DNA methylation of genes linked to retinoid signaling in squamous cell carcinoma of the esophagus: DNA methylation of *CRBP1* and *TIG1* is associated with tumor stage

Hirozumi Mizuiri,[1] Kazuhiro Yoshida,[1] Tetsuya Toge,[1] Naohide Oue,[2] Phyu Phyu Aung,[2] Tsuyoshi Noguchi[3] and Wataru Yasui[2,4]

[1]Department of Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University; [2]Department of Molecular Pathology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima 734-8551; and [3]Department of Oncological Science (Surgery II), Faculty of Medicine, Oita University, Oita 879-5593, Japan

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Hypermethylation of CpG islands is associated with the silencing of various tumor suppressor genes. *Retinoic acid receptor-beta* (*RAR-beta*), *cellular retinol-binding protein 1* (*CRBP1*), and *tazarotene-induced gene 1* (*TIG1*) have been linked to retinoic acid signaling. Little is known about the involvement of these three genes in esophageal squamous cell carcinoma (ESCC). In this study, we investigated the methylation status of these genes and analyzed the role of methylation of their DNA in ESCC. Methylation-specific polymerase chain reaction (PCR) was performed to study the methylation of CpG islands in 28 ESCC (stages I, II, and III) and 10 samples of corresponding non-neoplastic mucosa. The mRNA expression levels of the three genes were measured by quantitative reverse transcription-PCR. DNA hypermethylation of *RAR-beta* was found in seven (25.0%) of the 28 ESCC, of *CRBP1* in five (17.9%), and of *TIG1* in five (17.9%). DNA methylation of *RAR-beta* was identified in one of 10 samples of corresponding non-neoplastic mucosa (10.0%), whereas no DNA methylation of *CRBP1* or *TIG1* was detected. In total, at least one of the three genes was hypermethylated in 12 (42.9%) ESCC. Reduced expression of *RAR-beta*, *CRBP1*, and *TIG1* was found in 14 (50.0%), 15 (53.6%), and 13 (46.4%) ESCC, respectively. DNA methylation of each gene was significantly associated with reduced expression of the respective mRNA. No correlation was found between the DNA methylation status of *RAR-beta* and clinicopathological factors such as depth of invasion, lymph node metastasis, or tumor stage. In contrast, DNA methylation of both *CRBP1* and *TIG1* was observed only in stage III ESCC. These results show that inactivation of the retinoic acid signaling-associated genes *RAR-beta*, *CRBP1*, and *TIG1* by DNA methylation occurs frequently in ESCC. (*Cancer Sci* 2005; *96*: 571–577)

Esophageal squamous cell carcinoma (ESCC) is one of the most common cancers worldwide, but the prognosis for patients with this condition is extremely poor because of difficulties with early diagnosis and a lack of effective treatments.[1] The development of ESCC is a multi-step and progressive process, an early indicator of which is the increased proliferation of epithelial cells, including basal cell hyperplasia and dysplasia, which are regarded as precancerous lesions. Multiple genetic alterations are involved, including amplification/overexpression of the *epidermal growth factor* (*EGF*)/*EGF receptor* (*EGFR*)[2,3] and *cyclin D1*/*hst-1*/*int-2* genes,[4] abnormal retention of intron 9 in the *CD44* gene,[5] loss of heterozygosity (LOH) at multiple chromosomal loci,[6,7] microsatellite instability,[8] and mutation of the *TP53* gene.[9]

In addition to genetic alterations, epigenetic alterations, such as hypermethylation of CpG islands, are commonly observed in human cancers. Hypermethylation of CpG islands is associated with the silencing of several tumor-related genes, and has been proposed as an alternative way to inactivate tumor suppressor genes in cancer.[10,11] The expression of some tumor suppressor genes, such as $p16^{INK4a}$, *FHIT*, *CDH1*, *ECRG4*, *MGMT*, and *LRP1B*[12–17] is commonly downregulated by CpG island hypermethylation in ESCC. However, despite recent advances in DNA methylation studies of esophageal adenocarcinoma,[18,19] gastric cancer,[20,21] and colorectal cancer,[22,23] the extent of DNA methylation in ESCC is poorly understood.

Several lines of evidence suggest that retinoids suppress carcinogenesis and prevent the development of cancer. Retinoids regulate the growth, differentiation, and apoptosis of normal cells during embryonic development, and of premalignant and malignant cells during carcinogenesis. The effects of retinoids are mediated predominantly by retinoic acid receptors (RAR-alpha, -beta, and -gamma), which act as retinoic acid-dependent transcriptional activators in their heterodimeric forms with retinoid X receptors (RXR-alpha, -beta, and -gamma).[24,25] Among RAR and RXR, RAR-beta is thought to function as a tumor suppressor. Previous studies have shown that overexpression of RAR-beta induces growth arrest and apoptosis in several cancer cells.[26,27] In addition, the *RAR-beta* gene is hypermethylated in cancers of the stomach,[27] breast,[28] lung,[29] and head and neck.[30] Although diminished expression of RAR-beta in ESCC has been reported,[31] the DNA methylation status of *RAR-beta* in ESCC is unclear.

[4]To whom correspondence should be addressed. E-mail: wyasui@hiroshima-u.ac.jp

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Another key component of retinoid signaling is cellular retinol-binding protein 1 (CRBP1). Retinoic acid is present in the circulation, but most tissues rely on the uptake and cytosolic metabolism of retinoic acid to activate RAR and RXR. CRBP1 possesses high-affinity binding for retinoic acid, possibly functioning as a chaperone-like protein to regulate this prenuclear phase of retinoic acid signaling.[32] The *CRBP1* gene is known to be hypermethylated in various human cancers.[33] However, there are no reports on the role of CRBP1 in ESCC.

Tazarotene-induced gene 1 (*TIG1*) is one of the genes induced by tazarotene, a synthetic retinoid that binds RAR-beta and RAR-gamma.[34] TIG1 may function as a cell adhesion protein, and its expression on the cell surface may lead to increased cell–cell contact and reduced proliferation.[35] The *TIG1* gene is also known to be hypermethylated in various human cancers.[36,37] However, little is known about the role of *TIG1* in ESCC.

In the present study, we examined the methylation status of the *RAR-beta*, *CRBP1*, and *TIG1* genes, and the expression levels of these genes in 28 primary ESCC samples, as well as in samples of corresponding non-neoplastic mucosa. To determine whether hypermethylation causes transcriptional inactivation, we compared the methylation status with the mRNA expression levels of these genes. We also studied the relationship between the *RAR-beta*, *CRBP1*, and *TIG1* genes with respect to methylation status.

Materials and Methods

Tissue samples

Twenty-eight ESCC tissue specimens from 28 patients were analyzed for methylation of the *RAR-beta*, *CRBP1*, and *TIG1* genes. Ten samples of corresponding non-neoplastic mucosa were also analyzed. Total RNA was available for the 28 pairs of cancer tissues and corresponding non-neoplastic mucosa to study expression of these genes. Cancers and corresponding non-neoplastic samples were surgically removed, immediately frozen in liquid nitrogen, and stored at −80°C until use. We confirmed microscopically that the tumor specimens consisted mainly (> 50%) of cancer tissue and that the non-neoplastic samples did not exhibit any tumor cell invasion or significant inflammatory involvement. Tumors were evaluated according to the TNM staging system.[38] Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis; the procedure was in accordance with the Japanese Government's Ethical Guidelines for Human Genome/Gene Research.

Genomic DNA extraction and methylation analysis

To examine the DNA methylation patterns of the *RAR-beta*, *CRBP1*, and *TIG1* genes, we extracted genomic DNA with a Genomic DNA Purification Kit (Promega, Madison, WI, USA) and performed methylation-specific PCR (MSP).[39] In brief, 2 μg of genomic DNA was denatured by treatment with 2 M NaOH and modified with 3 M sodium bisulfite for 16 h. DNA samples were purified with Wizard DNA Purification Resin (Promega), treated with 3 M NaOH, precipitated with ethanol, and resuspended in 25 μL water. Two-microliter aliquots were used as templates for PCR reactions. The sequences of primers and the annealing temperature for *RAR-beta*, *CRBP1*, and *TIG1* MSP were as described previously (Table 1).[33,36,40] We determined the number of PCR cycles according to the correlation between the mRNA expression and DNA methylation of each gene in gastric cancer cell lines.[41] Hot-start PCR with a total cycle number of 30 was used in all MSP DNA amplifications.

Table 1. Primer sequences for methylation-specific polymerase chain reaction (MSP) and quantitative reverse transcription–polymerase chain reaction (RT-PCR)

Primer sequence	Annealing temperature (°C)
MSP (*RAR-beta*, methylated)	64
F: 5′-GGT TAG TAG TTC GGG TAG GGT TTA TC-3′	
R: 5′-CCG AAT CCT ACC CCG ACG-3′	
MSP (*RAR-beta*, unmethylated)	55
F: 5′-TTA GTA GTT TGG GTA GGG TTT ATT-3′	
R: 5′-CCA AAT CCT ACC CCA ACA-3′	
MSP (*CRBP1*, methylated)	70
F: 5′-TTG GGA ATT TAG TTG TCG TCG TTT C-3′	
R: 5′-AAA CAA CGA CTA CCG ATA CTA CGC G-3′	
MSP (*CRBP1*, unmethylated)	67
F: 5′-GTG TTG GGA ATT TAG TTG TTG TTG TTTT-3′	
R: 5′-ACT ACC AAA ACA ACA ACT ACC AAT ACT ACA-3′	
MSP (*TIG1*, methylated)	56
F: 5′-GCG GGG TTC GGG GAT TTC-3′	
R: 5′-GTA CGC GAA CAA ACA AAC G-3′	
MSP (*TIG1*, unmethylated)	55
F: 5′-GTG GGG TTT GGG GAT TTT GAT-3′	
R: 5′-ATA CAC AAA CAA ACA AAC ACA-3′	
Quantitative RT-PCR (*RAR-beta*)	60
F: 5′-ACC ACT GGA CCA TGT AAC TCT AGT GT-3′	
R: 5′-GGC ATC AAG AAG GGC TGG A-3′	
Quantitative RT-PCR (*CRBP1*)	60
F: 5′-CAA CAG TGA GCT GGG ACG G-3′	
R: 5′-GCC ACG CCC CTC CTT C-3′	
Quantitative RT-PCR (*TIG1*)	60
F: 5′-GGC CGC GCG TGG AT-3′	
R: 5′-GGT TGT AGC GCT CTG TGC TG-3′	
Quantitative RT-PCR (*ACTB*)	60
F: 5′-TCA CCG AGC GCG GCT-3′	
R: 5′-TAA TGT CAC GCA CGA TTT CCC-3′	

F, forward; R, reverse.

Quantitative reverse transcription-PCR analysis

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 1 μg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA). PCR was performed with a SYBR Green PCR Core Reagent Kit (Applied Biosystems, Foster City, CA, USA). Real-time detection of the emission intensity of SYBR green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems), as described previously.[42] Primer sequences are listed in Table 1. We calculated the ratio of target gene mRNA expression levels between ESCC tissue (T) and corresponding non-neoplastic mucosa (N). We considered T/N < 0.5 to represent reduced expression. *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls.

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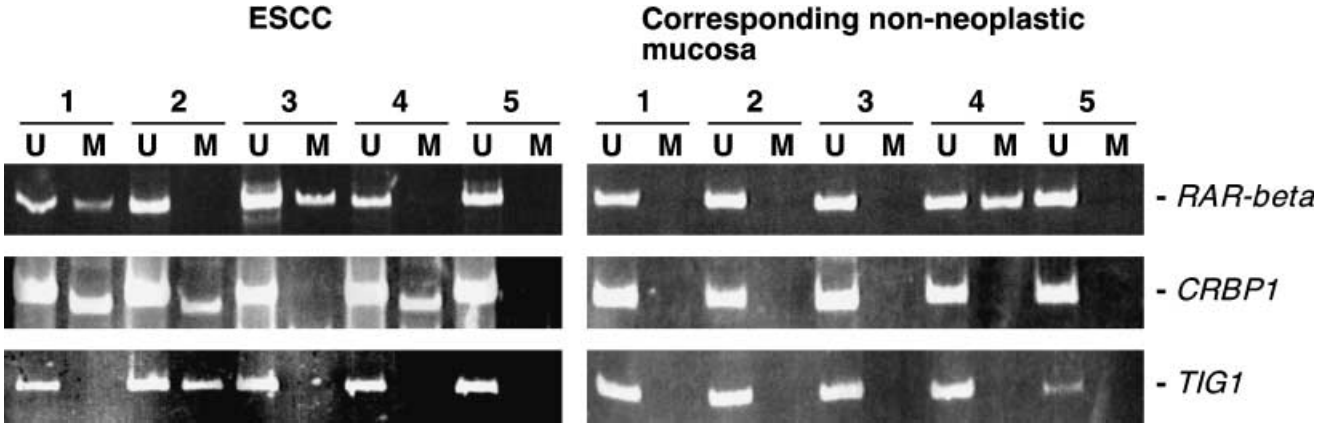

Fig. 1. Methylation-specific polymerase chain reaction (PCR) analysis of *RAR-beta*, *CRBP1*, and *TIG1* genes in esophageal squamous cell carcinoma and corresponding non-neoplastic mucosa. U, unmethylated PCR product; M, methylated PCR product. A methylated allele of the *RAR-beta* gene was detected in samples 1T and 3T. A methylated allele of the *CRBP1* gene was detected in samples 1T, 2T, and 4T. A methylated allele of the *TIG1* gene was detected in sample 2T. In corresponding non-neoplastic mucosa, a methylated allele of the *RAR-beta* gene was detected in sample 4N. Methylated alleles of *CRBP1* and *TIG1* were not detected.

Statistical methods

Statistical analysis was performed by using Fisher's exact test. *P*-values of less than 0.05 were regarded as statistically significant.

Results

Frequencies of *RAR-beta*, *CRBP1*, and *TIG1* methylation in ESCC and corresponding non-neoplastic esophageal mucosa

Representative MSP results for the *RAR-beta*, *CRBP1*, and *TIG1* genes in ESCC tissues and corresponding non-neoplastic samples are shown in Figure 1. Among the 28 ESCC, DNA hypermethylation was detected in seven (25.0%) for *RAR-beta*, five (17.9%) for *CRBP1*, and five (17.9%) for *TIG1*. The overall results are shown in Figure 2. Concordant hypermethylation of *RAR-beta* and *CRBP1* was found in only one ESCC sample, and concordant hypermethylation of *RAR-beta* and *TIG1* was found in only one ESCC sample. There was a tendency toward concordant methylation of *CRBP1* and *TIG1* ($P = 0.0269$, Fisher's exact test; Table 2). Among the 28 ESCC, at least one of the three genes was hypermethylated in 12 (42.9%). We analyzed the relationship between the methylation status of each gene and the clinicopathological factors. There was no correlation between the DNA methylation of *RAR-beta* and clinicopathological data (Table 3). However, DNA methylation of both *CRBP1* and *TIG1* was detected only in ESCC of advanced T grade, N grade, and tumor stage (Tables 4,5). The frequency of DNA methylation of *CRBP1* was significantly higher in stage III ESCC (five of 10, 50.0%) than in stage I/II ESCC (0 of 18, $P = 0.0026$, Fisher's exact test). The frequency of DNA methylation of *TIG1* was also significantly higher in stage III ESCC (five of 10, 50.0%) than in stage I/II ESCC (0 of 18, $P = 0.0026$, Fisher's exact test).

In samples of corresponding non-neoplastic mucosa, DNA methylation of *RAR-beta* was detected in one (10.0%) of 10 samples. On the other hand, the corresponding tumor sample

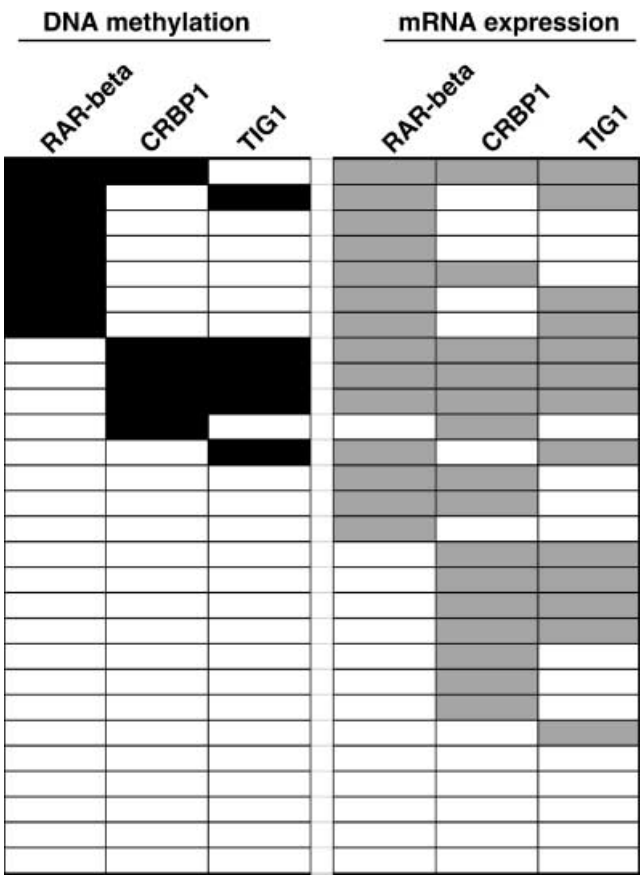

Fig. 2. Summary of DNA methylation and mRNA expression of *RAR-beta*, *CRBP1*, and *TIG1* in esophageal squamous cell carcinoma (ESCC) tissues. DNA methylation of each gene was associated with low expression of the respective mRNA. Concordant hypermethylation of *CRBP1* and *TIG1* was noted. Concordant hypermethylation of *RAR-beta* and *CRBP1* was found in only one ESCC sample, and concordant hypermethylation of *RAR-beta* and *TIG1* was found in only one ESCC sample. Black boxes represent samples with DNA methylation. Gray boxes represent samples with reduced expression.

Table 2. DNA methylation status of retinoic acid signaling-associated genes

		RAR-beta methylation status		*P*-value*
		Methylated	Unmethylated	
CRBP1 methylation status	Methylated	1 (20.0%)	4	1.000
	Unmethylated	6 (26.1%)	17	
TIG1 methylation status	Methylated	1 (20.0%)	4	1.000
	Unmethylated	6 (26.1%)	17	
		CRBP1 methylation status		
		Methylated	Unmethylated	
TIG1 methylation status	Methylated	3 (60.0%)	2	0.0269
	Unmethylated	2 (8.7%)	21	

*Fisher's exact test.

Table 3. Association between DNA methylation and mRNA expression of *RAR-beta* and clinicopathological parameters

		DNA methylation		*P*-value*	mRNA expression		*P*-value*
		M	U		Reduced†	Not reduced	
T grade	T1/2	3 (27.3%)	8	NS	6 (54.5%)	5	NS
	T3	4 (23.5%)	13		8 (47.1%)	9	
N grade	N0	2 (20.0%)	8	NS	2 (20.0%)	8	0.0461
	N1	5 (27.8%)	13		12 (66.7%)	6	
Stage	I/II	4 (22.2%)	14	NS	7 (38.9%)	11	NS
	III	3 (30.0%)	7		7 (70.0%)	3	
Differentiation§	W/M	4 (19.0%)	17	NS	10 (47.6%)	11	NS
	P	3 (42.9%)	4		4 (57.1%)	3	
DNA	M	–	–		7 (100%)	0	0.0058
methylation	U	–	–		7 (33.3%)	14	

*Fisher's exact test. †We considered T (tumor)/N (normal) < 0.5 to represent reduced expression. NS, not significant. §W, well-differentiated; M, moderately differentiated; P, poorly differentiated.

Table 4. Association between DNA methylation and mRNA expression of *CRBP1* and clinicopathological parameters

		DNA methylation		*P*-value*	mRNA expression		*P*-value*
		M	U		Reduced†	Not reduced	
T grade	T1/2	0 (0.0%)	11	NS	3 (27.3%)	8	NS
	T3	5 (29.4%)	12		12 (70.6%)	5	
N grade	N0	0 (0.0%)	10	NS	6 (60.0%)	4	NS
	N1	5 (27.8%)	13		9 (50.0%)	9	
Stage	I/II	0 (0.0%)	18	0.0026	9 (50.0%)	9	NS
	III	5 (50.0%)	5		6 (60.0%)	4	
Differentiation§	W/M	4 (19.0%)	17	NS	13 (61.9%)	8	NS
	P	1 (14.3%)	6		2 (28.6%)	5	
DNA	M	–	–		5 (100%)	0	0.0437
methylation	U	–	–		10 (43.5%)	13	

*Fisher's exact test. †We considered T (tumor)/N (normal) < 0.5 to represent reduced expression. NS, not significant. §W, well-differentiated; M, moderately differentiated; P, poorly differentiated.

(case no. 4) did not show *RAR-beta* methylation. Thus, the origin of this tumor may not be non-neoplastic mucosa with DNA methylation of *RAR-beta*. It is possible that tumor cells may be heterogeneous with regard to aberrant methylation, resulting in a lack of DNA methylation. DNA methylation of *CRBP1* or *TIG1* was not detected (Fig. 1).

MRNA expression of *RAR-beta*, *CRBP1*, and *TIG1* in ESCC

We used quantitative reverse transcription (RT)–PCR analysis to determine whether DNA methylation of the *RAR-beta*, *CRBP1*, and *TIG1* genes affects the expression of the their respective mRNA. Overall results are shown in Figure 2. Reduced expression of *RAR-beta*, *CRBP1*, and *TIG1* was found in 14 (50.0%), 15 (53.6%), and 13 (46.4%) of the 28 ESCC, respectively. Among the 14 ESCC with reduced expression of *RAR-beta*, seven (50.0%) had DNA methylation of *RAR-beta*, whereas of the 14 ESCC without reduced expression of *RAR-beta*, no *RAR-beta* methylation was detected ($P = 0.0058$, Fisher's exact test; Table 3). Reduced expression of *CRBP1* and of *TIG1* mRNAs was also associated with hypermethylation

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Table 5. Association between DNA methylation and mRNA expression of *TIG1* and clinicopathological parameters

		DNA methylation		*P*-value*	mRNA expression		*P*-value*
		M	U		Reduced†	Not reduced	
T grade	T1/2	0 (0.0%)	11	NS	3 (27.3%)	8	NS
	T3	5 (29.4%)	12		10 (58.8%)	7	
N grade	N0	0 (0.0%)	10	NS	5 (50.0%)	5	NS
	N1	5 (27.8%)	13		8 (44.4%)	10	
Stage	I/II	0 (0.0%)	18	0.0026	7 (38.9%)	11	NS
	III	5 (50.0%)	5		6 (60.0%)	4	
Differentiation§	W/M	4 (19.0%)	17	NS	11 (52.4%)	10	NS
	P	1 (14.3%)	6		2 (28.6%)	5	
DNA	M	–	–		5 (100%)	0	0.0131
methylation	U	–	–		8 (34.8%)	15	

*Fisher's exact test. †We considered T (tumor)/N (normal) < 0.5 to represent reduced expression. NS, not significant. §W, well-differentiated; M, moderately differentiated; P, poorly differentiated.

of respective genes ($P = 0.0437$ for *CRBP1*, $P = 0.0131$ for *TIG1*, Fisher's exact test; Tables 4 and 5). Among the 14 ESCC with reduced expression of *RAR-beta*, 12 (85.7%) were positive for lymph node metastasis ($P = 0.0461$, Fisher's exact test). There was no statistically significant association between clinicopathological factors and mRNA expression of *CRBP1* or *TIG1*.

Discussion

In this study, we analyzed the DNA methylation and mRNA expression status of three genes associated with retinoid signaling. DNA methylation of these genes was significantly associated with reduced gene expression, suggesting that DNA methylation plays an important role in transcriptional inactivation of these genes in ESCC. It is important to note that several samples showed reduced mRNA expression in the absence of DNA methylation. Alternative gene-inactivating mechanisms, such as hemizygous deletion or alteration of transcription factors, may account for the reduced gene expression in these samples. The *RAR-beta* gene is located on chromosome 3p24, the *CRBP1* gene is located on chromosome 3q23, and the *TIG1* gene is located on chromosome 3q25. LOH in chromosomes 3p and 3q has been reported in 35% and 30% of ESCC, respectively.[7] Previously, lack of correlation between expression of *RAR-beta* and LOH on 3p24 in ESCC has been reported,[31] thus only LOH on 3p24 does not cause the reduced gene expression of *RAR-beta*. In the present study, because the mRNA expression levels of the *RAR-beta* gene in tumor tissues were correlated with DNA methylation, it is possible that the *RAR-beta* gene may have monoallelic methylation in non-neoplastic tissue and biallelic methylation or monoallelic methylation plus LOH in tumors. High-level gains at 3q25–29 have been reported in ESCC by comparative genomic hybridization.[43]

Reduced expression of *RAR-beta* was detected in 50.0% of ESCC, and half of these cases showed DNA methylation of *RAR-beta*. Although DNA methylation of *RAR-beta* was detected in the corresponding non-neoplastic samples (10.0%), the frequency of methylation in ESCC (25.0%) was higher, suggesting that methylation of the *RAR-beta* gene may contribute to esophageal carcinogenesis. DNA methylation occurs in premalignant and histologically normal squamous epithelium of the esophagus.[19,44] The frequency of *RAR-beta* methylation did not differ significantly between early-stage and late-stage ESCC in this study. However, among the 14 ESCC with reduced expression of *RAR-beta*, 12 (85.7%) were positive for lymph nodes metastasis. A previous study indicated that retinoic acid induces the expression of *nm23-H1*,[45] which is known to reduce cell motility.[46,47] Reduced expression of *RAR-beta* followed by reduced expression of *nm23-H1* may occur frequently in ESCC with lymph node metastasis.

DNA methylation of *CRBP1* and *TIG1* was detected only in late-stage ESCC, and no methylation was detected in corresponding non-neoplastic mucosa, indicating that DNA methylation of these two genes may contribute not to carcinogenesis but to tumor progression. However, reduced expression of both *CRBP1* and *TIG1* was not associated with tumor stage. Therefore, the correlation between DNA methylation of these two genes and tumor stage may be a secondary effect of global changes in chromatin structure. In breast cancer, it has been reported that global DNA hypomethylation occurs during tumor progression.[48] Nevertheless, DNA methylation of both *CRBP1* and *TIG1* may be a marker of tumor progression.

Although concordant hypermethylation of *RAR-beta* and *CRBP1*[33] and of *RAR-beta* and *TIG1*[36] has been reported, there was no such tendency in ESCC in our study. Approximately half of the ESCC in our study had methylated DNA for at least one of the three genes, indicating that alterations of retinoic acid signaling are widely involved in ESCC and that inactivation of *RAR-beta* and *CRBP1* as well as of *TIG1* may not occur synergistically, but rather are random events. In contrast, concordant hypermethylation of *CRBP1* and *TIG1* was observed. Because both *CRBP1* and *TIG1* genes are located on chromosome 3q, it is possible that global DNA methylation effected this change.

In conclusion, our results show that inactivation of the retinoic acid signaling-associated genes *RAR-beta*, *CRBP1*, and *TIG1* due to DNA methylation occurs frequently in ESCC. Because methylated DNA can be induced by demethylating agents,[37] these three genes may be good molecular targets for effective therapeutic strategies for ESCC.

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