Original Article

DOI: 10.5582/bst.2016.01097

Promoter hypomethylation of RAR-related orphan receptor α 1 is correlated with unfavorable clinicopathological features in patients with colorectal cancer

Hisao Kano[1,2], Tadatoshi Takayama[2], Yutaka Midorikawa[2,*], Hiroki Nagase[1,3]

[1] *Department of Cancer Genetics, Nihon University School of Medicine, Tokyo, Japan;*
[2] *Department of Digestive Surgery, Nihon University School of Medicine, Tokyo, Japan;*
[3] *Division of Cancer Genetics, Chiba Cancer Center Research Institute, Chiba, Japan.*

Summary **Retinoic acid receptor-related orphan receptor α (*RORA*) is a tumor-specific differentially methylated region. *RORA* mRNA expression is frequently downregulated in colorectal cancer (CRC) due to promoter methylation, and this methylation is correlated with the development of CRC. Here we investigated the correlation between the methylation status of the *RORA* promoter region and clinical CRC stages. The methylation status of *RORA* isoform 1 (*RORA1*) and isoform 4 (*RORA4*) promoters was investigated in 43 paired CRC specimens and adjacent normal tissues by quantitative DNA methylation analysis using the Sequenom MassARRAY system and bisulfite sequencing. The relationship between the methylation status of the *RORA1* promoter and the CRC pathological stage was analyzed. *RORA1* expression was evaluated using quantitative PCR. Sixteen of 43 CRC specimens (37%) and three CRC cell lines (Caco2, HT29, and HCT116) showed increased levels of methylation in the *RORA1* promoter region compared with adjacent normal tissues, whereas no methylation was observed in the *RORA4* promoter. Quantitative PCR showed downregulation of *RORA1* expression both in CRC samples and cell lines. Furthermore, the *RORA1* promoter hypomethylation status showed a significant correlation with unfavorable CRC stages (stages III and IV) compared with favorable stages (stages I and II, $p = 0.014$). Hypomethylation of the *RORA1* promoter may have important clinical implications in unfavorable CRC development, and therefore, the methylation status of the *RORA1* promoter may constitute a useful biomarker to determine an indication for postoperative therapy such as adjuvant chemotherapy in highly advanced CRC patients.**

Keywords: DNA methylation, RORA, colorectal cancer, prognostic factor

1. Introduction

The identification of prognostic and predictive markers in colorectal cancer (CRC) pathogenesis is of great importance for developing new therapeutic strategies. CRC typically develops over decades and involves multiple genetic and epigenetic alterations in cancer-related genes during carcinogenesis. One of the most common epigenetic alterations is aberrant methylation of cytosine-guanine (CpG) islands that encompass the promoter and transcription start site. Aberrant methylation of these sites can be accompanied by transcriptional repression. A variety of tumor suppressor genes such as *Rb*, *CDKN2A/p16*, *MGMT*, $p14^{ARF}$, and *HLTF* are aberrantly methylated in CRC (*1*).

Previously, we performed restriction landmark genomic scanning to identify novel genomic regions of mouse skin tumor-specific differentially methylated regions (DMRs), and found 14 DMRs that were highly conserved between mouse and human (*2-5*). Among these, we focused on retinoic acid receptor-related orphan receptor α (*RORA*), which is frequently downregulated in

Released online in J-STAGE as advance publication June 10, 2016.

*Address correspondence to:
Dr. Yutaka Midorikawa, Department of Digestive Surgery, Nihon University School of Medicine, 30-1 Oyaguchi Kami-machi, Itabashi-ku, Tokyo 173-8610, Japan.
E-mail: mido-tky@umin.ac.jp

CRC and is correlated with tumor progression (*6*).

ROR alpha, -beta, and -gamma are evolutionarily related transcription factors that belong to the steroid hormone receptor superfamily. ROR was cloned by virtue of its strong homology with the retinoic acid receptor. *RORA* generates four isoforms (RORA1–4), which differ in their N-terminal region and demonstrate distinct transactivation properties (*7*). Notably, only two of the four isoforms, *RORA1* and *RORA4*, are actually transcribed (*8*). Although some putative ligands have been proposed for RORA, the nature of RORA ligands remains elusive (*9,10*).

Previous reports have demonstrated significantly lower *RORA* mRNA and protein levels in CRC samples compared with normal mucosa. Furthermore, an inverse correlation was found between RORA protein levels and progression in CRC patients (*6*). The functional significance of RORA in CRC was proposed to be RORA inhibition of canonical Wnt/β-catenin signaling to suppress CRC cell growth through protein kinase Cα-dependent phosphorylation (*11*), and RORA enhancement of p53 stability and transactivation ability to increase apoptosis (*12*). Several studies have described the possible mechanism through which RORA contributes to human disorders. For example, RORA stimulates transcription of the NF-kB inhibitor IkB (*13*), and *RORA* regulates hypoxia-inducible factor 1 transcription (*14*). A study in the human hepatoma HepG2 cell line showed that RORA directly upregulates secreted protein, acidic, cysteine-rich (SPARC), which is associated with a highly aggressive tumor phenotype, and behaves as a tumor suppressor in several specific cancer types (*15*). All these studies have implicated RORA as a functional tumor suppressor. However, whether these findings could be used in clinical applications, such as a prognostic biomarker, for evaluation of therapy, or as an indication for treatment, remains unclear.

In the present study, we investigated the methylation status of the *RORA* promoter in CRC patient samples compared with adjacent normal tissues. In sharp contrast to previous studies obtained from gene and protein expression analyses, our results suggested that the *RORA1* promoter may be hypermethylated in early, favorable stages in CRC and subsequently hypomethylated as cancer becomes more advanced. Furthermore, our results indicate a significant correlation between the *RORA1* promoter methylation status and the CRC stage. Thus, hypomethylation of the *RORA1* promoter may be of great diagnostic value for determining an indication for postoperative chemotherapy for advanced CRC.

2. Materials and Methods

2.1. *Human surgical specimens and cell lines*

CRC specimens and adjacent normal mucosa were obtained from 43 patients who underwent a curative operation at Nihon University School of Medicine. The study was approved by the Institutional Review Board, and informed consent was obtained from all patients. All tumors were pathologically diagnosed CRC and staged according to the TMN staging system; two patients were Stage I, 17 were Stage II, 16 were Stage III, and eight were Stage IV. CRC cell lines (Caco2, colo205, HT29, HCT116) and a breast cancer cell line (MCF7) were obtained from the RIKEN BioResource Center (Tsukuba, Japan). CRC cell lines were grown in RPMI1640 containing 10% fetal bovine serum, and MCF7 cells were grown in DMEM containing 5% fetal bovine serum. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

2.2. *Bisulfite treatment and promoter methylation analysis*

Genomic DNA was extracted with a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. All primers were designed using Methprimer (*http://www.urogene.org/methprimer/index1.html*) or Methyl Primer Express Software v1.0 (Applied Biosystems, Foster City, CA) and purchased from Operon Biotechnology (Tokyo, Japan). Bisulfite modification was performed by the sodium bisulfite method with the EZ DNA Methylation Kit (Zymo Research, Orange, CA). The bisulfite-treated genomic DNA was amplified with HotStar Taq Polymerase (Qiagen) (15 min at 94°C followed by 45 cycles of 20 s at 94°C, 30 s at 56°C, and 1 min at 72°C, with a 3-min final extension at 72°C). For MassARRAY EpiTYPER, the reverse primer has a T7 promoter tag for *in vitro* transcription (5′-cagtaatacgactcacta tagggagaaggct-3′), and the forward primer was tagged with a 10-mer to balance the Tm (5′-aggaagagag-3′). The primer sequences for *RORA* were as follows: Rora-1F: aggaagagagTTGT AGAAAAATTAAAGTTAGGGGG and Rora-1R: cagt aatacgactcactatagggagaaggctCAAACAAAACTATTCC AACACCAACA, Tm 56°C; Rora-4F: aggaagagagTGT TGGTGTTGGAATAGTTTTGT and Rora-4R: cagtaata cgactcactatagggagaaggctTTTTTTAATACCATAAAATT ACTCTAA, Tm 56°C (Operon). The PCR products were analyzed by gel electrophoresis and then examined by Sequenom MassARRAY quantitative analysis using the Mass ARRAY Compact System (Sequenom, San Diego, CA), as described previously (*2,16*). The bisulfite-treated DNA was also directly sequenced on an Applied Biosystems 3130xl Genetic Analyzer using a BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's instructions (Applied Biosystems).

2.3. *Quantitative PCR*

The mRNA expression levels were analyzed by quantitative PCR (qPCR). Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA)

and the RNeasy Mini Kit (Qiagen). RNA integrity and quality were assessed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. Single-stranded cDNA was synthesized from total RNA using the PrimeScript RT reagent Kit (Takara Bio, Tokyo, Japan). The generated cDNA was amplified on a Thermal Cycler Dice Real-Time System (Takara Bio) using SYBR Premix Ex Taq (Takara). The primer sequences and annealing temperatures for *RORA1* were as follows: HA057856-F: 5′-CAGAGCTATTCCAGCACCAGCA-3′ and HA057856-R: 5′-GGATTCCTGATGATTTGTCTCCAC-3′ (Takara Bio), Tm 63°C. The expression level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was measured using GAPDH-F: 5′-GCACCGTCAAGGCTGAGAA-3′ and GAPDH-R: 5′-TGGTGAAGACGCCAGTGGA-3′ primers at an annealing temperature of 60°C. *GAPDH* was amplified as a control to normalize the amount of input cDNA. The experiments were performed in triplicate.

2.4. *Statistical analysis*

Data collected from each experiment were statistically analyzed with the Pearson chi-square test and the ANOVA test. *p* values of less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. *Frequent methylation of the RORA1 promoter in colorectal tissue compared with RORA4*

To determine the methylation status of the *RORA* promoter in CRC, we used the Sequenom MassARRAY method and 43 fresh-frozen CRC surgical specimens, adjacent normal mucosa, four CRC cell lines, and a breast cancer cell line. Among the four different RORA isoforms, only *RORA1* and *RORA4* are actually transcribed, whereas the level of *RORA2* and *RORA3* are undetectable in normal tissue (*8*). Therefore, we focused our investigation on the methylation status of the *RORA1* and *RORA4* promoter regions. Methylation of the *RORA1* promoter was frequently observed in CRC samples and CRC cells (Figure 1). Comparing the epigrams of *RORA1* and *RORA4*, methylation of the *RORA1* promoter was more frequently observed than that of *RORA4* (Figure 2 and Figure 3). These results suggested that methylation of the *RORA1* promoter, but not the *RORA4* promoter, may occur as a distinct alteration in CRC. This finding prompted us to further analyze the methylation status of *RORA1* in CRC.

3.2. *Aberrant hypermethylation of the RORA1 promoter in CRC*

We then evaluated the methylation rate of the *RORA1* promoter in 43 paired CRCs and their adjacent normal mucosa. As shown in (Figure 4A), we defined hypermethylation as an average percent methylation of the entire target region of 38% or more, whereas hypomethylation was defined as less than 38%. Aberrant hypermethylation of the *RORA1* promoter was found in 16 of 43 CRC cases (37%). This finding was consistent with a previous study in which downregulation of *RORA* mRNA expression was frequently observed in CRCs (*6*). Three of the four CRC cell lines, Caco2, HT29, and HCT116, showed significantly increased *RORA1* promoter methylation compared with the MCF7 breast cancer cell line. To validate the *RORA1* promoter methylation data obtained from MassARRAY EpiTYPER, additional direct bisulfite sequencing analysis was performed using representative examples of the same samples. Consistent with the MassARRAY EpiTYPER data, direct bisulfite sequencing analysis clearly detected methylated cytosines in CRC samples. Representative examples of the paired samples of N9 and T9, and N11 and T11 are shown in (Figure 4B). Importantly, hypermethylation of N11 (normal mucosa), which was adjacent to T11 (tumor tissue), was also consistent with the MassARRAY EpiTYPER data showing peaks for both methylated cytosines and uracils at similar levels as T9.

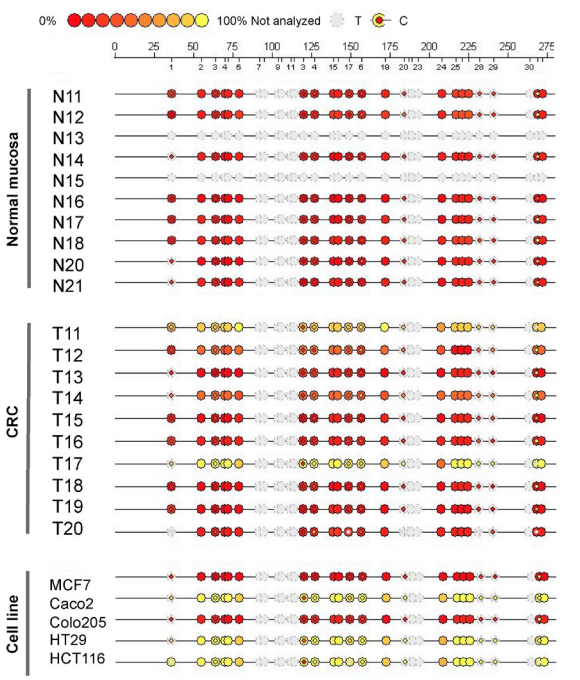

Figure 1. DNA methylation of the *RORA1* promoter region. The EpiTYPER program from Sequenom MassARRAY analysis presents the results of the percent of DNA methylation of the *RORA1* promoter region as an epigram. The epigram shows the percentage of DNA methylation at each CpG site of the target region. Different colors indicate relative methylation changes in 10% increments. The yellow circle indicates 100% methylation, and the red circle is 0% methylation at each CpG site. The number of CpG sites, target sequence length, and sample names are included in each epigram. *T*, primary tumor from a CRC patient; **N**, adjacent normal mucosa.

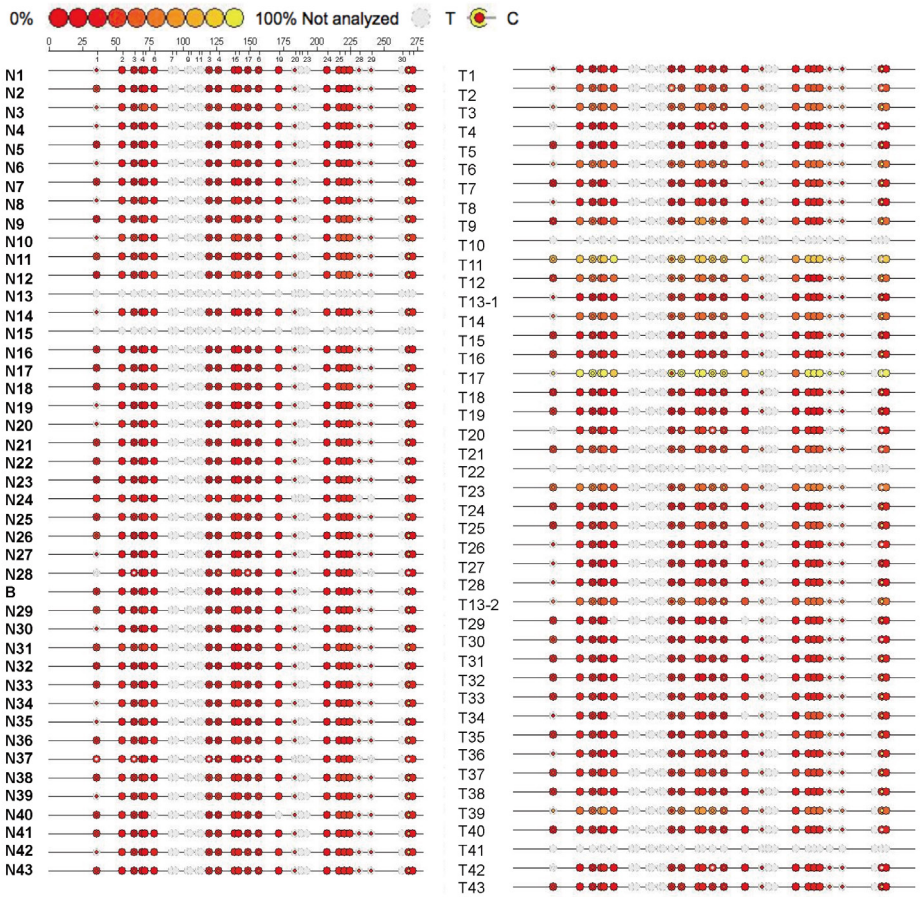

Figure 2. The rate of DNA methylation of the *RORA1* promoter in all CRC cases obtained from Sequenom MassARRAY analysis.

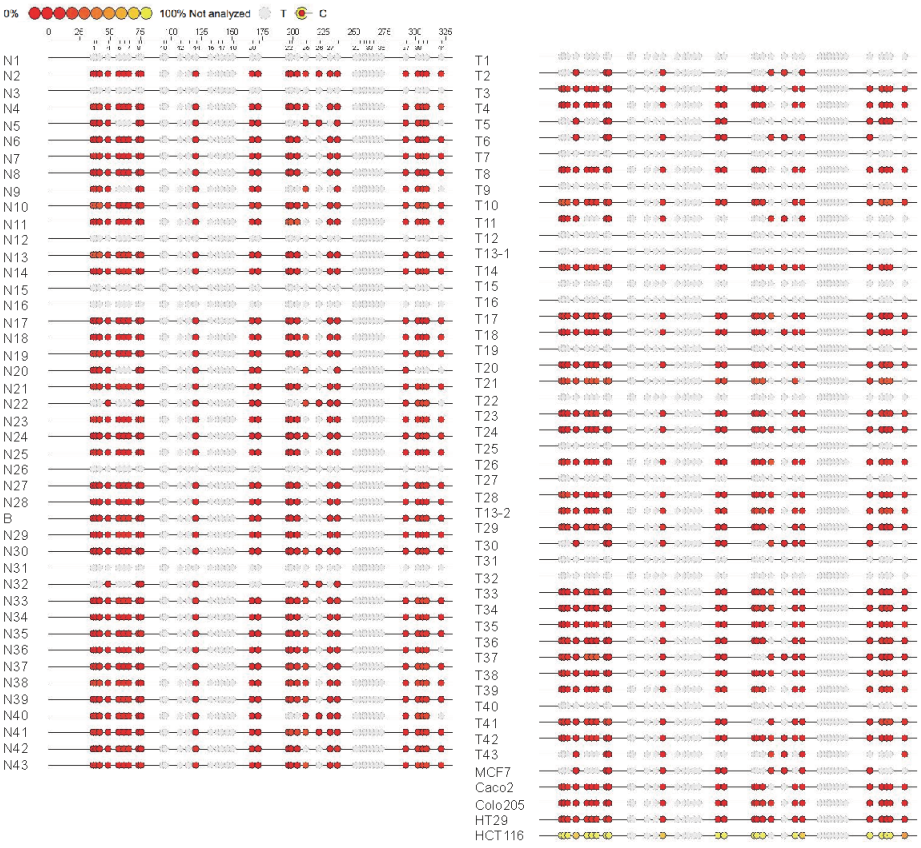

Figure 3. The rate of DNA methylation of the *RORA4* promoter in all CRC cases obtained from Sequenom MassARRAY analysis.

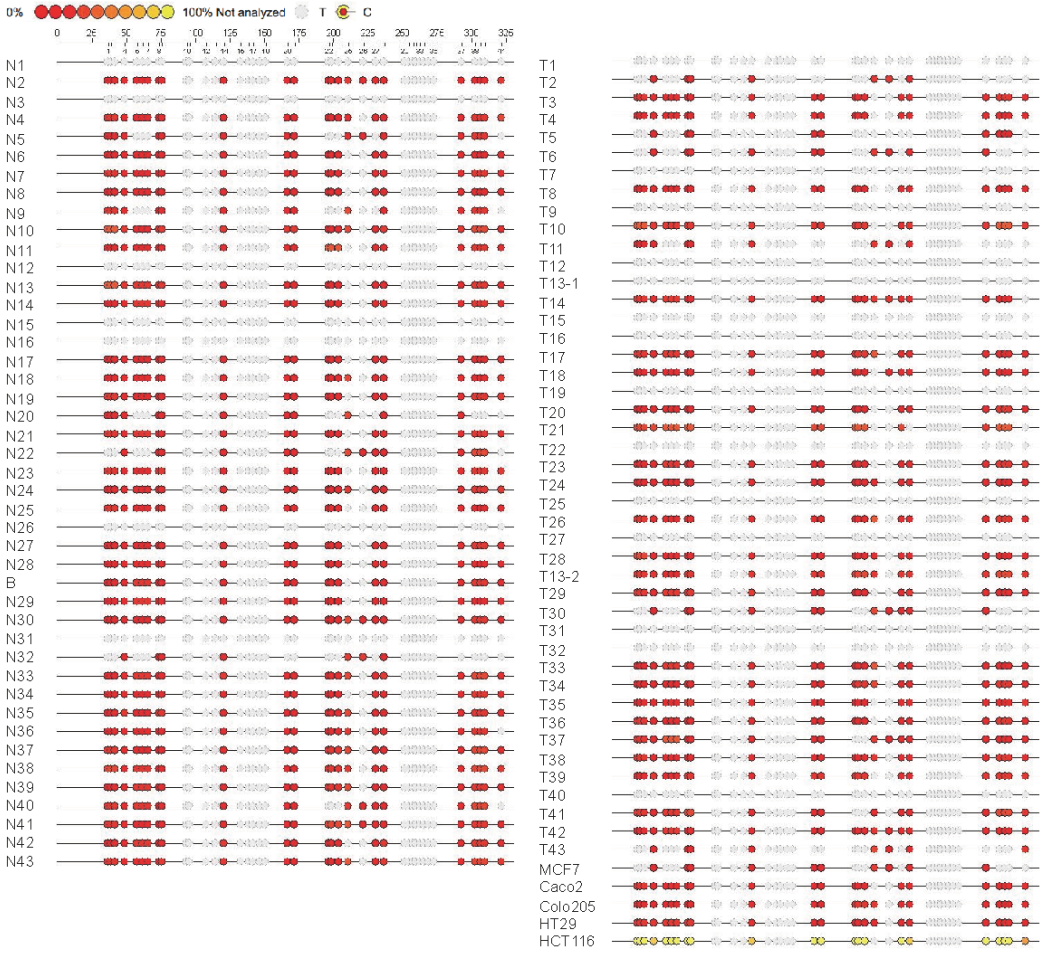

Figure 4. Promoter methylation of *RORA1* in CRC. (A) The y-axis represents the average methylation levels for the entire 354-bp target region of the bisulfite-treated fragment amplified by Rora-1F and Rora-1R primers. Error bars indicate the standard deviation of the mean. The black dashed line shows the average methylation levels for the entire target region of *RORA1*. **(B)** Histogram of bisulfite sequencing analysis depicting the methylation status of CpG sites. Blue arrows indicate the positions of methylated cytosine residues. Red arrows indicate the uracil residues converted from unmethylated cytosines by the bisulfite treatment. *T*, primary tumor from a CRC patient; *N*, adjacent normal mucosa.

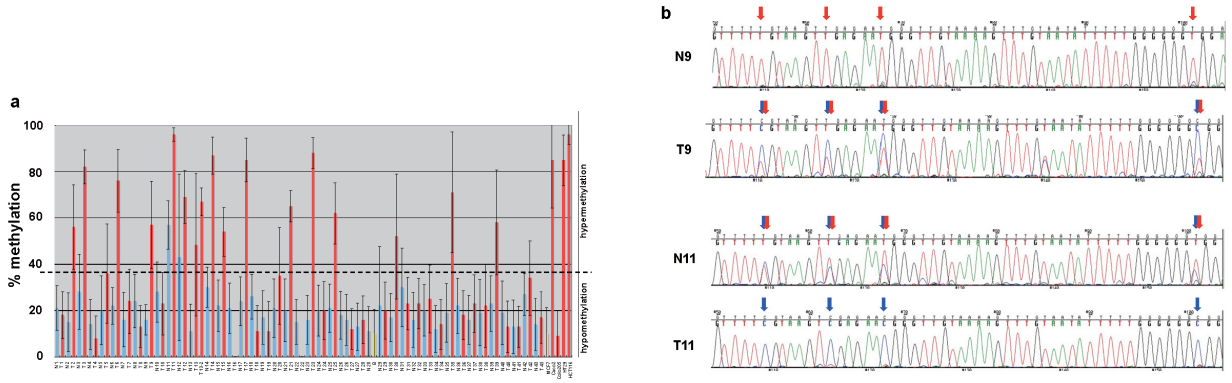

Figure 5. RORA1 mRNA levels in CRC cells and tumor tissues. Quantitative real-time PCR was performed to measure the expression of *RORA1* mRNA. The expression level was normalized to *GAPDH* expression. The y-axis represents the normalized value determined by the standard curve of each gene. The expression level was calculated as the mean of three independent experiments. Error bars indicate the standard deviation of the mean. **(A)** The relative expression of *RORA1* mRNA is shown as a histogram, and the methylation status of each cell line is shown below the graph. **(B)** Relative expression of *RORA1* in primary CRC and adjacent normal mucosa is shown as a histogram. *T*, primary tumor from a CRC patient; *N*, adjacent normal mucosa.

3.3. *Downregulation of RORA1 expression in CRCs*

To determine whether alteration in *RORA1* expression is associated with promoter methylation, we used qPCR to evaluate the level of *RORA1* mRNA in four CRC cell lines and the breast cancer cell line, as well as the 30 CRC surgical specimens and adjacent normal mucosa tissue for which RNA was available. *RORA1* expression

Table 1. Correlation between RORA1 methylation status and clinical CRC stage

Items	RORA1 methylation		*p*-value
	hypermethylation	hypomethylation	
Favorable CRC (*n* = 19)	9	10	0.014
Unfavorable CRC (*n* = 24)	7	17	

CRC, colorectal cancer.

was downregulated in the CRC cell lines (Caco2, colo205, HT29, HCT116) compared with the MCF7 breast cancer cell line (Figure 5A). Of note, although colo205 cells showed hypomethylation of the *RORA1* promoter, *RORA1* expression remained downregulated. Furthermore, low levels of *RORA1* expression were detected in almost every primary CRC compared with the adjacent normal mucosa, although we showed with MassARRY EpiTYPER that only 37% of the CRC samples were methylated (Figure 4B).

3.4. *3.4. Inverse correlation between methylation of the RORA1 promoter and the clinical stage of CRC*

Because promoter hypermethylation of *RORA1* was likely not correlated with its expression, we next assessed whether the methylation status of *RORA1* could be another prognostic indicator of CRC that is independent of gene expression. For this purpose, the 43 CRC patients were divided into two groups according to clinicopathological stage: 19 patients were grouped in favorable stages (stages I and II) and 24 patients in unfavorable stages (stages III and IV). We detected a significant difference between the two groups with respect to the methylation status of the *RORA1* promoter ($p = 0.014$) (Table 1). This result indicated that unfavorable, later stages of CRC were associated with decreased methylation of the *RORA1* promoter compared with favorable, earlier stages of CRC.

4. Discussion

Our study showed that the promoter region of *RORA1* was hypermethylated in CRC patients, and the methylation status of this gene was significantly associated with the clinical CRC stage. These results suggest that the methylation status of the *RORA1* promoter may constitute a useful biomarker to determine an indication for postoperative therapy such as adjuvant chemotherapy in highly advanced CRC patients.

We performed MassARRAY EpiTYPER analysis to quantitatively determine the methylation level at CpG sites in the *RORA1* promoter, which has been identified as a conserved tumor-specific DMR in a mouse model. A previous study showed that *RORA* expression is frequently inactivated in breast, prostate, and ovarian cancer. Notably, *RORA* is located in the middle of FRA15A, a common fragile site in chromosome 15q22.2 (*8*). Common fragile sites are highly unstable, and recombinogenic regions of the genome involve sister chromatid exchange, translocations, deletions, intrachromosomal gene amplification, and integration of DNA from tumor-associated viruses. Thus, *RORA* may behave as a tumor suppressor as described in the "two-hit" hypothesis, and promoter hypermethylation or a loss-of-function mutation coupled with loss of heterozygosity at the same locus may result in loss of tumor suppressive function. We assessed whether *RORA1* promoter methylation could play a causal role in *RORA1* gene silencing in CRCs. To the best of our knowledge, this is the first report describing the methylation status of the *RORA1* promoter in CRCs.

Consistent with a previous study, lower expression of *RORA1* was observed in CRCs compared with adjacent normal mucosa. Interestingly, *RORA1* was clearly downregulated in CRC cell lines compared with the MCF7 breast cancer cell line. Our study shows a slight discrepancy with a previous study that reported that *RORA* expression is frequently downregulated in breast cancer cell lines, including the MCF7 cell line, compared with the normal breast epithelium cell line MCF12F (8). This conflicting result may be due to the different primer sets used in each group. The previous study used primers that were designed to amplify a common region to detect all four isoforms of *RORA*, whereas we designed specific primers to amplify only *RORA1*.

CRC is well-known to result from multiple steps of genetic and epigenetic alterations that lead to the transformation of normal colonic epithelium to colon adenocarcinoma. Given the potential for functional involvement of RORA in canonical Wnt/β-catenin signaling, which is a genetic gatekeeper for CRC tumorigenesis, the increased methylation of *RORA1* and thus downregulation of expression may be required in an earlier step of the adenoma-carcinoma sequence. However, the "trigger" and mechanism that decrease *RORA1* methylation as CRC progresses are unknown. Regarding epigenetic alterations during CRC development, *RORA1* should be recognized not as part of the conventional two-hit model of "loss of heterozygosity (LOH) and methylation", but as an atypical tumor suppressor. Interestingly, a recent study using genome-wide single nucleotide polymorphism linkage arrays identified chromosome 15q22 as a novel CRC susceptibility locus (*17*). Additionally, an earlier linkage

study of autism spectrum disorder also demonstrated that chromosome 15q is an "epigenetic hotspot" or region that is susceptible to genomic imprinting that confers a risk for this disorder (*18*). As *RORA* is located on chromosome 15q, *RORA* gene expression likely involves a complex regulatory mechanism for expression, similar to that observed for loss of imprinting of *insulin-like growth factor 2*, a marker of the CpG island methylator phenotype in CRC (*19-21*). Indeed, a recent study revealed that RORA protein is destabilized by methyltransferase-mediated monomethylation of *zeste homolog 2* (*22*), which is frequently overexpressed in CRC (*23,24*). Taken together, we speculate that in favorable, early stages of CRC, *RORA1* expression is downregulated by methylation of CpG islands in the promoter region, and as CRC becomes advanced, along with accumulating genetic and epigenetic alterations in chromosome 15q, the methylation status of the *RORA1* promoter may become hypomethylated. *RORA1* hypomethylation is consistent with the observation of global hypomethylation in CRC from the discovery of large hypomethylated blocks in CRC that corresponds to more than half the genome (*25,26*). Additionally, decreasing *RORA1* expression may be no longer dependent on promoter hypermethylation but may be related to histone modification and/or posttranscriptional degradation in unfavorable CRC. Determining the acetylation and methylation of histone H3 at lysines 9 and 4, respectively, and methylation at lysine 9 in *RORA* promoter regions may provide mechanistic insight into the downregulation of *RORA1* expression in unfavorable, later stages of CRC.

In conclusion, we speculate that the methylation status of the *RORA* promoter may have important implications for prognosis of CRC that is independent of *RORA* expression. Identification of a useful marker for predicting the benefit of adjuvant chemotherapy for CRC patients is important. Therefore, we propose that hypomethylation of *RORA1* may be an attractive prognostic factor to identify patients with advanced CRC who require postoperative chemotherapy.

Acknowledgements

This work was mainly supported by a Grant-in-Aid for Scientific Research 21591883 (H.N.) and 15K10152 (Y.M.) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and the Academic Frontier Project for Private Universities: matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

1. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. Gastroenterology. 2008; 135:1079-1799.
2. Shinojima Y, Terui T, Hara H, *et al*. Identification and analysis of an early diagnostic marker for malignant melanoma: ZAR1 intra-genic differential methylation. J Dermatol Sci. 2010; 59:98-106.
3. Watanabe T, Yachi K, Ohta T, Fukushima T, Yoshino A, Katayama Y, Shinojima Y, Terui T, Nagase H. Aberrant hypermethylation of non-promoter zygote arrest 1 (ZAR1) in human brain tumors. Neurol Med Chir. 2010; 50:1062-1069.
4. Fujiwara K, Ghosh S, Liang P, Morien E, Soma M, Nagase H. Genome-wide screening of aberrant DNA methylation which associated with gene expression in mouse skin cancers. Mol Carcinog. 2015; 54:178-188.
5. Sugito K, Kawashima H, Uekusa S, *et al*. Identification of aberrant methylation regions in neuroblastoma by screening of tissue-specific differentially methylated regions. Pediatr Blood Cancer. 2013; 60:383-389.
6. Kottorou AE, Antonacopoulou AG, Dimitrakopoulos FI, Tsamandas AC, Scopa CD, Petsas T, Kalofonos HP. Altered expression of NFY-C and RORA in colorectal adenocarcinomas. Acta Histochem. 2012; 114:553-561.
7. Giguere V, Tini M, Flock G, Ong E, Evans RM, Otulakowski G. Isoform-specific amino-terminal domains dictate DNA-binding properties of ROR alpha, a novel family of orphan hormone nuclear receptors. Genes Dev. 1994; 8:538-553.
8. Zhu Y, McAvoy S, Kuhn R, Smith DI. RORA, a large common fragile site gene, is involved in cellular stress response. Oncogene. 2006; 25:2901-2908.
9. Kallen J, Schlaeppi JM, Bitsch F, Delhon I, Fournier B. Crystal structure of the human RORalpha Ligand binding domain in complex with cholesterol sulfate at 2.2 A. J Biol Chem. 2004; 279:14033-14038.
10. Wiesenberg I, Missbach M, Kahlen JP, Schrader M, Carlberg C. Transcriptional activation of the nuclear receptor RZR alpha by the pineal gland hormone melatonin and identification of CGP 52608 as a synthetic ligand. Nucleic Acids Res. 1995; 23:327-333.
11. Lee JM, Kim IS, Kim H, Lee JS, Kim K, Yim HY, Jeong J, Kim JH, Kim JY, Lee H, Seo SB, Kim H, Rosenfeld MG, Kim KI, Baek SH. RORalpha attenuates Wnt/beta-catenin signaling by PKCalpha-dependent phosphorylation in colon cancer. Mol Cell. 2010; 37:183-195.
12. Kim H, Lee JM, Lee G, Bhin J, Oh SK, Kim K, Pyo KE, Lee JS, Yim HY, Kim KI, Hwang D, Chung J, Baek SH. DNA damage-induced RORalpha is crucial for p53 stabilization and increased apoptosis. Mol Cell. 2011; 44:797-810.
13. Delerive P, Monté D, Dubois G, Trottein F, Fruchart-Najib J, Mariani J, Fruchart JC, Staels B. The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response. EMBO Rep. 2001; 2:42-48.
14. Chauvet C, Bois-Joyeux B, Berra E, Pouyssegur J, Danan JL. The gene encoding human retinoic acid-receptor-related orphan receptor alpha is a target for hypoxia-inducible factor 1. Biochem J. 2004; 384:79-85.
15. Chauvet C1, Vanhoutteghem A, Duhem C, Saint-Auret G, Bois-Joyeux B, Djian P, Staels B, Danan JL. Control of gene expression by the retinoic acid-related orphan receptor alpha in HepG2 human hepatoma cells. PloS One. 2011; 6:e22545.
16. Ehrich M, Nelson MR, Stanssens P, Zabeau M, Liloglou T, Xinarianos G, Cantor CR, Field JK, van den Boom D. Quantitative high-throughput analysis of DNA

methylation patterns by base-specific cleavage and mass spectrometry. Proc Natl Acad Sci U S A. 2005; 102:15785-15790.
17. Cicek MS, Cunningham JM, Fridley BL, *et al*. Colorectal cancer linkage on chromosomes 4q21, 8q13, 12q24, and 15q22. PloS One. 2012; 7:e38175.
18. Schanen NC. Epigenetics of autism spectrum disorders. Hum Mol Genet. 2006; 15:R138-R150.
19. Baba Y, Nosho K, Shima K, Huttenhower C, Tanaka N, Hazra A, Giovannucci EL, Fuchs CS, Ogino S. Hypomethylation of the IGF2 DMR in colorectal tumors, detected by bisulfite pyrosequencing, is associated with poor prognosis. Gastroenterology. 2010; 139:1855-1864.
20. Cheng YW, Idrees K, Shattock R, Khan SA, Zeng Z, Brennan CW, Paty P, Barany F. Loss of imprinting and marked gene elevation are 2 forms of aberrant IGF2 expression in colorectal cancer. Int J Cancer. 2010; 127:568-577.
21. Ogino S, Kawasaki T, Kirkner GJ, Kraft P, Loda M, Fuchs CS. Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J Mol Diag. 2007; 9:305-314.
22. Lee JM, Lee JS, Kim H, *et al*. EZH2 generates a methyl degron that is recognized by the DCAF1/DDB1/CUL4 E3 ubiquitin ligase complex. Mol Cell. 2012; 48:572-586.
23. Mimori K, Ogawa K, Okamoto M, Sudo T, Inoue H, Mori M. Clinical significance of enhancer of zeste homolog 2 expression in colorectal cancer cases. Eur J Surg Oncol. 2005; 31:376-380.
24. Wang CG, Ye YJ, Yuan J, Liu FF, Zhang H, Wang S. EZH2 and STAT6 expression profiles are correlated with colorectal cancer stage and prognosis. World J Gastroenterol. 2010; 16:2421-2427.
25. Hansen KD, Timp W, Bravo HC, Sabunciyan S, Langmead B, McDonald OG, Wen B, Wu H, Liu Y, Diep D, Briem E, Zhang K, Irizarry RA, Feinberg AP. Increased methylation variation in epigenetic domains across cancer types. Nat Genet. 2011; 43:768-775.
26. Timp W, Feinberg AP. Cancer as a dysregulated epigenome allowing cellular growth advantage at the expense of the host. Nat Rev Cancer. 2013; 13:497-510.

(Received May 28, 2016; Revised June 5, 2016; Accepted June 6, 2016)