugol's iodine in the mucosa surrounding resected esophageal carcinoma: an analysis of 147 cases. *Cancer.* 1998;82:1454-1459.
11. Miyazaki M, Ohno S, Futatsugi M, Saeki H, Ohga T, Watanabe M. The relation of alcohol consumption and cigarette smoking to the multiple occurrence of esophageal dysplasia and squamous cell carcinoma. *Surgery.* 2002; 131(1 suppl):S7-S13.
12. Slaughter DP, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer.* 1953;6:963-968.
13. Jones PA, Baylin SB. The epigenomics of cancer. *Cell.* 2007;128:683-692.
14. Sato F, Meltzer SJ. CpG island hypermethylation in progression of esophageal and gastric cancer. *Cancer.* 2006; 106:483-493.
15. Mandelker DL, Yamashita K, Tokumaru Y, et al. PGP9.5 promoter methylation is an independent prognostic factor for esophageal squamous cell carcinoma. *Cancer Res.* 2005;65:4963-4968.
16. Soma T, Kaganoi J, Kawabe A, Kondo K, Imamura M, Shimada Y. Nicotine induces the fragile histidine triad methylation in human esophageal squamous epithelial cells. *Int J Cancer.* 2006;119:1023-1027.
17. Belinsky SA, Palmisano WA, Gilliland FD, et al. Aberrant promoter methylation in bronchial epithelium and sputum from current and former smokers. *Cancer Res.* 2002; 62:2370-2377.
18. Soria JC, Rodriguez M, Liu DD, Lee JJ, Hong WK, Mao L. Aberrant promoter methylation of multiple genes in bronchial brush samples from former cigarette smokers. *Cancer Res.* 2002;62:351-355.
19. Zochbauer-Muller S, Lam S, Toyooka S, et al. Aberrant methylation of multiple genes in the upper aerodigestive tract epithelium of heavy smokers. *Int J Cancer.* 2003;107:612-616.
20. Chang HW, Ling GS, Wei WI, Yuen AP. Smoking and drinking can induce p15 methylation in the upper aerodigestive tract of healthy individuals and patients with head and neck squamous cell carcinoma. *Cancer.* 2004;101:125-132.
21. Maekita T, Nakazawa K, Mihara M, et al. High levels of aberrant DNA methylation in *Helicobacter pylori*-infected gastric mucosae and its possible association with gastric cancer risk. *Clin Cancer Res.* 2006;12:989-995.
22. Shimada Y, Imamura M, Wagata T, Yamaguchi N, Tobe T. Characterization of 21 newly established esophageal cancer cell lines. *Cancer.* 1992;69:277-284.
23. Moriguchi K, Yamashita S, Tsujino Y, Tatematsu M, Ushijima T. Larger numbers of silenced genes in cancer cell lines with increased de novo methylation of scattered CpG sites. *Cancer Lett.* 2007;249:178-187.
24. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc Natl Acad Sci U S A.* 2002;99:3740-3745.
25. Sugimachi K, Ohno S, Matsuda H, Mori M, Kuwano H. Lugol-combined endoscopic detection of minute malignant lesions of the thoracic esophagus. *Ann Surg.* 1988;208:179-183.
26. Takeshita T, Morimoto K, Mao X, Hashimoto T, Furuyama J. Characterization of the 3 genotypes of low Km aldehyde dehydrogenase in a Japanese population. *Hum Genet.* 1994;94:217-223.
27. Xing EP, Nie Y, Wang LD, Yang GY, Yang CS. Aberrant methylation of p16INK4a and deletion of p15INK4b are frequent events in human esophageal cancer in Linxian, China. *Carcinogenesis.* 1999;20:77-84.
28. Si HX, Tsao SW, Lam KY, Srivastava G, Liu Y, Wong YC, et al. E-cadherin expression is commonly downregulated by CpG island hypermethylation in esophageal carcinoma cells. *Cancer Lett.* 2001;173:71-78.
29. Kuroki T, Trapasso F, Yendamuri S, et al. Promoter hypermethylation of RASSF1A in esophageal squamous cell carcinoma. *Clin Cancer Res.* 2003;9:1441-1445.
30. Enomoto S, Maekita T, Tsukamoto T, et al. Lack of association between CpG island methylator phenotype in human gastric cancers and methylation in their background non-cancerous gastric mucosae. *Cancer Sci.* 2007;98:1853-1861.
31. Ogino S, Cantor M, Kawasaki T, et al. CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. *Gut.* 2006;55:1000-1006.
32. Ishii T, Murakami J, Notohara K, et al. Oesophageal squamous cell carcinoma may develop within a background of accumulating DNA methylation in normal and dysplastic mucosa. *Gut.* 2007;56:13-19.
33. Nakajima T, Maekita T, Oda I, et al. Higher methylation levels in gastric mucosae significantly correlate with higher risk of gastric cancers. *Cancer Epidemiol Biomarkers Prev.* 2006;15:2317-2321.
34. Kondo Y, Kanai Y, Sakamoto M, Mizokami M, Ueda R, Hirohashi S. Genetic instability and aberrant DNA