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

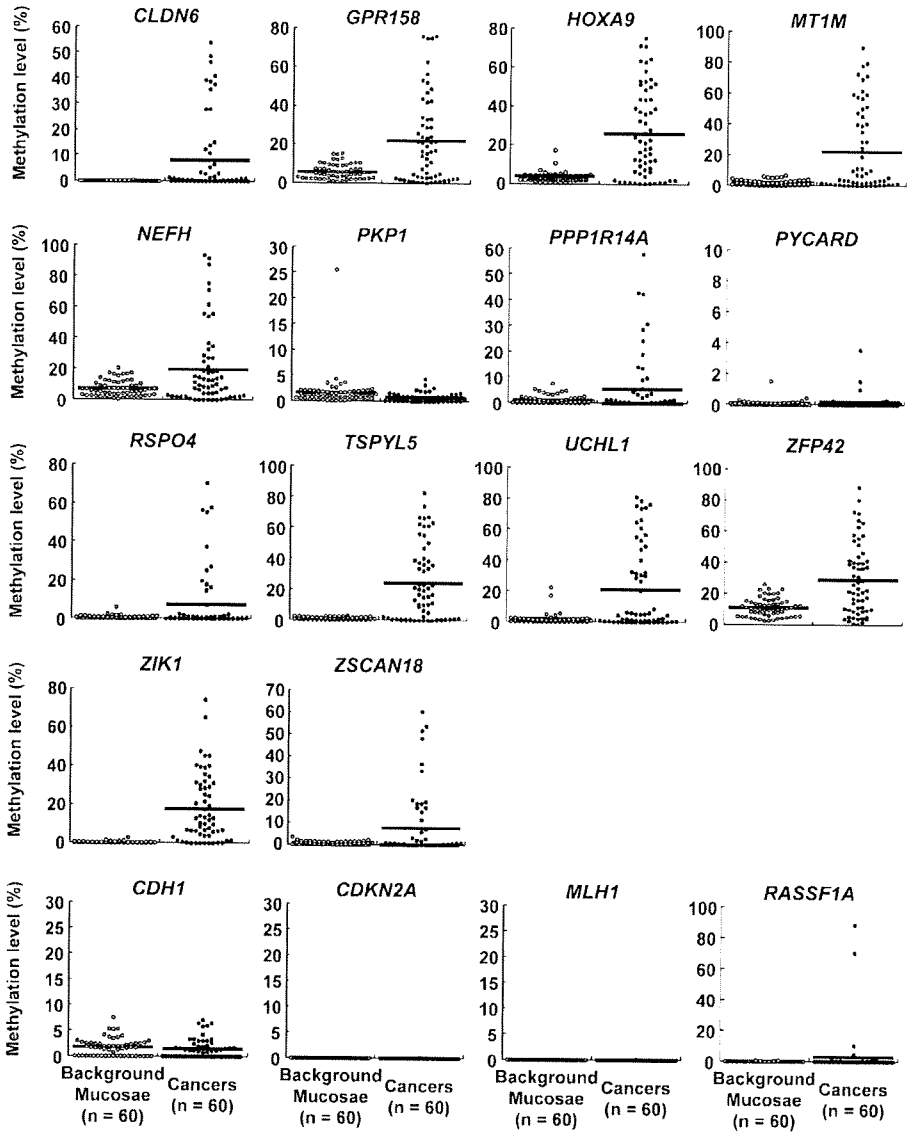

FIGURE 1. Methylation levels of the 14 selected genes and 4 tumor suppressor genes in esophageal squamous cell carcinomas (ESCCs) and their background mucosae are shown. Distinct distributions of methylation levels were observed in the background mucosae and in ESCCs. Mean methylation levels of individual 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).

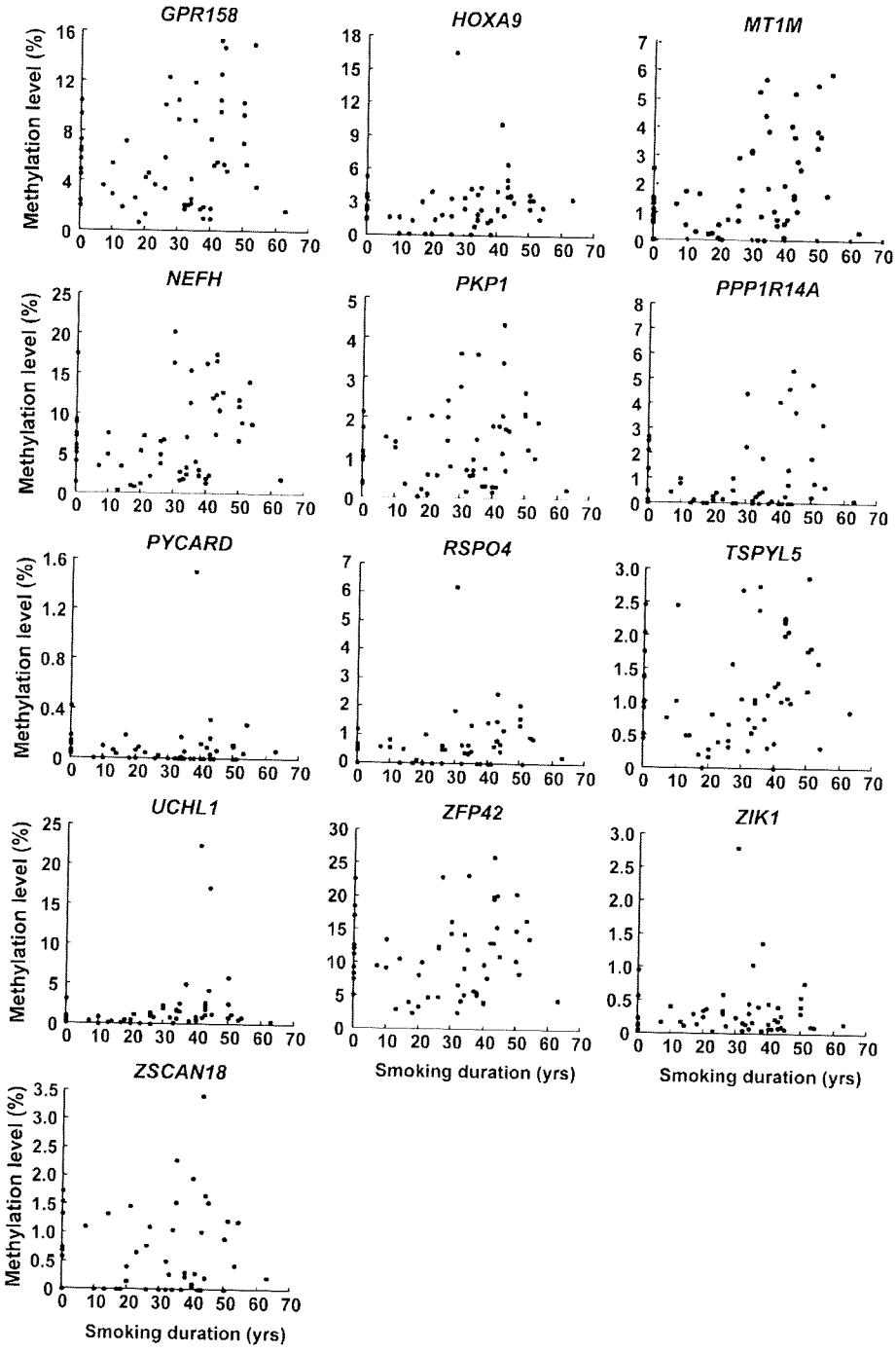

FIGURE 2. Correlations between smoking duration and methylation levels of the 13 selected genes in the background mucosae are illustrated. A positive correlation was observed for homeobox A9 (*HOXA9*) ($P = .044$), metallothionein 1M (*MT1M*) ($P = .002$), neurofilament, heavy polypeptide 200 kDa (*NEFH*) ($P = .032$), 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.

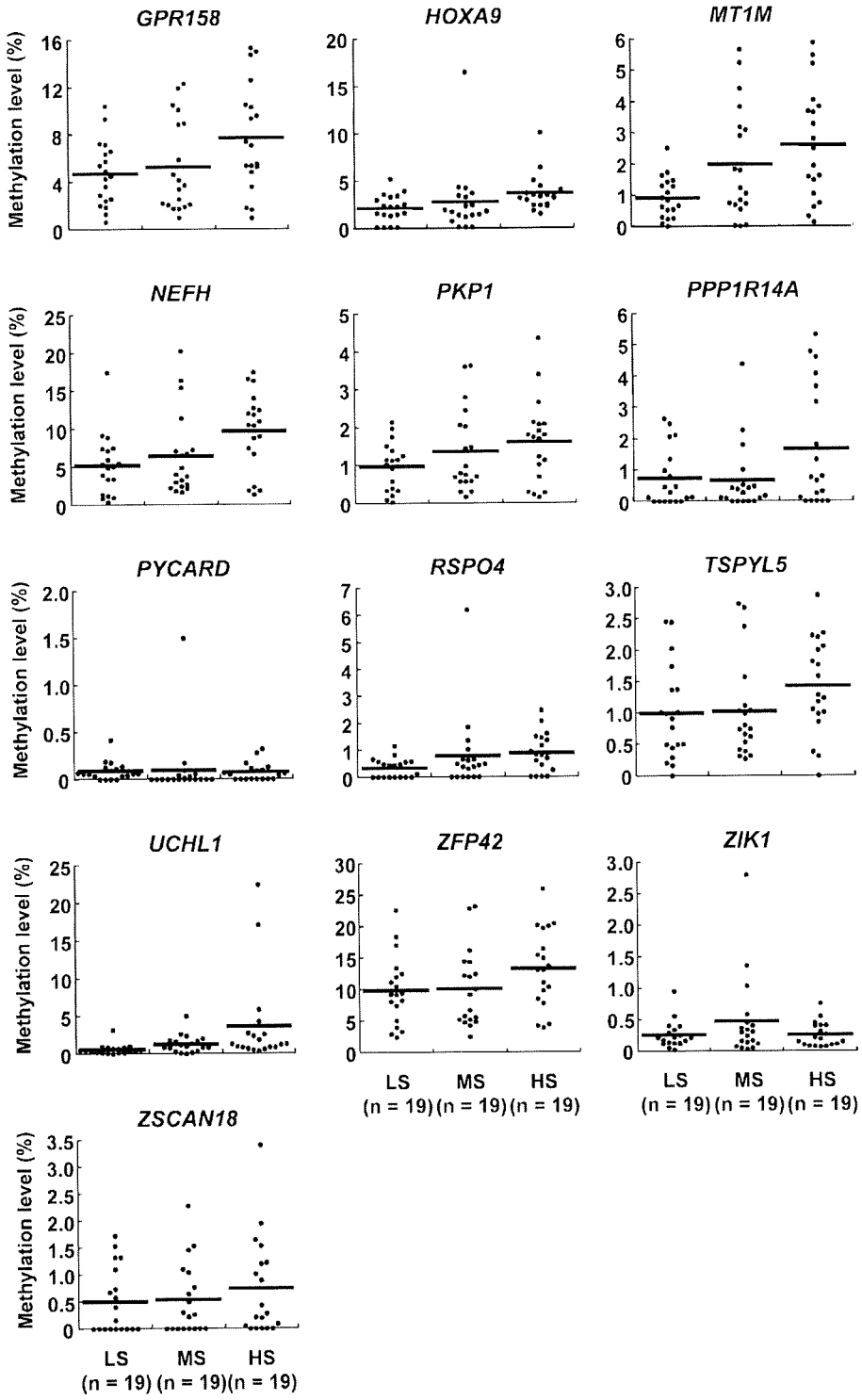

FIGURE 3. Methylation levels of the 13 selected genes in tertiles of smoking duration are shown light smokers (LS) (no or short smoking duration; <21 years); middle smokers (MS) (smoking duration <40 years but ≥21 years); and heavy smokers (HS) (smoking duration ≥40 years). Significantly increasing trends of methylation levels were observed for homeobox A9 (*HOXA9*), metallothionein 1M (*MTIM*), 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, $\leq$6%). No significant correlation between the methylation levels in the background mucosae and the methylation frequencies in the ESCCs (n = 60) was observed ($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

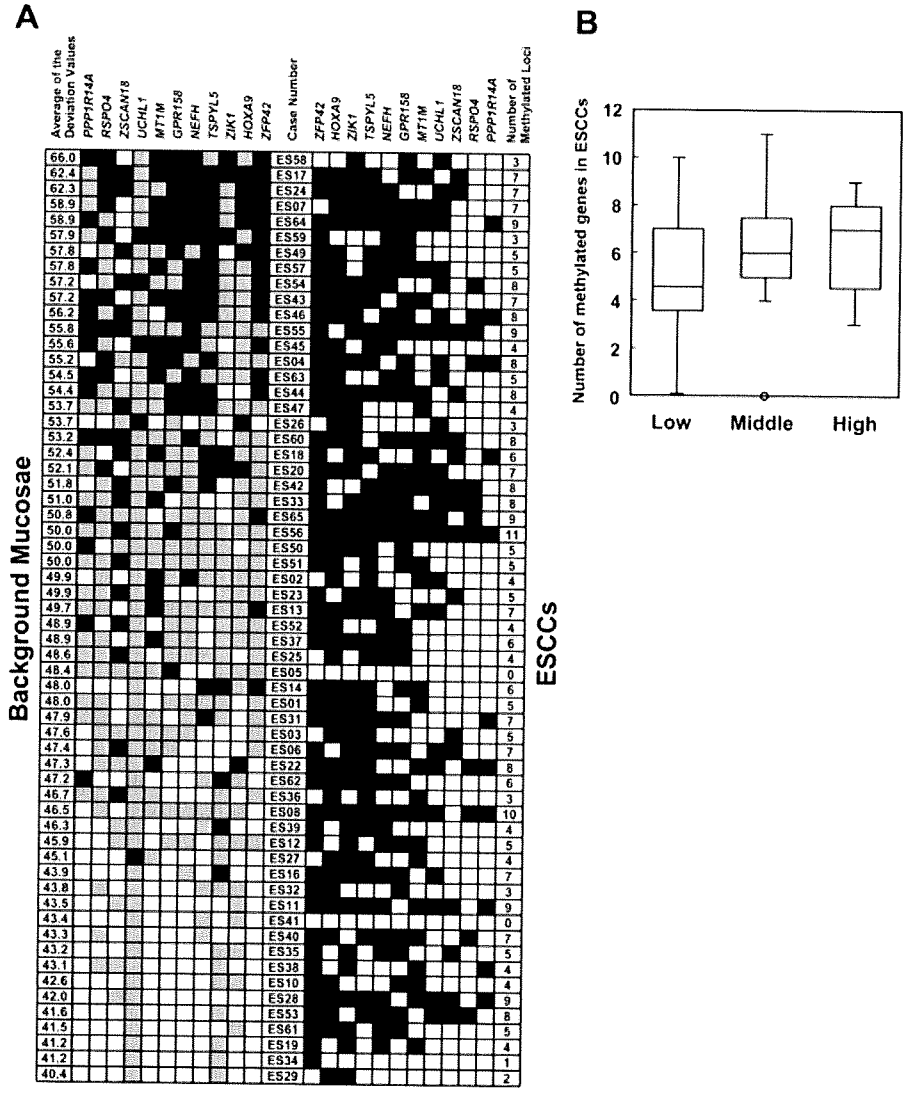

FIGURE 4. Correlations between methylation levels in the background mucosae and frequent methylation in esophageal squamous cell carcinomas (ESCCs) are illustrated. (A) Color coded representation of deviation values in the background mucosae and presence of methylation in ESCCs. The analysis was performed 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

methylation in chronic hepatitis and cirrhosis—A comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. *Hepatology.* 2000;32:970-979.

35. Shen L, Kondo Y, Rosner GL, et al. MGMT promoter methylation and field defect in sporadic colorectal cancer. *J Natl Cancer Inst.* 2005;97:1330-1338.
36. Yan PS, Venkataramu C, Ibrahim A, et al. Mapping geographic zones of cancer risk with epigenetic biomarkers in normal breast tissue. *Clin Cancer Res.* 2006;12:6626-6636.
37. Arai E, Kanai Y, Ushijima S, Fujimoto H, Mukai K, Hirohashi S. Regional DNA hypermethylation and DNA methyltransferase (DNMT) 1 protein overexpression in both renal tumors and corresponding nontumorous renal tissues. *Int J Cancer.* 2006;119:288-296.
38. Nakajima T, Yamashita S, Maekita T, Niwa T, Nakazawa K, Ushijima T. The presence of a methylation fingerprint of *Helicobacter pylori* infection in human gastric mucosae. *Int J Cancer.* 2009;124:905-910.
39. Ushijima T, Okochi-Takada E. Aberrant methylations in cancer cells: where do they come from? *Cancer Sci.* 2005;96:206-211.
40. Issa JP, Ahuja N, Toyota M, Bronner MP, Brentnall TA. Accelerated age-related CpG island methylation in ulcerative colitis. *Cancer Res.* 2001;61:3573-3577.
41. van der Vaart H, Postma DS, Timens W, ten Hacken NH. Acute effects of cigarette smoke on inflammation and oxidative stress: a review. *Thorax.* 2004;59:713-721.
42. Wolff EM, Liang G, Cortez CC, et al. RUNX3 methylation reveals that bladder tumors are older in patients with a history of smoking. *Cancer Res.* 2008;68:6208-6214.
43. Garro AJ, McBeth DL, Lima V, Lieber CS. Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. *Alcohol Clin Exp Res.* 1991;15:395-398.
44. Choi SW, Stickel F, Baik HW, Kim YI, Seitz HK, Mason JB. Chronic alcohol consumption induces genomic but not p53-specific DNA hypomethylation in rat colon. *J Nutr.* 1999;129:1945-1950.
45. Herceg Z. Epigenetics and cancer: towards an evaluation of the impact of environmental and dietary factors. *Mutagenesis.* 2007;22:91-103.
46. Ushijima T, Nakajima T, Maekita T. DNA methylation as a marker for the past and future. *J Gastroenterol.* 2006; 41:401-407.
47. Graff JR, Gabrielson E, Fujii H, Baylin SB, Herman JG. Methylation patterns of the E-cadherin 5′ CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. *J Biol Chem.* 2000;275:2727-2732.
48. Shames DS, Minna JD, Gazdar AF. Methods for detecting DNA methylation in tumors: from bench to bedside. *Cancer Lett.* 2007;251:187-198.
49. Hu M, Yao J, Cai L, et al. Distinct epigenetic changes in the stromal cells of breast cancers. *Nat Genet.* 2005;37: 899-905.

Carcinogenesis vol.30 no.3 pp.466–471, 2009
doi:10.1093/carcin/bgp021
Advance Access publication January 23, 2009

Association between frequent CpG island methylation and *HER2* amplification in human breast cancers

Kotoe Terada[1,2], Eriko Okochi-Takada[1], Sadako Akashi-Tanaka[3], Kazuaki Miyamoto[4], Kiyomi Taniyama[4], Hitoshi Tsuda[5], Kiyoshi Asada[1], Michio Kaminishi[2] and Toshikazu Ushijima[1,*]

[1]Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan, [2]Department of Surgical Metabolic Care and Endocrine Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo 113-8655, Japan, [3]Department of Surgery, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan, [4]Institute for Clinical Research, National Hospital Organization Kure Medical Center/ Chugoku Cancer Center, Hiroshima 737-0023, Japan and [5]Pathology Laboratory, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

*To whom correspondence should be addressed. Tel: +81 3 3547 5240; Fax: +81 3 5565 1753;
Email: tushijim@ncc.go.jp

The presence of frequent methylation of CpG islands (CGIs), designated as the CpG island methylator phenotype in some cancers, is associated with distinct clinicopathological characteristics, including gene amplification, in individual tumor types. Amplification of *HER2* in human breast cancers is an important prognostic and therapeutic target, but an association between *HER2* amplification and frequent CGI methylation is unknown. To clarify the association, we here quantified methylation levels of promoter CGIs of 11 genes, which are unlikely to confer growth advantage to cells, in 63 human breast cancers. The number of methylated genes in a cancer did not obey a bimodal distribution, and the 63 cancers were classified into those with frequent methylation (n = 16), moderate methylation (n = 26) and no methylation (n = 21). The incidence of *HER2* amplification was significantly higher in the cancers with frequent methylation (11 of 16) than in those with no methylation (2 of 21, P = 0.001). Also, the number of methylated genes correlated with the degree of *HER2* amplification (r = 0.411, P = 0.002). Correlation analysis with clinicopathological characteristics and methylation of *CDKN2A*, *BRCA1* and *CDH1* revealed that frequent methylation had significant correlation with higher nuclear grades (P = 0.001). These showed that frequent methylation had a strong association with *HER2* amplification in breast cancers and suggested that frequent methylation can be a determinant of various characteristics in a fraction of human breast cancers.

Introduction

Aberrant DNA methylation is deeply involved in the development and progression of human cancers (1–4). Methylation of CpG islands (CGIs) in promoter regions is a major mechanism for inactivation of tumor suppressor genes. At the same time, maintenance of appropriate DNA methylation levels is known to be important for maintenance of genome integrity. DNA hypomethylation can lead to genomic instability and increased tumor incidence in mice (5,6) and is associated with loss of heterozygosity in human cancers (7,8). On the other hand, aberrant DNA methylation precedes loss of heterozygosity in human liver cancers (9).

The presence of frequent methylation of CGIs in a cancer was first described in colorectal cancers and designated as the CpG island methylator phenotype (CIMP) (10). Depending upon tumor tissue types, the presence of frequent CGIs methylation, or CIMP, can be clearly observed and is associated with distinct clinicopathological features. For example, by careful selection of marker genes and their quantitative methylation analysis, CIMP in colorectal cancers was shown to be strongly associated with *BRAF* mutations (11). In neuroblastomas, both in Japan and Germany, CIMP was observed as a distinct entity associated with poor prognosis and *MYCN* amplification (12,13). Remarkably, all the cases with *MYCN* amplification had frequent methylation, with only one exception. Cases with CIMP but without *MYCN* amplification had a better prognosis than those that had both and a worse prognosis than those that had neither. This complete containment of tumors with *MYCN* amplification within CIMP-positive tumors suggested that CIMP could precede gene amplification or that at least the presence of frequent aberrant DNA methylation was associated with gene amplification.

Gene amplification of *HER2*, which is a member of the epidermal growth factor receptor family (14), is very important in human breast cancers. Initially, *HER2* amplification was found to be present in 15–30% of newly diagnosed breast cancer cases and to be associated with increased metastatic potential and decreased overall survival (15). Suppression of *HER2* activity was shown to have antitumor activity, and antibodies against HER2 were developed as a therapeutic agent against breast cancers. Now, it is well known that a humanized antibody against HER2, such as trastuzumab, is very effective against breast cancers with *HER2* amplification (16,17). Nevertheless, inducers of *HER2* amplification remain unknown.

In this study, we aimed to clarify whether or not the presence of frequent CGI methylation was associated with *HER2* amplification in human breast cancers. For this end, from the genes silenced in human cancers (18,19), we selected genes whose silencing is unlikely to confer growth advantage and avoided selection bias of cells with methylation. Also, we performed quantitative methylation analysis of their putative nucleosome-depleted regions (20), which are most resistant to DNA methylation (21). Association between frequent CGI methylation and clinicopathological characteristics, including silencing of three tumor-suppressor genes (*CDKN2A*, *BRCA1* and *CDH1*), was also analyzed.

Materials and methods

Patients and tissue samples

Sixty-three breast cancer tissue specimens were obtained from patients who underwent mastectomy or breast-conserving surgery (stage I 22 cases; stage II 26 cases; stage III 15 cases and stage IV 0 case). Informed consent was obtained from all the patients, and analysis was approved by the institutional review boards. Cancer tissues were frozen after resection and stored at −80°C until extraction of genomic DNA. High-molecular weight DNA was extracted by the phenol–chloroform method. Histological types were evaluated according to the criteria of the Japanese Breast Cancer Society (22).

Bisulfite modification and quantitative methylation-specific polymerase chain reaction

Completely methylated DNA and completely unmethylated DNA were prepared by methylating genomic DNA with *Sss*I methylase (New England Biolabs, Beverly, MA) and amplifying genomic DNA with the GenomiPhi amplification system (GE Healthcare, Buckinghamshire, UK), respectively. Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as described previously (23). The modified DNA was suspended in 40 μl of Tris-EDTA buffer, and an aliquot of 1 μl was used for polymerase chain reaction (PCR) with a primer set specific to methylated or unmethylated sequences (supplementary Table 1 is available at *Carcinogenesis* Online). Using the completely methylated DNA and completely unmethylated DNA, an annealing temperature specific for each primer set was determined. Real-time PCR was performed using SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The number of DNA molecules with methylated sequences and

Abbreviations: CGI, CpG island; CIMP, CpG island methylator phenotype; ESR, estrogen receptor; PCR, polymerase chain reaction; PGR, progesterone receptor.

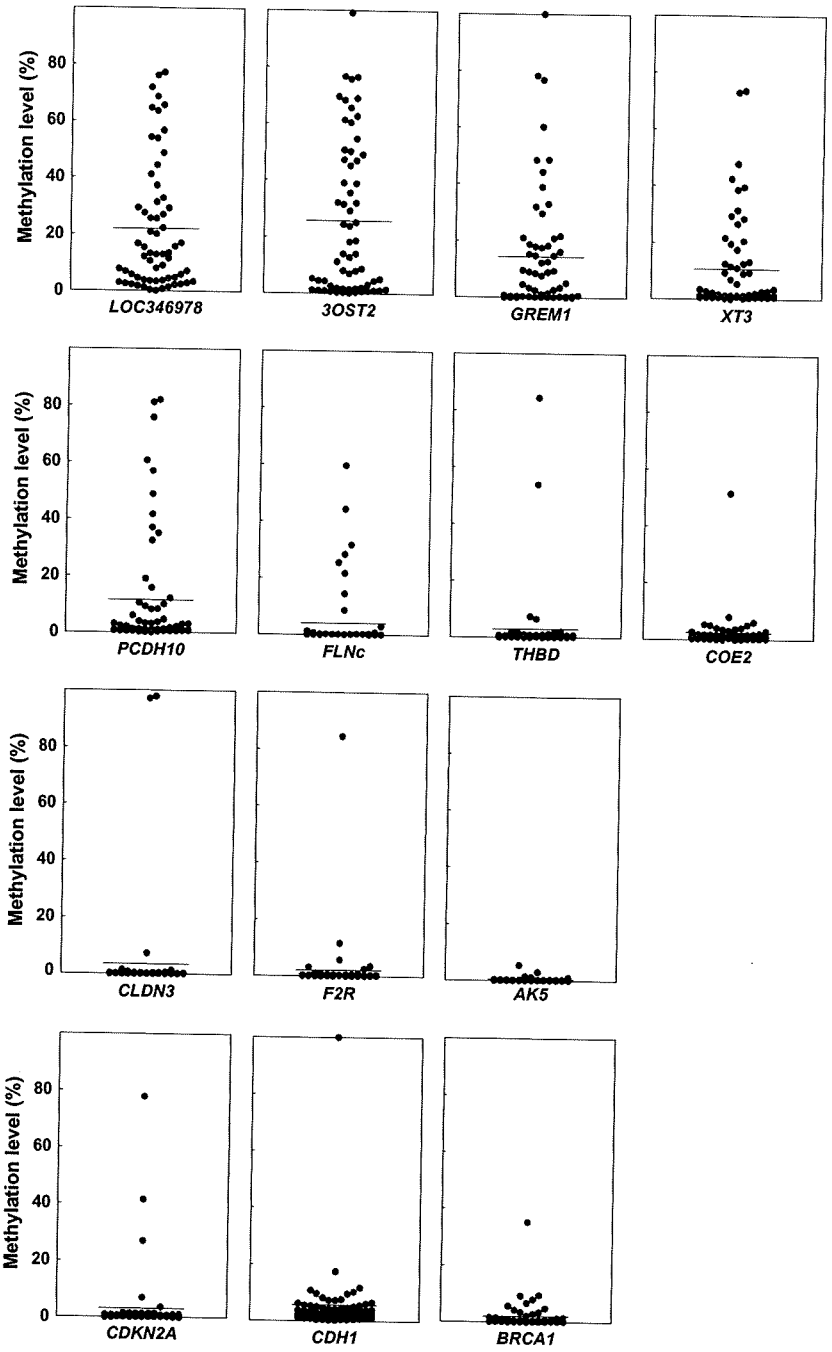

Fig. 1. Methylation levels in the 63 breast cancer samples. The numbers of DNA molecules methylated and unmethylated in promoter CGIs were obtained by quantitative methylation-specific PCR, and a methylation level was calculated as a fraction of methylated DNA molecules among the total DNA molecules. Some cancers had no methylation and the others had various levels of methylation. The methylation level in cancers was considered to represent the fraction of cancer cells in a sample and less occasionally the fraction of cells with methylation among cancer cells. We confirmed that we detected dense methylation of promoter CGIs by sequencing the quantitative methylation-specific PCR products obtained using primers specific to methylated DNA molecules (suppplementary Figure 3 is available at *Carcinogenesis* Online).

that with unmethylated sequences in a test sample were measured by comparing its amplification with those of standard samples that contained 10–10^6 DNA molecules. The standard samples were prepared by cloning PCR products of methylated and unmethylated sequences into the pGEM-T Easy vector (Promega, Madison, WI) or by purifying their PCR products using the Wizard SV Gel and PCR clean-up system (Promega). The 'methylation level' was calculated as the fraction of methylated DNA molecules among the total DNA molecules.

Fluorescence in situ hybridization analysis of the HER2 amplification

Fluorescence *in situ* hybridization was performed using a PathVysion kit (Abbot Molecular, Des Plaines, IL) with our modification (24). The *HER2* locus

and centromere of chromosome 17 (CEP17) were labeled by SpectrumOrange and SpectrumGreen fluorescence, respectively, and nuclei were counterstained with 4′, 6-diamidino-2-phenylindole. *HER2* and CEP17 signals were counted in 60 nuclei under a fluorescence microscope. Cancers with *HER2*:CEP17 ratio ≥ 2 were determined as *HER2* amplification positive.

Analysis of 3OST2 expression on cell growth

MCF7 Tet-Off cell line was purchased from Clontech Laboratories (Mountain View, CA). Full-length *3OST2* complementary DNA, cloned from human mammary epithelial cells, was inserted into the multiple cloning site of pTRE2hyg vector (Clontech Laboratories). The MCF7 Tet-Off cell line was transfected with the vector, and a stable clone was obtained by selection using hygromycin. Growth curves were analyzed by counting the cell numbers for the parental cell line, stable clones transfected with *3OST2*-expressing vector and with empty vector (without doxycycline). Overexpression of *3OST2* complementary DNA was confirmed by real-time reverse transcription–PCR analysis.

Sequencing analysis of quantitative methylation-specific PCR products

Quantitative methylation-specific PCR products of seven genes, *3OST2*, *FLNc*, *GREM1*, *THBD*, *PCDH10*, *XT3* and *LOC346978*, were cloned into pGEM-T Easy Vector (Promega). For each sample, ~10 clones were cycle sequenced using T7 primer, 5′-TAATACGACTCACTATAGGG-3′ and an Applied Biosystems 310 sequencer (Applied Biosystems, Foster City, CA).

Statistical analysis

Increasing or decreasing trends in no methylation, moderate methylation and then frequent methylation cancers were analyzed by the Mantel–Haenszel chi-square test. Differences between the frequent methylation *HER2*-positive cancers and moderate methylation (or no methylation) *HER2*-positive cancers were analyzed by the chi-square test. Correlation between the degree of *HER2* amplification and the number of methylated genes was analyzed using Pearson's correlation coefficient. All the analyses were performed using SPSS (SPSS, Chicago, IL).

Results

Quantitative methylation analysis of breast cancers

From 20 and 14 genes that were methylated in human breast and gastric cancers, respectively (18,19), we selected 11 genes (*LOC346978*, *3OST2*, *GREM1*, *XT3*, *PCDH10*, *FLNc*, *THBD*, *COE2*, *CLDN3*, *F2R* and *AK5*) and quantified their methylation levels in 63 breast cancers. These genes, except for *3OST2* and *CLDN3*, were not expressed in normal human mammary epithelial cells (18,19,25,26), and their silencing was unlikely to confer growth advantage to cells. Also, introduction of *3OST2* complementary DNA into MCF7 cells did not cause growth suppression (supplementary Figure 1 is available at *Carcinogenesis* Online), and its silencing was unlikely to confer growth advantage. Therefore, the majority of the 11 genes were considered to be suitable to detect the presence of a cellular environment that tends to induce methylation of promoter CGIs. We also analyzed methylation of three tumor suppressor genes (*CDKN2A*, *BRCA1* and *CDH1*) for clinicopathological analysis.

Quantitative methylation analyses of the 14 genes showed that some cancers had no methylation and the others had various levels

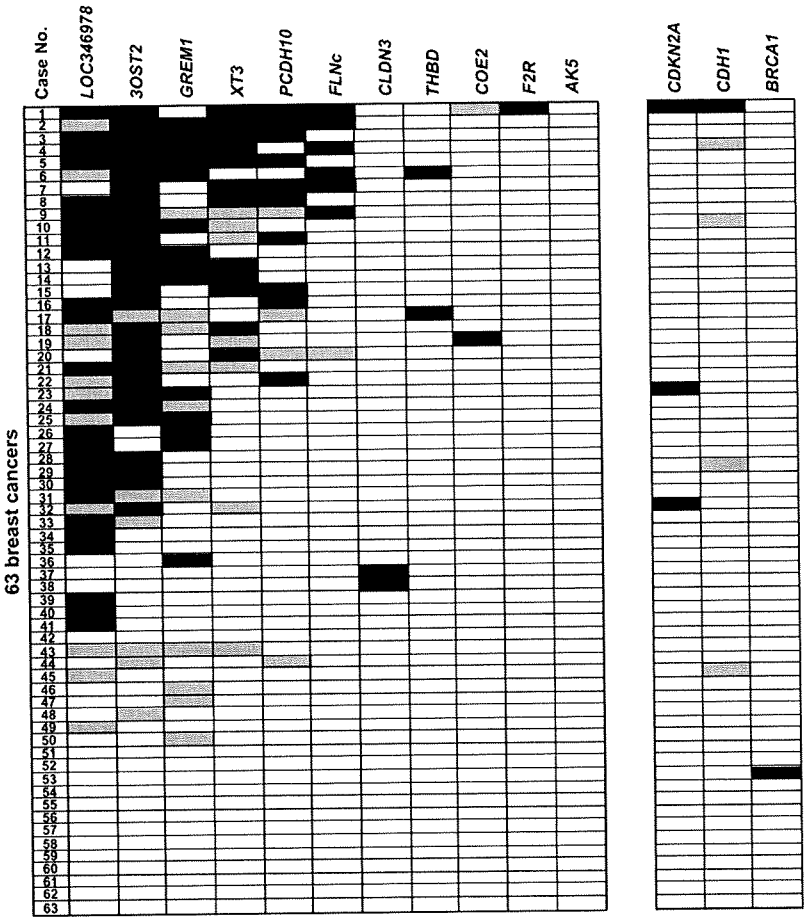

Fig. 2. Methylation profile of the 11 marker genes and three tumor suppressor genes in 63 breast cancers. Methylation in each sample was scored as positive or negative using two different cutoff values, and the 63 samples were aligned by the number of methylated CGIs. Methylation-positive samples using 10 and 20% as cutoff values are shown by gray and black boxes, respectively.

of methylation (Figure 1). Such distribution of methylation levels was typically observed for *FLNc*, *THBD*, *CLDN3*, *F2R* and *CDKN2A*. The presence of such distribution confirmed previous findings that cancer samples could essentially be classified into two groups: cancers with methylation of a specific gene and those without (11,19,27). Counting cancer cells in the tissue section samples showed that two samples with least cancer cells contained cancer cells with fractions of 19.8 ± 5.2% and 22.9 ± 0.3% (mean ± SD). Based on these data, we adopted two cutoff values 10 and 20% to score each cancer sample as positive or negative. When overall distribution of methylation was examined, similar patterns of cancers with methylation were observed using the two cutoff values (Figure 2). Using either value, the number of methylated genes in a cancer did not obey bimodal distribution and looked quite similar (Figure 3). Therefore, we adopted a cutoff value of 20% to score individual cancers as positive or negative for methylation.

Then, the 63 cancers were classified by the frequency of CGI methylation. To avoid biases due to a cutoff number of methylated genes, we classified the cancers into three groups, those with no methylation, moderate methylation and frequent methylation, using two different cutoff numbers for frequent methylation. Using a cutoff number of three methylated genes or more, 16, 26 and 21 cases were classified into cancers with frequent methylation, moderate methylation and no methylation, respectively. Using a cutoff number of four methylated genes or more, 8, 34 and 21 cases were classified into those with frequent methylation, moderate methylation and no methylation, respectively.

Association between frequent CGI methylation and the HER2 amplification

The presence of *HER2* amplification was analyzed by fluorescence *in situ* hybridization, and 24 of 63 (38%) cancers had *HER2* amplification (supplementary Figure 2 is available at *Carcinogenesis* Online). The extent of amplification ranged from 2.0- to 16.8-fold. Using a cutoff number of three for frequent methylation, the fractions of cancers with *HER2* amplification were 11/16, 11/26 and 2/21 in cancers with frequent methylation, moderate methylation and no methylation, respectively (Figure 4A). Using a cutoff number of four, it was 6/8, 16/34 and 2/21, respectively (Figure 4B).

When correlation between the degree of CGI methylation and fraction of cancers with *HER2* amplification was examined by trend analysis, a highly significant increasing trend was observed from cancers with no methylation, to those with moderate methylation and then to those with frequent methylation ($P < 0.001$ for both of cutoff numbers). When cancers with frequent methylation and those with no methylation were compared, the former had a significantly higher fraction ($P = 0.003$ and 0.001 for cutoff numbers of four and three, respectively). Also, the degree of *HER2* amplification showed a correlation with the number of methylated genes (correlation coefficient = 0.411, $P = 0.002$) (Figure 5 and supplementary Table 2 is available at *Carcinogenesis* Online). This demonstrated that frequent CGI methylation had an association with *HER2* amplification.

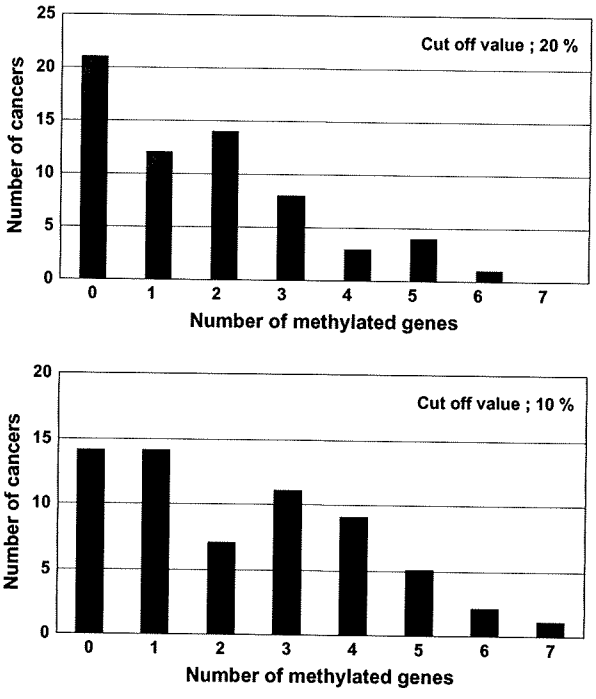

Fig. 3. Distribution of the number of methylated genes in a cancer. Similar distribution patterns were observed using two different cutoff values. The distribution was not bimodal, and involvement of multiple mechanisms in frequent methylation in breast cancers was suggested.

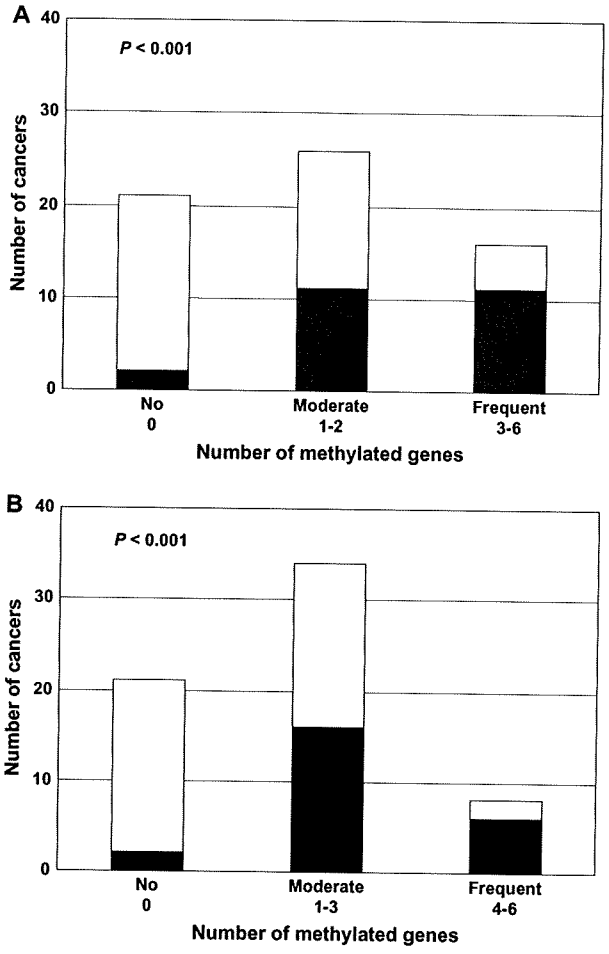

Fig. 4. The correlation between the degree of frequent methylation and *HER2* amplification. Two different cutoff numbers were used to define frequent methylation. These analyses adopted a cutoff value of 20% for methylation-positive. **(A)** Frequent methylation was defined as cancers with methylation of three or more genes. **(B)** Frequent methylation was defined as cancers with methylation of four or more genes. Whichever cutoff number was used, a clear increasing trend of *HER2*-positive cancers in no methylation, moderate methylation and then frequent methylation groups was observed ($P < 0.001$ for both of the two cutoff numbers). Closed and open boxes represent cancers with and without *HER2* amplification, respectively.

Association between frequent CGI methylation and other clinicopathological features, including methylation of tumor suppressor genes

The correlation between frequent CGI methylation and methylation of three tumor suppressor genes, *CDKN2A*, *CDH1* and *BRCA1*, was analyzed (Table I). However, none of the three genes showed any correlation (P = 0.557, 0.157 and 0.232, respectively). Regarding other clinicopathological characteristics, the degree of frequent CGI methylation correlated with higher nuclear grades (P = 0.001). The degree of frequent CGI methylation tended to show correlations with advanced pathological stage (P = 0.068) and post-menopausal status (P = 0.044). However, no association was observed with lymph node metastasis and negative expression of estrogen receptor (ESR) or progesterone receptor (PGR).

Discussion

The present study demonstrated for the first time that frequent CGI methylation in breast cancers had a highly significant association with *HER2* amplification. Regarding DNA methylation and HER2 overexpression, Fiegl *et al.* (28) previously found that methylation levels of four genes (*CDH13*, *PGR*, *HSD17B4* and *MYOD1*) and one gene (*BRCA1*), which were selected from 35 genes, correlated with HER2 expression positively and inversely, respectively (P = 0.01–0.04). Methylation levels of individual genes in cancers are affected by the content of cancer cells, and, also, the correlation observed in the study was considered to be due to interaction between a function of an individual gene and HER2 overexpression. In contrast, here, we focused on the abnormality in epigenetic regulation in cancers. To estimate its degree, we used marker genes that were unlikely to confer growth advantages even if methylated, scored their methylation as positive or negative and integrated the information from the 11 marker genes into the frequency of methylation in a cancer sample. The cancers were classified into three groups, namely those with frequent methylation, moderate methylation and no methylation. As a result, a very strong association between frequent methylation and *HER2* amplification ($P < 0.001$) was demonstrated. Also, the degree of frequent methylation showed a clear correlation with the degree of *HER2* amplification. *BRCA1* methylation did not correlate with the degree of frequent methylation or *HER2* amplification (P = 0.806).

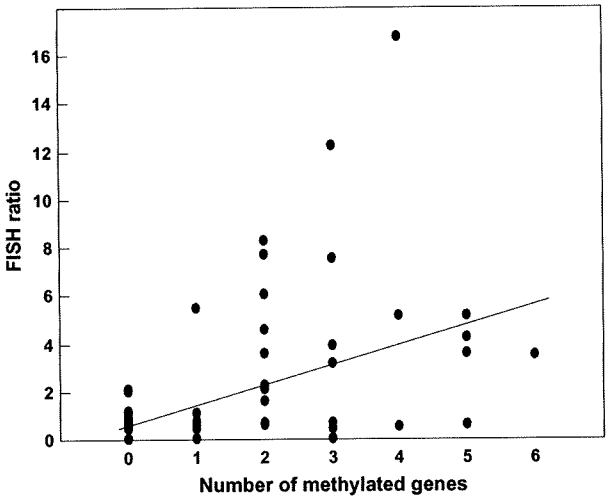

Fig. 5. The correlation between the number of methylated genes and degree of *HER2* amplification. The degree of *HER2* amplification showed a strong correlation with the number of methylated genes (correlation coefficient = 0.411, P = 0.002).

Table I. Association between frequent CGI methylation and clinicopathological features, including methylation of tumor suppressor genes

	No methylation	Moderate methylation	Frequent methylation	*P* value
CDKN2A methylation (+/−)	2/19	0/26	1/15	0.557
BRCA1 methylation (+/−)	1/20	0/26	0/16	0.232
CDH1 methylation (+/−)	0/21	0/26	1/15	0.157
Menopausal (pre/post)	12/9	9/17	4/12	0.044
Stage (I/II/III)	9/11/1	9/8/9	4/7/5	0.068
Lymph node metastasis (positive/negative)	6/15	13/13	7/9	0.308
ESR (positive/negative)	15/6	14/12	11/5	0.779
PGR (positive/negative)	17/4	14/12	9/7	0.100
Nuclear grade (1/2/3)	4/10/7	2/7/17	0/2/14	0.001

Frequent methylation was defined as breast cancers with methylation of three or more genes. Increasing or decreasing trends were tested by Mantel–Haenszel chi-square.

The association between frequent methylation and *HER2* amplification has clinical implications. It is known that *HER2* amplification status can show a discrepancy between primary and metastatic sites in a small fraction of patients (29). There is a possibility that HER2-negative breast cancers at initial diagnosis change into HER2 positive at their recurrence and that the presence of frequent methylation at the initial diagnosis can be used to predict such cases. Since accurate detection of HER2-positive cancers is very important to implement appropriate treatment, including trastuzumab (17), future studies to predict the *HER2* amplification status using frequent methylation and to clarify the mechanism of the association are warranted. Also, the effect of frequent methylation on long-term survival is important. So far, only 5 of 63 cases suffered from recurrence (one frequent methylation, three moderate methylation and one no methylation cases), and the effect cannot be statistically analyzed. Since the association between *HER2* amplification and poor survival (without trastuzumab) is well established, the effect of frequent methylation on long-term survival seems worth being analyzed in the future.

Some breast cancers with *HER2* amplification belonged to the moderate methylation or no methylation groups although the majority of cancers with *HER2* amplification belonged to the frequent methylation group. This was in contrast with the case of neuroblastomas, where all the neuroblastomas with *MYCN* amplification had frequent methylation, CIMP, with only one exception (12,13). Therefore, the relationship between frequent methylation and *HER2* amplification in breast cancers seems more complex than the relationship between CIMP and *MYCN* amplification in neuroblastomas. Not only frequent methylation could lead to *HER2* amplification through chromosomal instability (9), which was our initial expectation, but also *HER2* amplification could lead to frequent methylation or they might have common inducers.

The degree of frequent methylation also correlated with higher nuclear grades. It also tended to show association with advanced stages and post-menopausal status. It has been reported that *CDH1* methylation was associated with negative ESR and PGR expressions (P = 0.06 and 0.09, respectively) and that frequent methylation of seven tumor suppressor genes was associated with poor differentiation (30). It has also been reported that PGR expression was negatively associated with *ESR1*, *TGFBR2*, *PPTGS2* and *CDH13* methylation (P = 0.01–0.04) (31) and that ESR and PGR expressions were positively and negatively associated with *HIN-1/RASSF1A* and *RIL/CDH13* methylation, respectively (32). Taken together, the frequent methylation in breast cancers was weakly associated with advanced stages, negative PGR and ESR expressions and poor differentiation (higher nuclear grades). Nevertheless, the correlation between frequent methylation and *HER2* amplification was much stronger than these associations in our study. It was considered that quantitative analysis of marker genes was advantageous to clarify the strong association.

The cutoff value of methylation levels to score cancer samples as positive or negative for methylation was determined based upon the fraction of cancer cells in two samples with their smallest contents (20%). To count methylation in a fraction of cancer cells, we also tested a cutoff value, 10%, but quite similar results were obtained (Figure 3). Regarding the cutoff number for frequent methylation, we tried three and four but observed a highly significant association using both numbers (Figure 4). This excluded a possibility that a false-positive association between frequent methylation and *HER2* amplification was observed due to arbitrary cutoff values or numbers. Also, we confirmed that we detected dense methylation of promoter CGIs by our quantitative methylation-specific PCR analysis by sequencing the PCR products. Almost all the CpG sites in the products were densely methylated (supplementary Figure 3 is available at *Carcinogenesis* Online). Finally, we confirmed that the methylation detected in cancer tissues originated from cancer cells. Methylation levels of nine genes that showed high methylation levels (≥10%) in some cancer samples were measured in 11 pairs of non-cancerous breast and cancer tissues (supplementary Figure 4 is available at *Carcinogenesis* Online). The methylation levels of all the genes were elevated only in cancer tissues, and the methylation we detected was considered to originate from cancer cells.

In summary, frequent methylation in breast cancers had a strong association with *HER2* amplification.

Supplementary material

Supplementary Tables 1 and 2 and Figures 1–4 can be found at http://carcin.oxfordjournals.org/

Funding

Third-term Comprehensive Cancer Control Strategy and Pioneering Basic Research from the Ministry of Health, Labor and Welfare, Japan.

Acknowledgements

The authors are grateful to Dr S. Yamamoto for his advice in statistical analyses.

Conflict of Interest Statement: None declared.

References

1. Herman,J.G. *et al.* (2003) Gene silencing in cancer in association with promoter hypermethylation. *N. Engl. J. Med.*, **349**, 2042–2054.
2. Baylin,S.B. *et al.* (2006) Epigenetic gene silencing in cancer—a mechanism for early oncogenic pathway addiction? *Nat. Rev. Cancer*, **6**, 107–116.
3. Esteller,M. (2007) Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat. Rev. Genet.*, **8**, 286–298.
4. Jones,P.A. *et al.* (2007) The epigenomics of cancer. *Cell*, **128**, 683–692.
5. Chen,R.Z. *et al.* (1998) DNA hypomethylation leads to elevated mutation rates. *Nature*, **395**, 89–93.
6. Gaudet,F. *et al.* (2003) Induction of tumors in mice by genomic hypomethylation. *Science*, **300**, 489–492.
7. Nakagawa,T. *et al.* (2005) DNA hypomethylation on pericentromeric satellite regions significantly correlates with loss of heterozygosity on chromosome 9 in urothelial carcinomas. *J. Urol.*, **173**, 243–246.
8. Rodriguez,J. *et al.* (2006) Chromosomal instability correlates with genome-wide DNA demethylation in human primary colorectal cancers. *Cancer Res.*, **66**, 8462–9468.
9. Kondo,Y. *et al.* (2000) Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis—a comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. *Hepatology*, **32**, 970–979.
10. Toyota,M. *et al.* (1999) CpG island methylator phenotype in colorectal cancer. *Proc. Natl Acad. Sci. USA*, **96**, 8681–8686.
11. Weisenberger,D.J. *et al.* (2006) CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with *BRAF* mutation in colorectal cancer. *Nat. Genet.*, **38**, 787–793.
12. Abe,M. *et al.* (2005) CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. *Cancer Res.*, **65**, 828–834.
13. Abe,M. *et al.* (2007) Marked and independent prognostic significance of the CpG island methylator phenotype in neuroblastomas. *Cancer Lett.*, **247**, 253–258.
14. King,C.R. *et al.* (1985) Amplification of a novel v-*erb*B-related gene in a human mammary carcinoma. *Science*, **229**, 974–976.
15. Slamon,D.J. *et al.* (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science*, **235**, 177–182.
16. Arteaga,C.L. (2003) Trastuzumab, an appropriate first-line single-agent therapy for HER2-overexpressing metastatic breast cancer. *Breast Cancer Res.*, **5**, 96–100.
17. Hudis,C.A. (2007) Trastuzumab—mechanism of action and use in clinical practice. *N. Engl. J. Med.*, **357**, 39–51.
18. Miyamoto,K. *et al.* (2005) Identification of 20 genes aberrantly methylated in human breast cancers. *Int. J. Cancer*, **116**, 407–414.
19. Enomoto,S. *et al.* (2007) Lack of association between CpG island methylator phenotype in human gastric cancers and methylation in their background non-cancerous gastric mucosae. *Cancer Sci.*, **98**, 1853–1861.
20. Li,B. *et al.* (2007) The role of chromatin during transcription. *Cell*, **128**, 707–719.
21. Ushijima,T. (2005) Detection and interpretation of altered methylation patterns in cancer cells. *Nat. Rev. Cancer*, **5**, 223–231.
22. Sakamoto,G. *et al.* (2005) General rules for clinical and pathological recording of breast cancer 2005. *Breast Cancer*, **12** (suppl.), S1–S27.
23. Kaneda,A. *et al.* (2004) Decreased expression of the seven *ARP2/3 complex* genes in human gastric cancers. *Cancer Lett.*, **212**, 203–210.
24. Taniyama,K. *et al.* (2008) Tyrosine1248-phosphorylated HER2 expression and HER2 gene amplification in female invasive ductal carcinomas. *Breast Cancer*, **15**, 231–240.
25. Van Rompay,A.R. *et al.* (1999) Identification of a novel human adenylate kinase. cDNA cloning, expression analysis, chromosome localization and characterization of the recombinant protein. *Eur. J. Biochem.*, **261**, 509–517.
26. Reference database for gene Expression Analysis (RefExA). http://www.Isbm.org/database/index.html.
27. Ogino,S. *et al.* (2006) CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. *Gut*, **55**, 1000–1006.
28. Fiegl,H. *et al.* (2006) Breast cancer DNA methylation profiles in cancer cells and tumor stroma: association with HER-2/neu status in primary breast cancer. *Cancer Res.*, **66**, 29–33.
29. Simon,R. *et al.* (2001) Patterns of her-2/neu amplification and overexpression in primary and metastatic breast cancer. *J. Natl Cancer Inst.*, **93**, 1141–1146.
30. Li,S. *et al.* (2006) DNA hypermethylation in breast cancer and its association with clinicopathological features. *Cancer Lett.*, **237**, 272–280.
31. Widschwendter,M. *et al.* (2004) Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen. *Cancer Res.*, **64**, 3807–3813.
32. Feng,W. *et al.* (2007) Correlation between CpG methylation profiles and hormone receptor status in breast cancers. *Breast Cancer Res.*, **9**, R57.

Received August 20, 2008; revised December 16, 2008; accepted January 11, 2009

Int. J. Cancer: **124,** 2367–2374 (2009)

DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: Its possible involvement in the formation of epigenetic field defect

Takayuki Ando[1,2], Takeichi Yoshida[1,3], Shotaro Enomoto[3], Kiyoshi Asada[1], Masae Tatematsu[4], Masao Ichinose[3], Toshiro Sugiyama[2] and Toshikazu Ushijima[1]*

[1]*Carcinogenesis Division, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan*
[2]*Third Department of Internal Medicine, Toyama University, Toyama, Japan*
[3]*Second Department of Internal Medicine, Wakayama Medical University, Wakayama, Japan*
[4]*Division of Oncological Pathology, Aichi Cancer Center Research Institute, Chikusa, Nagoya, Japan*

Accumulation of aberrant DNA methylation in normal-appearing gastric mucosae, mostly induced by *H. pylori* infection, is now known to be deeply involved in predisposition to gastric cancers (epigenetic field defect), and silencing of protein-coding genes has been analyzed so far. In this study, we aimed to clarify the involvement of microRNA (miRNA) gene silencing in the field defect. First, we selected three miRNA genes as methylation-silenced after analysis of six candidate "methylation-silenced" tumor-suppressor miRNA genes. Methylation levels of the three genes (*miR-124a-1, miR-124a-2* and *miR-124a-3*) were quantified in 56 normal gastric mucosae of healthy volunteers (28 volunteers with *H. pylori* and 28 without), 45 noncancerous gastric mucosae of gastric cancer patients (29 patients with *H. pylori* and 16 without), and 28 gastric cancer tissues (13 intestinal and 15 diffuse types). Among the healthy volunteers, individuals with *H. pylori* had 7.8–13.1-fold higher methylation levels than those without ($p < 0.001$). Among individuals without *H. pylori*, noncancerous gastric mucosae of gastric cancer patients had 7.2–15.5-fold higher methylation levels than gastric mucosae of healthy volunteers ($p < 0.005$). Different from protein-coding genes, individuals with past *H. pylori* infection retained similar methylation levels to those with current infection. In cancer tissues, methylation levels were highly variable, and no difference was observed between intestinal and diffuse histological types. This strongly indicated that methylation-silencing of miRNA genes, in addition to that of protein-coding genes, contributed to the formation of a field defect for gastric cancers.

Key words: field for cancerization; microRNA; methylation; gastric cancer; *Helicobacter pylori*

Metachronous occurrence of gastric cancers is becoming an important issue as localized resection of early gastric cancers by endoscopic submucosal dissection (ESD) has become common.[1] The incidence of secondary primary gastric cancers after ESD reaches as high as 2.0% per year[2] whereas the incidence of gastric cancer in the general Japanese population is 0.14% per year.[3] This indicates that noncancerous gastric mucosae are already predisposed to developing gastric cancers, forming a field defect (field for cancerization). High incidences of metachronous cancers have been known not only for gastric cancers but also for bladder, liver, and esophageal cancers[4–6] and are becoming recognized for lung, breast and colorectal cancers.[7–9]

A molecular basis for the field defect has been considered as an accumulation of genetic and epigenetic alterations in normal-appearing tissues. Traditionally, cells with a genetic alteration were considered to form a physically continuous patch, producing a genetically altered field.[10] Recently, we found that aberrant DNA methylation of specific genes can be induced in as high as several percentage of cells in noncancerous gastric mucosae (thus in multiple independent gastric glands), and the degree of methylation is associated with gastric cancer risks.[11,12] Importantly, *Helicobacter pylori* infection, a major carcinogenic factor in the stomach, was shown to potently induce aberrant DNA methylation in gastric epithelial cells.[11] In addition to gastric cancers, the presence of aberrant DNA methylation in noncancerous tissues and possible association with cancer risks have been reported for liver,[13] colon,[14] esophageal,[15] breast[16] and renal cancers.[17]

Genes so far analyzed in noncancerous gastric mucosae are those methylated in gastric cancers, including tumor-suppressor genes, such as *CDKN2A*, *MLH1*, *CDH1*, *LOX* and *APC*,[2,11,18,19] and genes with little or no expression in normal gastric mucosae, such as *FLNc*, *HAND1* and *THBD*. The latter group of genes is methylated in parallel with tumor-suppressor genes but with higher frequencies, and is considered as a good marker to detect the presence of an epigenetic field defect.[11] In contrast with these protein-coding genes, involvement of microRNA (miRNA) silencing in field defect formation has not been clarified yet. Since the role of aberrant expression or reduction of various miRNAs in human multistep carcinogenesis is now clear,[20,21] there is a possibility that miRNAs silencing by aberrant DNA methylation is involved in field defect formation. Indeed, several tumor-suppressor miRNAs, including *miR-124a*,[22] *miR-137*, *miR-193a*[23] and *miR-127*,[24] are reported to be silenced by aberrant DNA methylation of their promoter CpG islands (CGI) in cancers.

In this study, we aimed to clarify whether or not miRNA silencing by DNA methylation can be involved in the formation of a field defect for gastric cancers. First, we searched for miRNAs that are reported to have tumor-suppressive functions and be controlled by DNA methylation, and confirmed methylation-silencing of these candidate genes. Then, we quantified their methylation levels in gastric mucosae of healthy volunteers, noncancerous gastric mucosae of gastric cancer patients, and primary gastric cancer tissues.

Material and methods

Cell lines and tissue samples

Six gastric cancer cell lines, AGS, KATOIII, MKN28, MKN45, MKN74 and NUGC3 were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and the American Type Culture Collection (Manassas, VA). Three gastric cancer cell lines, HSC39, HSC44 and HSC57 were gifted by Dr. K. Yanagihara, National Cancer Center Research Institute, Tokyo, Japan. GC2 was developed by M. T. TMK1 was gifted by Dr. W. Yasui, Hiroshima University, Hiroshima, Japan. 5-Aza-2′-deoxycitidine (5-aza-dC) treatment was performed with AGS, HSC57 and MKN28. Cells were seeded on day 0, media was added with freshly prepared 5-aza-dC on days 1 and 3, and cells were harvested on day 5. The concentrations of 5-aza-dC were determined as minimum concentrations that deplete DNMT1.[25]

Gastric mucosae were obtained by endoscopic biopsy of antral regions from 56 healthy volunteers (25 male and 31 female; aver-

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Ministry of Health, Labour and Welfare, Japan.

***Correspondence to:** Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo, Japan.
Fax: +81-3-5565-1753. E-mail: tushijim@ncc.go.jp

Received 5 August 2008; Accepted after revision 25 November 2008
DOI 10.1002/ijc.24219
Published online 11 December 2008 in Wiley InterScience (www.interscience.wiley.com).

age age 53, ranging from 27 to 91) and 45 gastric cancer patients (35 male and 10 female; average age 66, ranging from 38 to 89). Gastric cancer tissues were obtained from 28 gastric cancer patients (21 male and 7 female; average age 66, ranging from 49 to 81; 13 intestinal and 15 diffuse types) who underwent gastrectomy due to gastric cancers. Gastric epithelial cells were obtained by the gland isolation technique from eight noncancerous gastric tissues. Informed consents were obtained from all the patients and healthy volunteers. Gastric mucosae, noncancerous mucosae and cancer tissues were frozen in liquid nitrogen immediately after biopsy or resection, and stored at −80°C until extraction of genomic DNA. High molecular weight DNA was extracted by the phenol/chloroform method. RNA was isolated with ISOGEN (Nippon Gene, Tokyo, Japan).

H. pylori infection status was analyzed by a serum anti-*H. pylori* IgG antibody test (SRL, Tokyo, Japan), rapid urease test (Otsuka, Tokushima, Japan), or culture test (Eiken, Tokyo, Japan). All cancers were histologically diagnosed according to the Japanese classification of gastric carcinoma,[26] and classified according to the Lauren classification system.[27]

Sodium bisulfite modification, methylation-specific PCR (MSP), quantitative real-time MSP and bisulfite sequencing

Fully methylated DNA and fully unmethylated DNA were prepared by methylating genomic DNA with *Sss*I methylase (New England Biolabs, Beverly, MA) and by amplifying genomic DNA with the GenomiPhi amplification system (GE Healthcare, Buckinghamshire, UK), respectively. Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as previously described,[28] and the modified DNA was suspended in 40 μl of Tris-EDTA buffer. An aliquot of 1 μl was used for methylation-specific PCR (MSP) and Quantitative real-time MSP (qMSP) with a primer set specific to methylated (M) or unmethylated (U) sequences.

For MSP, the fully methylated and unmethylated DNA was used to determine an annealing temperature that specifically amplifies only methylated or unmethylated DNA. A minimum number of PCR cycles to obtain visible bands was determined using the fully (un)methylated DNA, and four cycles were added for analysis of gastric cancer cell lines. The primers were designed just upstream of reported transcription start sites within the CGI (Table I; Fig. 1*a*), whose methylation statuses are now known to be critical for induction of gene silencing.[29,30]

qMSP was performed by real-time PCR using SYBR® Green I (BioWhittaker Molecular Applications, Rockland, ME) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Although the same primer set was used for qMSP, a specific annealing temperature in the presence of SYBR® Green I was redetermined using the fully methylated and unmethylated DNA. The number of molecules in a sample was determined by comparing its amplification with those of standard DNA that contained exact numbers of molecules (10^1–10^6 molecules). Based on the numbers of M molecules and U molecules for a genomic region, a methylation level of the region was calculated as the fraction of M molecules in the total number of DNA molecules (# of M molecules + # of U molecules). The standard DNA samples were prepared by cloning PCR products of methylated and unmethylated sequences into the pGEM-T Easy vector (Promega, Madison, WI), respectively, or by purifying the PCR products using the Wizard SV Gel and PCR clean-up system (Promega).

For bisulfite sequencing, an aliquot of 1 μl of the sodium bisulfite-treated DNA was amplified by PCR with the primers common to methylated and unmethylated DNA sequences (Table I). The PCR product was cloned into pGEM-T Easy vector (Promega), and 15 clones or more were cycle-sequenced for each sample.

Quantitative real-time reverse transcription (RT)-PCR

For quantitative RT-PCR, cDNA was synthesized from 10 ng of total RNA using TaqMan® MicroRNA-specific primers and a

TABLE I – PRIMERS AND CONDITIONS FOR MSP AND REAL-TIME MSP

Gene	M/U	Primer sequence		Length (bp)	Anneal (°C)		Number of cycles for MSP
		Forward (5′→3′)	Reverse (5′→3′)		MSP	Real-time MSP	
miR-124a-1	M	AGAGTTTTTGGAAGACGTCG	AAAAAAATAAAAAAACGACGC	155	58	58	36
	U	AATAAAGAGTTTTTGGAAGATGTT	CAAAAAAAAAAAAAACAACAC	166	58	58	36
miR-124a-2	M	GGTTTATGTATGTTTTTAGGCG	TCCGTAAAAATATAAACGATAG	93	59	56	32
	U	TAGGTTTATGTATGTTTTTAGGTG	CTATTCCATAAAAATATAAACAATACA	99	52	50	36
miR-124a-3	M	GATAGTATAGTCGGTTGAGCGTAGC	CCTCAAAACTAAAACGAACGACG	152	61	59	31
	U	TAGTTGGTTGAGTGTAGTGTTTTTG	CAAAACTAAAACAAACAACAAACATC	142	61	59	36
miR-137	M	TAGGGGCGGGTTAGCG	TACCGCTACCGCTACTACC	99	57	–	36
	U	TTTTGGTGGTGGTGGT	ACCCAAAAATACCATCACCA	113	63	–	35
miR-193a	M	GAGGTAGTTTCGGAAGCGTAC	GACCCCCGAAACCAACG	86	61	–	36
	U	ATTGATTTATATTTTTGAGAGTGTTG	TCCCAAACTAACATACACTCCA	153	58	–	35
miR-127	M	GTTTGGGGAGAGCGTAAACG	GTAAACGAACACCGCACCG	96	63	–	34
	U	GTTTTGTGAATTATTTGGTTTTG	TTACAAATATCCCTCCACCAC	176	58	–	38

Gene	Primers for bisulfite sequencing		Length (bp)	Anneal (°C)	Number of cycles
	Forward (5′→3′)	Reverse (5′→3′)			
miR-124a-1	AAGGATGGGGGAGAATAAAGAGTTT	CTCAACCAACCCCATTCTTAACATT	354	60	32
miR-124a-2	ATTAGATTTATAGGTTTATGTATGTTTTAGG	ACTCTTCTCTCCCTACCCATC	235	54	30
miR-124a-3	GAAAGGGAGAAGTGTGGGTTTTT	CTCTTAACATTCACCGCGTACCTTAAT	268	54	32

M, Primers specific to methylated DNA; U, Primers specific to unmethylated DNA.

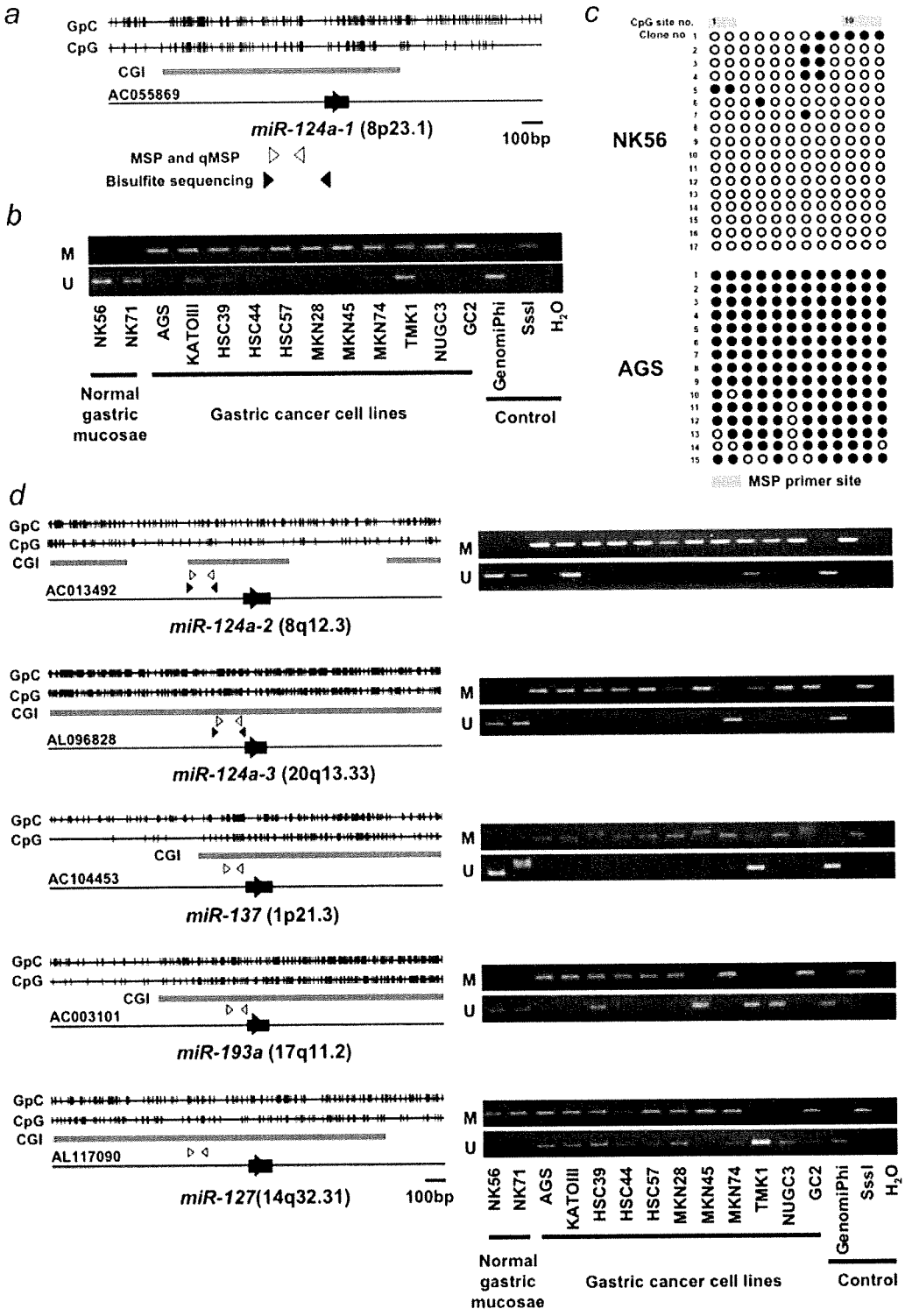

FIGURE 1 – Genomic structures and methylation statuses of the six miRNA genes in gastric cancer cell lines. (*a*) Structures of the *miR-124a-1* gene. Vertical ticks, individual GpC (top) and CpG sites (bottom); gray box, CGI; closed box, genomic location of *miR-124a-1*; open arrowheads, locations of the primers for MSP and real-time MSP; and closed arrowheads, locations of the primers for bisulfite sequencing. (*b*) Methylation statuses of *miR-124a-1* in normal gastric mucosae and 11 gastric cancer cell lines analyzed by MSP. SssI, genomic DNA methylated by *Sss*I methylase; GenomiPhi, genomic DNA amplified by GenomiPhi; and M and U, primer sets specific to methylated and unmethylated DNA, respectively. (*c*) The methylation status of a CGI around *miR-124a-1* analyzed by bisulfite sequencing. Twelve CpG sites were analyzed in NK56 normal and AGS gastric cancer cell lines, and 15 clones or more were sequenced for each sample. Closed circle, methylated CpG site; and open circle, unmethylated CpG site. (*d*) Genomic structures of five other miRNA genes and their methylation statuses in normal gastric mucosae and 11 gastric cancer cell lines analyzed by MSP.