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DNA methylation status of REIC/Dkk-3 gene in human malignancies

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	cancer cell lines was significantly heavier than that in the other cell lines that we tested. REIC/Dkk-3 type-a methylation was inversely correlated with the expression of REIC/Dkk-3. REIC/Dkk-3 type-a expression was restored in MDA-MB-231 cells using 5-aza-2'-deoxycytidine treatment. We found that estrogen receptor (ER)-positive breast cancers were significantly more common among the methylated group than among the non-methylated group. Conclusions REIC/Dkk-3 type-a methylation was frequently detected in a broad range of cancers, including breast cancers, NSCLCs, MPMs, gastric cancers and colon cancers, and appeared to play a key role in silencing REIC/Dkk-3 type-a expression in these malignancies.
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DNA methylation status of *REIC/Dkk-3* gene in human malignancies

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Abstract

Purpose The *REIC (Reduced expression in immortalized cells)/Dkk-3* is down-regulated in various cancers and considered to be a tumor suppressor gene. *REIC/Dkk-3* mRNA has two isoforms (type-a,b). *REIC* type-a mRNA has shown to be a major transcript in various cancer cells and its promoter activity was much stronger than that of type-b. In this study, we examined the methylation status of *REIC/Dkk-3* type-a in a broad range of human malignancies.

Methods We examined *REIC/Dkk-3* type-a methylation in breast cancers, non-small cell lung cancers (NSCLCs), gastric cancers and colorectal cancers, and malignant pleural mesotheliomas using a quantitative combined bisulfite restriction analysis assay and bisulfate sequencing. *REIC/Dkk-3* type-a and type-b expression was examined using reverse-transcriptional PCR. The relationships between the methylation and clinicopathological factors were analyzed.

Results The rate of *REIC/Dkk-3* type-a methylation ranged from 26.2% to 50.0% in the various primary tumors that were examined. *REIC/Dkk-3* type-a methylation in breast cancer cells was significantly heavier than that in the other cell lines that we tested. *REIC/Dkk-3* type-a methylation was inversely correlated with *REIC/Dkk-3* type-a expression. There was a correlation between *REIC/Dkk-3* type-a and type-b mRNA expression. *REIC/Dkk-3* type-a expression was restored in MDA-MB-231 cells using 5-aza-2'-deoxycytidine treatment. We found that estrogen receptor-positive breast cancers were significantly more common among the methylated group than among the non-methylated group.

Conclusions *REIC/Dkk-3* type-a methylation was frequently detected in a broad range of cancers and appeared to play a key role in silencing *REIC/Dkk-3* type-a expression in these malignancies.

Keywords DNA methylation - *REIC/Dkk-3* - breast cancer - lung cancer - mesothelioma

Introduction

Accumulating evidence suggests that tumor progression is governed not only by genetic changes intrinsic to cancer cells but also by epigenetic changes. In cancer epigenetics, aberrant CpG methylation in the promoter region is a key mechanism for gene inactivation resulting in tumorigenesis in human malignancies (Toyooka et al. 2004).

The *REIC (Reduced expression in immortalized cells)/Dkk-3(Dickkopf-3)* cDNA, which was expressed in human normal cells and was down-regulated in human immortalized cells and human tumor-derived cells, was identified using a representative difference analysis system (Tsuji et al. 2000). The amino acid sequence revealed that the *REIC* gene product was human *Dkk-3*, one of the Dkk family members. The Dkk family of secreted proteins consists of four members, which share two conserved cysteine-rich domains (Glinka et al. 1998; Krupnik et al. 1999). Dkk-1, the best-characterized member of the Dkk family, functions as a Wnt antagonist or agonist by binding to and inhibiting or activating the Wnt coreceptor LRP6 (Bafico et al. 2001). Unlike Dkk-1, Dkk-2, and Dkk-4, however, REIC/Dkk-3 was recently shown to inhibit TCF-4 receptor activity in lung cancer cells (Yue et al. 2008). TCF-4 activates c-Myc and cyclinD1 through the Wnt/beta-catenin pathway and promotes tumor invasion and metastasis. Because *REIC/Dkk-3* is down-regulated in a variety of malignancies and the overexpression of *REIC/Dkk-3* suppresses cell growth, *REIC/Dkk-3* has been proposed to act as a tumor suppressor (Tsuji et al. 2001; Kurose et al. 2004). Hypermethylation and the down-regulation of *REIC/Dkk-3* were observed in a variety of malignancies including non-small cell lung cancers (NSCLCs) (Kobayashi et al. 2002; Licchesi et al. 2008), gastrointestinal cancers (Maehata et al. 2008), renal clear cell carcinoma (Kurose et al. 2004), acute lymphoblastic leukemia (Roman-Gomez et al. 2004) and osteosarcomas (Hoang et al.

2004). We previously showed the therapeutic effect of REIC/Dkk-3 in prostate cancers (Abarzua et al. 2005; Edamura et al. 2007) and malignant pleural mesothelioma (MPM) (Kashiwakura et al. 2008). In addition, tumor suppression by REIC/Dkk-3 has also been confirmed in other malignant tumors (Hsieh et al. 2004; Hoang et al. 2004).

REIC/Dkk-3 mRNA has two isoforms (type-a,b; GeneBank accession AB057804). Many papers have described the methylation status in the promoter of *REIC/Dkk-3* type-b (Licchesi et al. 2008; Maehata et al. 2008; Veeck et al. 2009). However, the promoter of *REIC/Dkk-3* type-a also seems to be important, since Kobayashi et al. (2002) (the group that first identified the *REIC/Dkk-3* in immortalized cells) have demonstrated that the promoter activity of *REIC/Dkk-3* type-a (major promoter) had an approximately 26-fold stronger effect than that of *REIC/Dkk-3* type-b (minor promoter) in a luciferase assay and major transcript was *REIC/Dkk-3* type-a in various cancer cell they tested. They suggested that hypermethylation of the major promoter (type-a) was a major mechanism for down-regulation of *REIC* expression. They also suggested the methylation of the minor promoter (type-b) was accompanied with that of major promoter (type-a) in most case except four lung cancer cells that they tested. Regardless those four lung cancer cells had type-b hypermethylation, *REIC/Dkk-3* type-b expression was detected in those four lung cancer cells. So they discussed the possibility that minor promoter (type-b) was utilized

for the expression in a tissue specific manner, as seen in dual promoter of APC gene.

In this study, we examined the DNA methylation of *REIC/Dkk-3* type-a in various kinds of cancers by quantitative combined bisulfite restriction analysis (qCOBRA) and investigated the correlation between the *REIC/Dkk-3* type-a methylation and *REIC/Dkk-3* type-a expression. The qCOBRA assay can provide more reliable results because the conventional methylation-sensitive restriction enzyme assay that Kobayashi et al. (2002) performed was recently known to be prone to false positive results due to spurious incomplete digestion (Xinog et al. 1997). We also analyzed the correlation between *REIC/Dkk-3* type-a and type-b expression in various cancer cell lines. Furthermore, we examined the correlation between *REIC/Dkk-3* type-a methylation and the clinicopathological features of primary tumors.

Materials and Methods

Clinical samples and cell culture

Surgically resected specimens of 37 primary breast cancers, 42 primary NSCLCs, 21 primary gastric cancers, 20 primary colon cancers and 7 MPMs were obtained from Okayama University Hospital (Okayama, Japan), 6 MPMs were obtained from Okayama Rousai Hospital (Okayama, Japan), 5 MPMs were obtained from National Sanyo Hospital (Yamaguchi, Japan), 27 MPMs were obtained from

Karmanos Cancer Center (MI). Ten corresponding non-malignant breast tissues and 10 non-malignant lung tissues were also examined. All tissues were frozen with the liquid nitrogen immediately after surgery and were stored at -80°C until extraction of DNA. Institutional Review Board permission and informed consent were obtained for all cases.

Seven breast cancer cell lines (HCC70, HCC1599, HCC1806, MDA-MB-231, MDA-MB-361, MCF7 and ZR75-1) and 11 lung cancer cell lines (NCI-H23, NCI-H44, NCI-H125, NCI-H157, NCI-H1299, NCI-H1819, NCI-H1963, NCI-H1975, NCI-H2009, NCI-H358 and A549), 4 MPM cell lines (NCI-H2052, NCI-H2373, NCI-H2452, NCI-H290), 6 prostate cancer cell lines (PC3, LNCap-FGC, Du145, Caki-1, Caki-2 and KPK) were examined in this study. MCF7, ZR-75-1, MDA-MB-231, MDA-MB-361 were obtained from Cell Resource Center for Biomedical Research Institute of Development Aging and Cancer Tohoku University (Miyagi, Japan). Seven cell lines (HCC70, HCC1599, HCC1806, H2052, H2373, H290, H2452) were kind gifts from Adi F. Gazdar (Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX). Six cell lines (PC3, LNCap-FGC, Du145, Caki-1, Caki-2, KPK) were kind gifts from the department of urology (Okayama university, Okayama, Japan). The other cell lines were obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in RPMI-1640 medium (Sigma Chemical Co., Saint Louis, MO) supplemented

with 10% FBS and were incubated in 5% CO_2.

DNA extraction and DNA methylation modification

Genomic DNA was extracted from the surgically resected frozen samples and cultured cells by digestion with SDS/proteinase K followed by phenol:chloroform (1:1) extraction and ethanol precipitation. Two µg of each DNA was treated with EZ DNA Methylation Kit (ZYMO RESEARCH, Orange, CA), following the manufacturer's instructions and was stored at –20 °C until use.

Quantitative COBRA Assay

Nested PCR was carried out using bisulfite-treated DNA followed the restriction enzyme digestion. First round touchdown PCR was performed under the following conditions: 95°C for 12 min, 40 cycles of 94°C 45 sec, annealing temperature between 58°C to 56°C for 1 min, 72°C for 3 min, followed by final extension step at 72°C for 7 min in a 25 µl reaction mixture containing with 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mm $MgCl_2$, 10 mM β-mercaptoethanol, 1.25mM of each deoxynucleotide triphophate (dNTP) mixture, 0.5 µM of each primer, 0.5 unit of HotStar Taq DNA Polymerase (Qiagen, Valencia, CA) and 100 ng of bisulfite-treated DNA. Second round touchdown PCR was performed using 0.4 µl of the first round PCR products as a template under same condition, but 47 cycles. Universal methylated DNA and universal unmethylated DNA were used for positive control and negative control,

respectively. The location of the CpG dinucleotides in the exon1 and in the 5'-flanking region of *REIC/Dkk-3* is shown in Fig.1. Primers were designed using Primer Express Software ver.1.0 in the promoter region of *REIC/Dkk-3* type-a. Primers for the first round PCR were *REIC*-COBRA-F1 5’-TGGGTTGTTGTAAGTTTGAAGGT-3’, *REIC*-COBRA-R1 5’-CTCACCCACCCCRACTAAAC-3’, Primers for the second round PCR were as follows: *REIC*-COBRA-F2 5’-TGAAGGTTAGATAAGAYGGGTTTAGG-3’, *REIC*-COBRA-R2 5’-ACCCACCCCRACTAAACCRAAT-3’. These primers were designed to ensure amplification of both methylated and unmethylated forms. Two µl of second PCR products were digested with 3 units of BstUI (which restriction site is CGCG) for the restriction fragment length polymorphism analysis. The amplicon of second PCR was named RRCOBRA (Region for REIC COBRA) and the 5 restriction sites of BstUI are shown in Fig.1. The digested PCR products were visualized on 3% agarose gels stained with ethidium bromide. The percentages of digested band were analyzed by NIH ImageJ 1.37V Software (http://rsb.info.nih.gov/ij) as described previously (Xiong and Laird 1997). We performed linear regression analysis of qCOBRA with nested PCR using serial dilution to examine if qCOBRA with nested PCR really reflected % methylation. We diluted unmethylated DNA amplicon with methylated amplicon to make serial dilution (% methylated DNA; 0, 10, 20, 30, 50, 70, 80, 90, 100 %) and performed

qCOBRA, as described above.

Bisulfited DNA Sequencing Analysis

RRCOBRA was cloned into pCR2.1-TOPO Vector using TOPO TA cloning kit (Invitrogen Life Technologies, Carlsbad, CA) following manufacturer's instructions. To determine the methylation status in the promoter lesion of *REIC/Dkk-3* gene, five breast cancer cell lines (MCF-7, MDA-MB-231, ZR75-1, HCC1806 and HCC1599) and a lung cancer cell lines (H1299) were examined. Individual 7 clones from each cell line were sequenced using the dGTP BigDye terminator v3.1 Cycle Sequencing Kit with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

RNA extraction and Reverse Transcriptional (RT)-PCR

Total RNA was extracted from cultured cells using RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instruction. Oligo(dT)-primed cDNA was synthesized using Super-Script II (Qiagen, Valencia, CA) with DNase treatment. RT-PCR was carried out in a 20 µl of reaction mixture with 1xPCR buffer, 200 µM of dNTP, 0.3 µM of each primer, 0.5 units of HotStarTag DNA Polymerase and 100 ng of cDNA. A touchdown PCR was performed for *REIC/Dkk-3* type-a and type-b under the following conditions: 95°C for 12 min, 35 cycles of 94°C 30 sec, annealing temperature between 62°C to 58°C for 1 min, 72°C for 3 min, followed by final extension step at 72°C for 7 min. As an internal control, RT-PCR

for *GAPDH* was carried out under the following conditions: 95°C for 12min, 35 cycles of 94°C for 45 sec, 55°C for 90 sec, 72°C for 90 sec followed by final extension step at 72°C for 7 min. The primers for *REIC/Dkk-3* type-a expression were *REIC*(a)-F 5'-GGGAGCGAGCAGATCCAGT-3' (exon1a), *REIC*(a)-R 5'-TTTGTCCAGTCTGGTTGTTGGT-3' (exon3). The primers for *REIC/Dkk-3* type-b expression were *REIC*(b)-F 5'-TGGGAGCTATTAGCGTAGAGGAT-3' (exon1b), *REIC*(b)-R 5'-CATTGTGATAGCTGGGAGGTAAG-3' (exon3). The PCR products were visualized on 2% agarose gels stained with ethidium bromide. The bands were analyzed using NIH ImageJ 1.37V Software. The expression ratio in each cell line was defined as the ratio of particular sample when compared to those of H1299. To confirm the responsibility of DNA methylation for *REIC/Dkk-3* silencing, we treated heavily methylated cell lines (MDA-MB-231) with 5-Aza-2'-deoxycytidine (5-Aza-CdR) at the concentration of 5 μM and 8 μM for 6 days with medium changes on days1, 3 and 5. Treated and untreated cells from individual triplicate flasks were harvested on day 6 to determine the *REIC/Dkk-3* type-a expression using RT-PCR.

ER, PgR and HER2 status in primary breast cancers

Estrogen receptor (ER), Progesterone receptor (PgR) and HER2 status in primary breast cancers was obtained from patient medical records. HER2 positive was defined as a score of 2+ and 3+ by

immunohistochemistry.

Statistical analyses

The frequencies of *REIC/Dkk-3* methylation between two groups were compared using the Fisher's exact test or Mann-Whitney's U test when appropriate. Probability value less than 0.05 was defined as being statistically significant. All data was analyzed by JMP9 for Windows (SAS Institute, Cary, NC).

Results

DNA methylation status in the promoter region of *REIC/Dkk-3* type-a

The result of bisulfite genomic DNA sequencing of RRCOBRA are shown in Fig.1. Each CpG in the 5'-flanking region and in exon1a was heavily methylated in MCF7, MDA-MB-231 and ZR75-1. The CpGs in the 5'-flanking region of exon 1a were lightly to moderately methylated, but the CpGs in exon1a were rarely methylated in HCC1806 and HCC1599. In contrast, most of the CpGs were rarely methylated in H1299.

We performed the linear regression analysis using the nested qCOBRA and confirmed the quantitative capacity (data not shown). Representative examples of the COBRA assay in breast cancer cell lines are shown in Fig. 2a. The percentages of *REIC/Dkk-3* type-a methylation were calculated by

qCOBRA in each cell line and primary tumor (Fig. 2b and 2c, respectively) and summarized in Table1. We decided the samples with more than 10% of digested bands as methylation positive in this study. Aberrant methylation was detected in 7 of the 7 (100%) breast cancer cell lines and 16 of the 37 (43.2%) primary breast cancers; 5 of the 11 (45.4%) lung cancer cell lines and 11 of the 42 (26.2%) primary lung cancers; 0 of 4 (0%) in MPM cell lines and 7 of the 27 (25.9%) USA primary MPMs; 7 of the 18 (38.9%) Japanese primary MPMs, 8 of the 21 (38.1%) primary gastric cancers; and 10 of the 20 (50.0%) primary colon cancers. *REIC/Dkk-3* methylation was not detected in 10 normal breast tissues and 10 normal lung tissues (data not shown). The methylation of *REIC/Dkk-3* type-a in the breast cancer cell lines was more frequent than that in the lung, MPM and prostate cancer cell lines ($p = 0.02$, $p = 0.01$ and $p = 0.04$, respectively). However, no significant differences in methylation were observed among the primary breast, lung, MPMs, gastric and colon cancers (Fig.2c). The results of qCOBRA in five breast cancer cell lines and a lung cancer cell line (H1299) were corresponded with the results of bisulfite sequencing.

***REIC/Dkk-3* mRNA expression in cell lines and correlation with qCOBRA assay.**

Representative example of RT-PCR for *REIC/Dkk-3* type-a and type-b in the breast cancer cell lines was shown in Fig.2d. Expression of *REIC/Dkk-3* type-a was only detected in HCC1599 and HCC1806 cells, which rarely to moderately harbored *REIC/Dkk-3* methylation. Expression of *REIC/Dkk-3* type-b was also

detected in HCC1599 and HCC1806 cells. The results of expression of *REIC/Dkk-3* type-a and type-b in all cell lines (n=28) were summarized in Table 2. There was a correlation between the expression of *REIC/Dkk-3* type-a and type-b ($p<0.01$). The relative expressions of *REIC/Dkk-3* type-a mRNA and the percentages of *REIC/Dkk-3* type-a methylation are shown in Fig.3. The expression of *REIC/Dkk-3* type-a and *REIC/Dkk-3* type-a methylation was inversely correlated in the cell lines that were examined ($p<0.01$). To confirm that methylation was responsible for the gene silencing, heavily methylated MDA-MB-231 cells were treated with 5-Aza-CdR. *REIC/Dkk-3* type-a mRNA expression was restored by the treatment of 5-Aza-CdR in a dose-dependent manner (Fig.4).

***REIC/Dkk-3* methylation and clinicopathological correlation.**

We next examined the relationships between the *REIC/Dkk-3* methylation status and the clinicopathological factors described in Table 3. For the breast cancers, we observed that ER-positive cases were more common in the methylated group than in the nonmethylated group ($p=0.03$). No significant relationships between *REIC/Dkk-3* methylation and the other clinicopathological factors were observed.

Discussion

In this study, we demonstrated that arbitrary CpG methylation in *REIC/Dkk-3* type-a promoter region was frequently observed in solid malignancies. Regarding qCOBRA in this study, we confirmed the accuracy of this assay by linear regression analysis because we performed nested PCR. We decided the samples with more than 10% of digested bands as methylation positive to maintain compatibility with conventional COBRA assay, as we could distinguish 10% of digested band on the agarose gel electrophoresis. Collolla et al. (2003) also used a 10% threshold to declare methylation when compared qCOBRA with pyrosequencing methylation analysis. So a 10% threshold seems to be reasonable criteria to distinguish methylation positive. We examined cell lines using qCOBRA assay, and the accuracy of the qCOBRA was also confirmed by bisulfate genomic DNA sequencing and linear regression analysis.

The *REIC/Dkk-3* type-a methylation, that was determined using qCOBRA assay, revealed to be inversely correlated with the *REIC/Dkk-3* type-a mRNA expression among the cell lines ($p<0.01$), and the restoration of *REIC/Dkk-3* type-a expression by 5-Aza-CdR treatment was observed in a *REIC/Dkk-3* type-a methylated cell line. These results indicate that DNA methylation of *REIC/Dkk-3* type-a was responsible for silencing *REIC/Dkk-3* type-a expression. As reported previously, we confirmed that there was a correlation between *REIC/Dkk-3* type-a expression and type-b expression in the cell lines that we

examined ($p<0.01$). Discrepancy of the expression level between *REIC/Dkk-3* type-a and type-b was observed in lung cancer cell lines, although the expressions of *REIC/Dkk-3* type-a were completely corresponded with the expression of *REIC/Dkk-3* type-b in other cell lines, indicating that *REIC/Dkk-3* type-b might be utilized for the expression in a tissue specific manner, as Kobayashi et al. (2002) described.

Among the cancers that were examined, *REIC/Dkk-3* type-a methylation was more frequently detected in breast cancer cell lines, although moderate methylation was also observed in other cancers. A previous report showed that the introduction of REIC/Dkk-3 into some breast cancer cells had an anti-tumor effect (Kawasaki et al. 2009). In addition, the introduction of REIC/Dkk-3 into cancer cells had a direct effect on the induction of apoptosis and an indirect effect on the activation of tumor immunity in NK cells through the up-regulation of IL-7 (Sakaguchi et al. 2009). Furthermore, REIC/Dkk-3 induces the differentiation of human CD14+ monocytes into a novel cell type, resembling immature dendritic cells generated with IL-4 and GM-CSF (Watanabe et al. 2009). These findings support the possible utility of *REIC/Dkk-3* gene therapy for not only breast cancers but also a broad range of human malignancies. Indeed, *REIC/Dkk-3* gene therapy is ongoing for prostate cancer (http://clinicaltrials.gov/ct2/show/NCT01197209).

Regarding the clinicopathological factors, we found that ER-positive breast cancer was more common in the methylated group than in the non-methylated group in the present study. In contrast, Veeck et al. (2009) reported that *REIC/Dkk-3* methylation was not correlated with the ER and PgR statuses. In other cancers, no significant relationship was observed between the *REIC/Dkk-3* methylation status and any of the clinicopathological factors that were examined. Previous study has shown that *REIC/Dkk-3* methylation was associated with poor survival in primary breast cancers (Veeck et al. 2009). We could not compare these results directly because they examined the *REIC/Dkk-3* type-b methylation, which has a lower promoter activity than that of *REIC/Dkk-3* type-a. In addition, the number of cases in this study may have been too small for the survival analysis, since the primary purpose of this study was to detect *REIC/Dkk-3* type-a methylation and to examine the correlation between methylation and expression. In lung cancer, reduced expression of *REIC/Dkk-3* was previously shown to be frequent in poorly differentiated adenocarcinoma and squamous cell carcinoma (Nozaki et al. 2001). Further investigations are needed to determine the clinicopathological impact of *REIC/Dkk-3* type-a methylation.

In conclusion, we found that the promoter region of *REIC/Dkk-3* type-a was frequently methylated in breast, lung, gastric, colon, prostate cancers and MPMs. *REIC/Dkk-3* type-a methylation and *REIC/Dkk-3* type-a mRNA expression were inversely correlated in the cell lines that were examined.

Our results suggest that *REIC/Dkk-3* type-a methylation is an important mechanism in the pathogenesis of various types of malignancies. Since gene therapy using REIC/Dkk-3 expressing adenovirus vectors is currently on going for the treatment of prostate cancer, similar therapeutic modalities may be applicable for other types of cancers.

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Conflict of interest statement None.

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Table 1 Rate of *REIC/Dkk3* methylation in each human cancer by quantitative COBRA assay

Organ	Number of *REIC/Dkk3* methylated sample (%)	
	Cell lines	Primary tumors
Breast cancer	7 of 7 (100%)	16 of 37 (43.2%)
Lung cancer	5 of 11 (45.4%)	11 of 42 (26.2%)
Malignant pleural mesothelioma		
(USA)	0 of 4 (0%)	7 of 27 (25.9%)
(Japanese)	ND	7 of 18 (38.9%)
Gastric cancer	ND	8 of 21 (38.1%)
Colon cancer	ND	10 of 20 (50.0%)
Prostate cancer	3 of 6 (50%)	ND

ND: not determined

Table 2 Expression of *REIC/Dkk-3* type-a and type-b in various cancer cells

Organ		mRNA expression	
		type-a	type-b
Breast cancers	HCC70	-	-
	HCC1599	+	+
	HCC1806	+	+
	MDA-MB-231	-	-
	MDA-MB-361	-	-
	MCF7	-	-
	ZR75-1	-	-
Lung cancers	H23	+	-
	H44	-	-
	H125	+	-
	H157	+	+
	H1299	+	+
	H1819	+	+
	H1963	+	+
	H1975	-	+
	H2009	-	-
	H358	-	-
	A549	+	+
MPMs	H2052	+	+
	H2373	+	+
	H2452	-	-
	H290	+	+
Prostate cancers	Caki-1	+	+
	Caki-2	+	+
	Du145	+	+
	KPK	+	+
	LNCapFGC	-	-
	PC3	-	-

Table 3 Clinicopathological factors and *REIC/Dkk-3* methylation in various primary cancers

A. primary breast cancers

Variables	Number of methylation positive samples (%)
Total (n=38)	16 (39)
Age	
<65 (n=32)	14 (44)
65≤ (n= 6)	1 (17)
Histology	
papillotubular (n=8)	2 (25)
Solid-tubular (n=11)	4 (36)
Scirrhous (n=17)	9 (53)
Others (n=2)	0
T categories	
1 (n=11)	6 (55)
2 (n=16)	5 (31)
3 (n=3)	1 (33)
4 (n=8)	3 (38)
N categories	
0 (n=15)	6 (40)
1 (n=23)	9 (39)
M categories	
0 (n=36)	14 (23)
1 (n=2)	1 (50)

Stage	
I (n=9)	5 (56)
II (n=15)	5 (33)
III (n=12)	4 (33)
IV (n=2)	1 (50)
Estrogen receptor *	
Positive (n=19)	11 (58)
Negative (n=18)	4 (22)
Progesterone receptor	
Positive (n=18)	9 (50)
Negative (n=15)	6 (40)
HER2 status	
Positive (n=11)	3 (27)
Negative (n=19)	10 (53)

*: $p<0.05$

B. Primary lung cancers

Variables	Number of methylation positive samples (%)
Total (n=41)	11 (27)
Age	
<65 (n=17)	3 (18)
65≤ (n=24)	8 (33)
Histology	
Adenocarcinoma (n=27)	7 (26)
Squamous cell carcinoma (n=14)	4 (29)
T categories	
1 (n=27)	7 (26)

2 (n=12)	4 (33)
3 (n=0)	0
4 (n=2)	0
N categories	
0 (n=29)	8 (28)
1 (n=10)	2 (20)
M categories	
0 (n=39)	11 (28)
1 (n=2)	0
Stage	
I (n=28)	8 (29)
II (n=4)	1 (25)
III (n=7)	1 (14)
IV (n=2)	0

C. Primary gastric cancers

Variables	Number of methylation positive samples (%)
Total (n=21)	8 (38)
Age	
<65 (n=10)	5 (50)
65≤ (n=11)	3 (27)
Histology	
Intestinal (n=10)	5 (50)
Diffuse (n=11)	3 (27)
T categories	
1 (n=3)	1 (55)
2 (n=8)	4 (50)

3 (n=7)	2 (29)
4 (n=3)	0
N categories	
0 (n=7)	4 (57)
1≤ (n=14)	4 (29)
Stage	
I (n=6)	4 (67)
II (n=3)	1 (33)
III (n=5)	3 (60)
IV (n=7)	0

D. Primary colon cancers

Variables	Number of methylation positive samples (%)
Total (n=20)	10 (50)
Age	
<65 (n=9)	5 (56)
65≤ (n=11)	5 (46)
Histology	
Well (n=5)	1 (20)
Moderately (n=11)	6 (55)
Poorly (n=3)	2 (67)
Others (n=1)	1 (100)
T categories	
1 (n=2)	1 (50)
2 (n=0)	0
3 (n=14)	6 (43)
4 (n=3)	3 (100)
N categories	
0 (n=8)	4 (50)

1≤ (n=12)	6 (50)
M categories	
0 (n=12)	6 (50)
1 (n=8)	4 (50)
Stage	
I (n=3)	2 (67)
II (n=4)	1 (25)
III (n=5)	3 (60)
IV (n=8)	4 (50)
Location	
Right (n=8)	5 (63)
Left (n=11)	4 (36)

Figures legends

Fig.1 Map of the 5'-flanking region of *REIC/Dkk-3* and the bisulfite genomic DNA sequence

Upper figure demonstrates the promoter region of *REIC/Dkk-3*. Gray bars indicate exons and the bent arrow indicates the transcription start site (TSS) (+1). Thin vertical lines on the horizontal line indicate the sites of CpG dinucleotides. Allow heads indicate the restriction sites of BstUI. COBRA primer sets are indicated by pairs of bold arrows. Dark gray bar under the COBRA primer sets indicates RRCOBRA. Methylation status of individual subcloned DNA fragments of each cell line is shown below. Each circle represents a CpG dinucleotide in 5'-flanking region of *REIC/Dkk-3* (for -213 to +205 nt). The numbers at the top indicate the CpG dinucleotide in the RRCOBRA (from 5' to 3'). These numbers correspond to those depicted in upper figures. Open circle represents non-methylated CpG dinucleotide; Black circle represents methylated CpG dinucleotide.

Fig.2 Quantitative COBRA assay

The results of COBRA assay for *REIC/Dkk-3* type-a in breast cancer cell lines were shown in Fig.2a. Methylated alleles were fragmented with restriction enzyme modification and

unmethylated alleles were uncut. Percentages of digested band in cell lines (Fig.2b) and primary tumors (Fig.2c) were quantitated by NIH ImageJ 1.37V software. The horizontal bars indicate the average in each group. The mRNA expression of *REIC/Dkk-3* type-a and type-b in breast cancer cell lines was shown in Fig.2d. PC, universal methylated DNA as positive control; NC, universal unmethylated DNA as negative control.

Fig.3 Relative expression of *REIC/Dkk-3* type-a mRNA and percentages of *REIC/Dkk-3* methylation in each cell line

Columns show the relative expression of *REIC/Dkk-3* type-a mRNA in each sample. The expression ratio was defined as the ratio of particular sample when compared to those of H1299. % methylation was calculated by qCOBRA assay.

Fig.4 Restoration of *REIC/Dkk-3* type-a mRNA expression in MDA-MB-231 cells

The effect of 5-Aza-CdR on the restoration of *REIC/Dkk-3* type-a mRNA expressions in heavily methylated breast cancer cells (MDA-MB-231). *REIC/Dkk-3* type-a mRNA was detected by RT-PCR. GAPDH was used as an internal control.

colour figure

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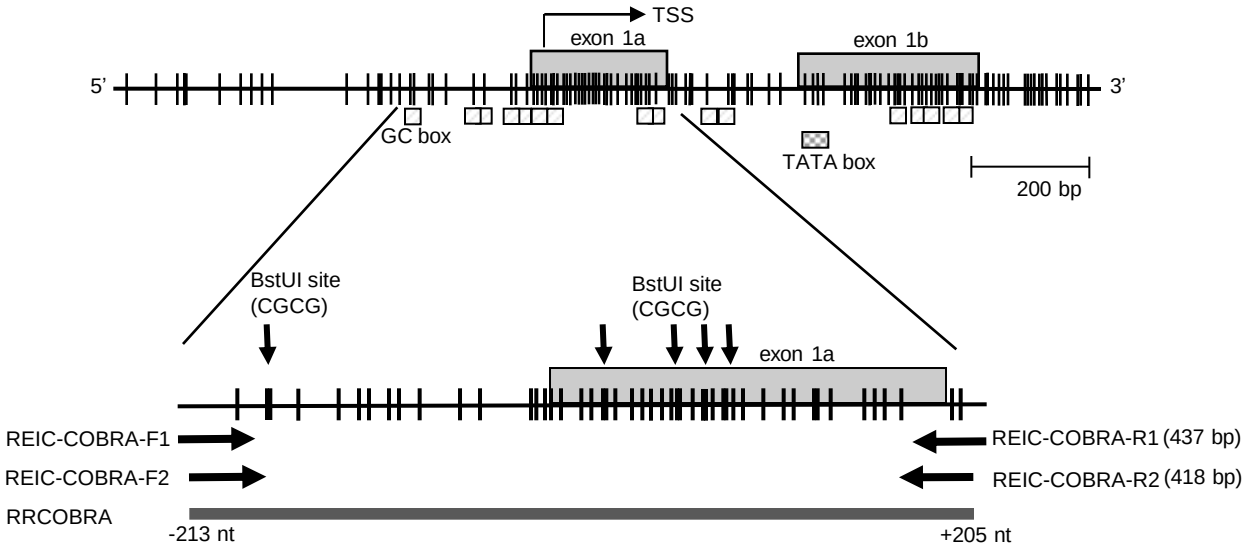

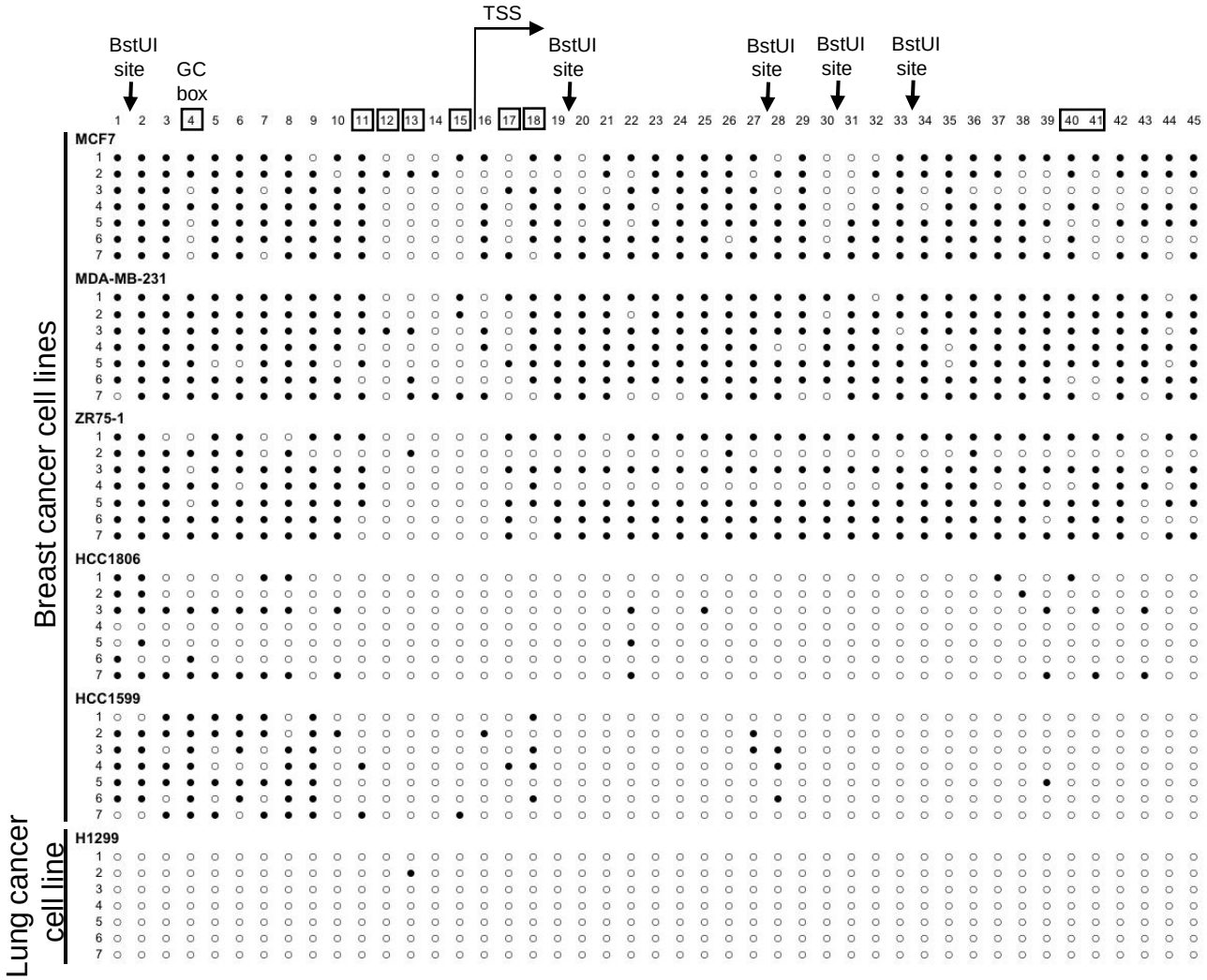

colour figure

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Fig.2a

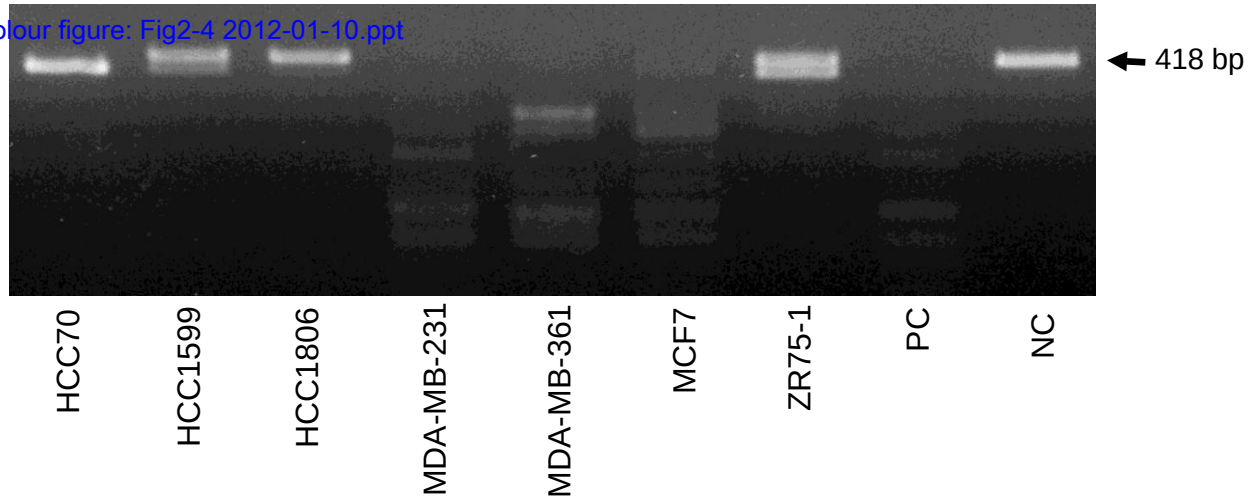


Fig. 2b

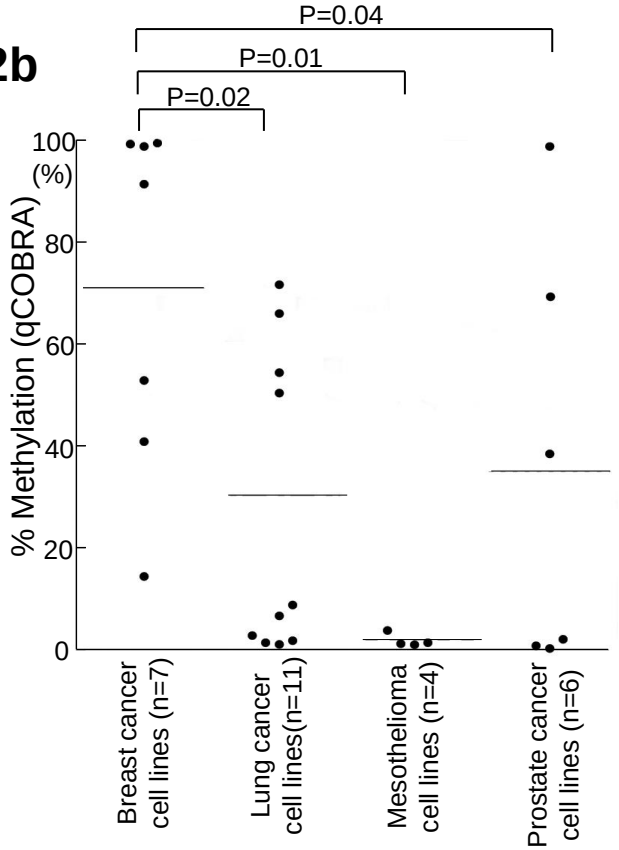


Fig. 2c

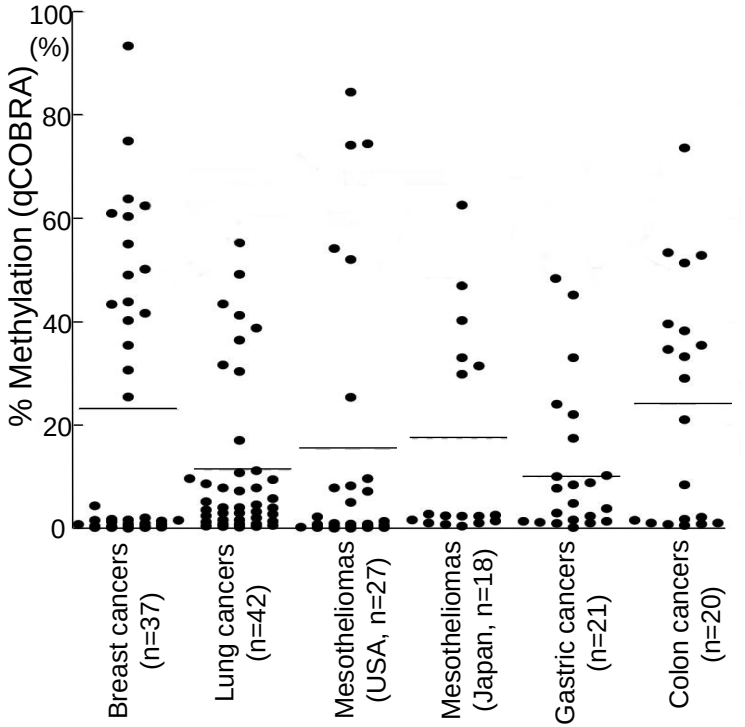


Fig.2d

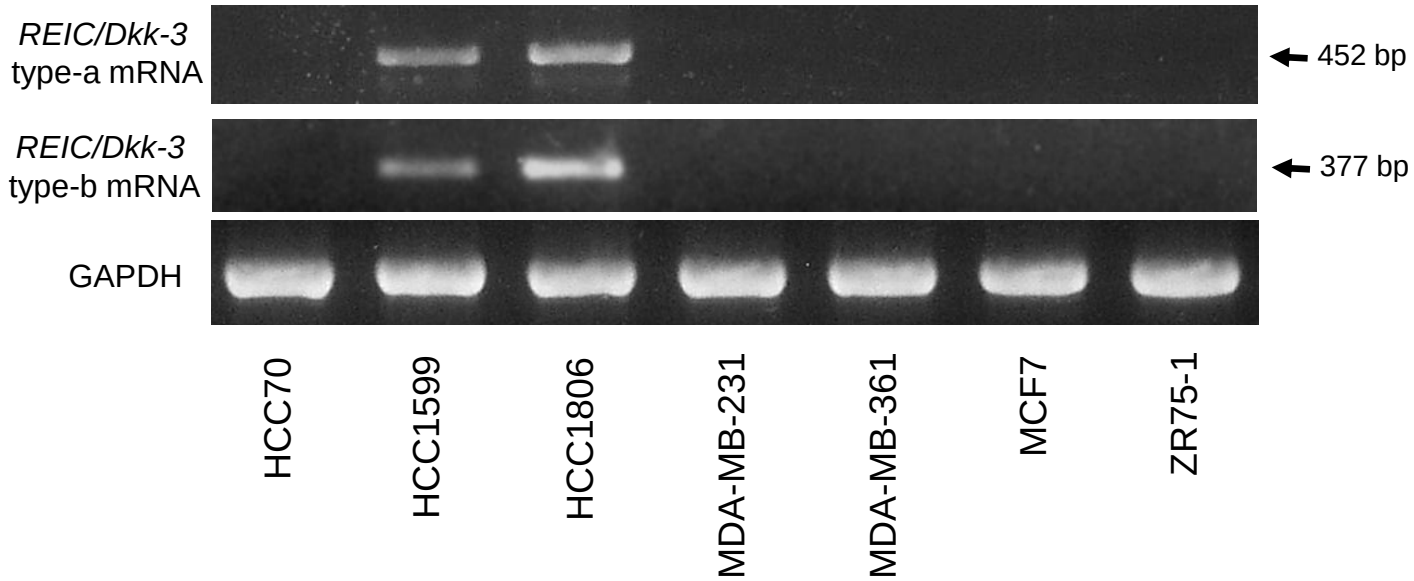

REIC/Dkk-3 type-a mRNA
452 bp
REIC/Dkk-3 type-b mRNA
377 bp
GAPDH
HCC70
HCC1599
HCC1806
MDA-MB-231
MDA-MB-361
MCF7
ZR75-1

Fig.3

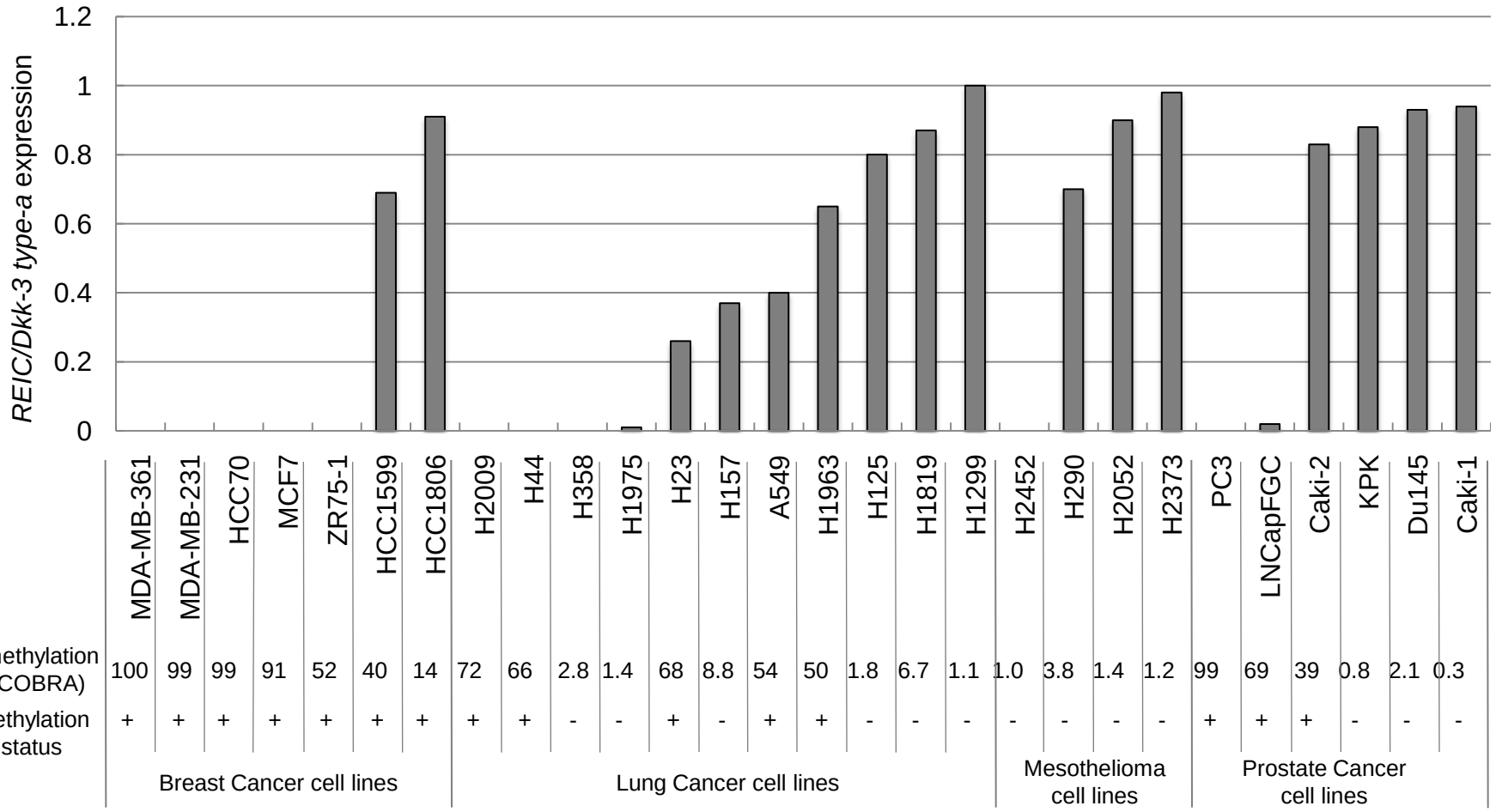
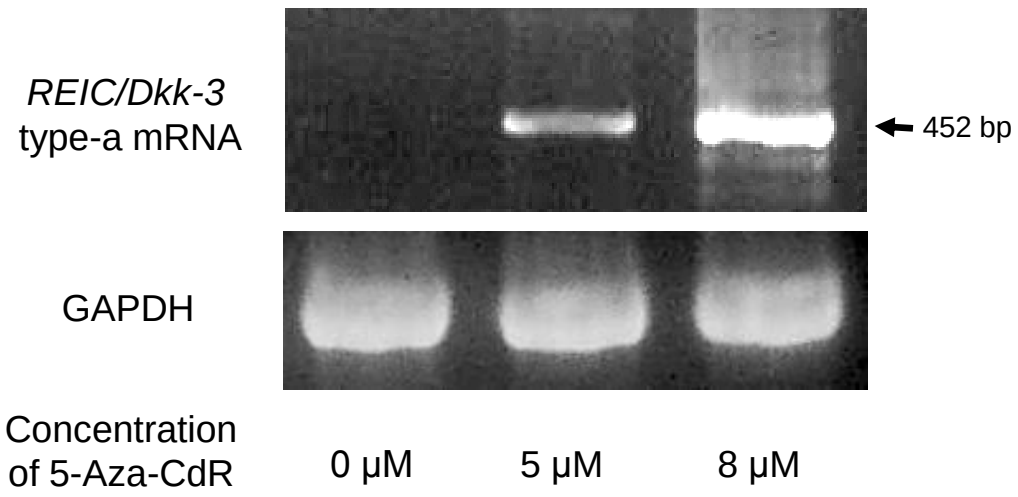

1.2
1
0.8
0.6
0.4
0.2
0
REIC/Dkk-3 type-a expression
MDA-MB-361
MDA-MB-231
HCC70
MCF7
ZR75-1
HCC1599
HCC1806
H2009
H44
H358
H1975
H23
H157
A549
H1963
H125
H1819
H1299
H2452
H290
H2052
H2373
PC3
LNCapFGC
Caki-2
KPK
Du145
Caki-1
%methylation (qCOBRA)
100 99 99 91 52 40 14 72 66 2.8 1.4 68 8.8 54 50 1.8 6.7 1.1 1.0 3.8 1.4 1.2 99 69 39 0.8 2.1 0.3
methylation status
+ + + + + + + + + - - + - + + - - - - - - - + + + - - -
Breast Cancer cell lines
Lung Cancer cell lines
Mesothelioma cell lines
Prostate Cancer cell lines

Fig.4


REIC/Dkk-3
type-a mRNA
452 bp
GAPDH
Concentration
of 5-Aza-CdR
0 μM
5 μM
8 μM

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Authors Tatsuro Hayashi, Hiroaki Asano, Shinichi Toyooka, Junichi Soh, Tadahiko Shien, Naruto Taira

Yuho Maki, Norimitsu Tanaka, Hiroyoshi Doihara, Yasutomo Nasu, Nam-ho Huh, Shinichiro Miyoshi

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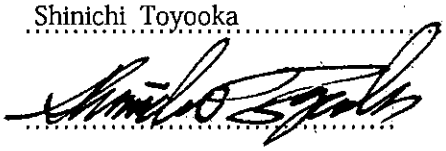
Signature  Date Aug 17 2011

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Oct 31, 2011

Birgit Roselt,
Managing Editor
Journal of Cancer Research and Clinical Oncology

Dear Ms. Roselt,

We are willing to re-submit the manuscript JCRCO3123 entitled "DNA methylation status of REIC/Dkk-3 gene in human malignancies" to be considered publication in the Journal of Cancer Research and Clinical Oncology.

We have carefully reviewed the reviewers' comments and have addressed their concerns as detailed on the following page. Consequently, several changes have been made to our manuscript, which we believe significantly strengthen it. We hope the revised manuscript is acceptable for publication in "Journal of Cancer Research and Clinical Oncology" as a regular article.

Yours sincerely

Shinichi Toyooka, M.D.

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Reviewer #1: Hayashi T et al demonstrated frequent methylation at the promoter region of REIC/Dkk-3 in various cancers by using quantitative COBRA and bisulfite sequencing. Among them, ER-positive breast cancers were significantly methylated compared with its negative ones. Here, the authors focused on Dkk-3 type-a methylation, because it has already been shown that the promoter activity of type-a was much stronger than that of type-b (Kobayashi K, Gene, 2002). Nevertheless, it has been reported that methylation of Dkk-3 type-b was detected in various cancers (Introduction, page 5, 2nd paragraph). Therefore, additional data are necessary in order to show the importance of Dkk-3 type-a in cancers.

Comments:

1) The authors should study methylation and expression of Dkk-3 type-a in primary tissues, and analyze whether or not these show inverse correlation in cancer tissues like cancer cell lines. It is also important to compare the methylation status between type-a and -b in cancers.

2) Nested PCR with 40 cycles was used for COBRA analysis in this study. It is not likely that such conditions really reflect % methylation in cancers. Many data on COBRA consist of standard PCR but not of nested PCR. The authors should perform linear regression analysis using the nested PCR with % control DNA (% methylation: 0- 100%), which was shown in the previous report (Xiong and Laird 1997).

3) In Figure 4 and page 10, the authors should indicate the concentration of 5-Aza-CdR. It is widely used 1-10 μM as a final concentration.

4) No information on 27 mesotheliomas from USA (Figure 2d) was seen in the text.

Response:

1. We usually look up cell lines to examine the relation between DNA methylation and mRNA expression (Tomii et al. Int.J.Cancer 2006), because cancerous tissue sometimes contain normal counterparts (stroma, lymphocyte, etc) or mRNA degradation may occur even in frozen samples. We have examined the relation between *REIC/Dkk-3* methylation and *REIC/Dkk-3* mRNA expression in cell lines and showed the inverse correlation in our manuscript.

As for the importance of *REIC/Dkk-3* type-a, Kobayashi et al.(Gene 282, 151-158, 2002) described not only the promoter activity of type-a was much stronger than that of type-b, but also they confirmed major transcript was type-a isoform in the various cancer cells that they tested. They also suggested hyper-methylation of major promoter (type-a) was major mechanism for the down-regulation of *REIC/Dkk-3* expression. Further more, they described type-a methylation was accompanied with the type-b methylation in most case except four lung cancer cells that they tested. *REIC/Dkk-3* expression was detected in those four lung cancers, regardless those four lung cancers have type-b hypermethylation. So they discussed the possibility that minor promoter (type-b) was utilized for the expression in a tissue specific manner, as seen in dual promoters in APC gene.

But there is some weakness in the method for detecting DNA methylation in their paper. They examined DNA methylation by methylation-specific restriction enzyme that was no longer in use, recently. Take into consideration these results, it seems to be

reasonable to examine the major promoter (type-a) methylation by quantitative COBRA in various cancers. We can provide reliable methylation status of REIC/Dkk-3 type-a.
We added the explanation why we focused on REIC/Dkk-3 type-a in page 5 L11-16.

2. We agreed with the comment. We decided to use 10% threshold, because we could recognize 10% of digested bands on the agarose gel electrophoresis and we assume those sample are positive in conventional COBRA assay. On the other hand, the digested bands less than 10% are so faint that we can't easily recognize those bands on agarose gel electrophoresis. 10% of digested bands seem to be reasonable criteria to maintain compatibility with conventional COBRA assay. We could observe the inversely correlation between *REIC/Dkk-3* methylation and *REIC/Dkk-3* expression using this criteria. To support this idea, Collella et al. (BioTechniques 2003) described that qCOBRA and pyrosequencing methylation analysis (PyroMethA) always gave concordant calls for the methylation status when a 10% threshold to declare methylation was used.

We performed the linear regression analysis using the nested qCOBRA and confirmed the quantitative capability as shown below.
We added the methodology of linear regression analysis in page 9.
We added "we performed the linear regression analysis using nested qCOBRA and confirmed the quantitative capacity" in page 12.

%methylated DNA	% unmethylated DNA	qCOBRA(% digested bands)
100	0	99.7%
90	10	84.7%
80	20	75.2%
70	30	69.2%
50	50	39.4%
30	70	33.2%
20	80	26.9%
10	90	17.9%
0	100	2.5%

3. We indicated the concentration of 5-Aza-CdR in Figure 4 and page 11.
4. We described the information about 27 mesotheliomas from USA (page 6, page 12 and Table1).

Reviewer #2: In this manuscript, Hayashi et al. analyzed the DNA methylation status of the promoter region of REIC/Dkk-3 type-a in various malignancies, including breast cancers, non-small cell lung cancer (NSCLCs), gastric cancers and colorectal cancers, and malignant pleural mesotheliomas. The authors describe i) that the methylation status of REIC/Dkk-3 type-a was varied among cancers, ii) that the methylation status was inversely correlated with REIC/Dkk-3 type-a expression, and iii) that estrogen receptor (ER) positive breast cancers were significantly methylated compared with ER negative breast cancers.

REIC/Dkk-3 has two alternative tissue-specific promoters, type-a and type-b, and the silencing of REIC/Dkk-3 and hypermethylation of REIC/Dkk-3 type-b promoter have already been reported in a variety of malignancies. Thus, in this study, the authors analyzed methylation status of type-a promoter using other types of malignancies. This is a potentially important study to understand the silencing mechanism of REIC/Dkk-3 more deeply. However, the authors should address the following concerns to make their data solid.

Major Comments:

1. The authors should investigate which is the major transcript of REIC/Dkk-3, type-a or type-b, in normal counterparts of cancers analyzed in this study, or in cancer cell lines expressing REIC/Dkk-3. The authors claimed that the reason why they focused on REIC/Dkk-3 type-a promoter was that a previous report showed that promoter activity of REIC/Dkk-3 type-a was stronger than that of type-b in 293 cells (Kobayashi et al., 2002). However, it is known that the promoter activity is dependent on cell types used for promoter assay, and to reveal the major transcript in normal cells is important for the decision of target promoter for DNA methylation analysis.

2. The novelties of this study should be clarified. The previous study already has reported DNA methylation status of REIC/Dkk-3 type-a in gastric cancers and NSCLC (Kobayashi et al., 2002). The authors should clearly describe which cancer types are newly analyzed in this study.

3. The authors should describe the reason why they used 10% as the criteria for methylation-positive cancers. The criteria should be determined for each cancer types because the contents of stroma are different among cancer types.

4. The authors should investigate the association of DNA methylation status and HER-2 amplification for breast cancers because HER-2 amplification is an important factor of breast cancer characteristics, and HER2 amplification is reported to be associated with frequent DNA methylation at multiple CpG islands (Terada et al, 2009).

Minor Comments:

1. The authors should add a photo of the positive control, such as GAPDH mRNA, to Figure 4.

Response;

1. With regard to cancers, Kobayashi et al. (2002) already described main promoter was type-a and the methylation of main promoter is the major mechanism for down-regulation of *REIC/Dkk-3* expression. We commented the reason why we had focused on major promoter (type-a) in the response to reviewer1 as discussed above.

With the regard to the *REIC* expression (type-a and b) in primary tumors and normal counterparts, we won't examine the mRNA in frozen samples, because cancerous tissue sometimes contain normal counterparts (stroma, lymphocyte, etc) or mRNA degradation may occur even in frozen samples.

2. Kobayashi et al. (2002) described the *REIC* methylation in mainly lung and gastric cancer cells, but there is a weakness in their study, because they examined DNA methylation by methylation-specific restriction enzyme that was no longer in use, recently.

This manuscript will be the first paper that quantified the *REIC/Dkk-3* main promoter (type-a) methylation by reliable qCOBRA in many cell lines and primary cancers, such as breast, lung, gastric, colorectal cancers and pleural mesothelioma. There is no paper described the *REIC/Dkk-3* methylation in mesotheliomas. Those are novelties of our study.

3. We responded why we used a 10% threshold in our manuscript, as noted above. We could not detect any *REIC/Dkk-3* type-a methylation in corresponding 10 non-malignant breast tissues and 10 non-malignant lung tissues.

4. As reviewer says, it is true that HER-2 amplification is an important factor in breast cancers. But we are focused on the relation between *REIC/Dkk-3* type-a methylation and its mRNA expression. We think that *REIC/Dkk-3* methylation does not directly involve in HER-2 amplification.

Minor Comments;
1. We add internal control (GAPDH) to Figure 4.

Reviewer #2: Hayashi et al. addressed this Reviewer's comments as in the "Responses to reviewers". However, some responses seem to have failed to address the concerns appropriately. At least, the authors should reflect their responses in the revised manuscript to help the readers of Journal of Cancer Research and Clinical Oncology. Comments: 1. This Reviewer commented that expression of REIC/Dkk-3 type-b should be analyzed, in addition to that of type-a, to identify the major transcript in the samples analyzed in this study. Even if type-a was the main promoter of REIC/Dkk-3 in NIH/3T3 cells (Kobayashi et al., 2002), there is a possibility that the minor promoter (type-b) is used in a tissue-specific manner, as the authors mentioned in Introduction (page 5, ll. 16). The authors must analyze expression of type-b in their own samples. 2. The authors should clearly describe the novelty of this study in the revised manuscript, not only in their response to the Reviewer. 3. The reason why the authors used a threshold of 10% for methylation-positive samples should be added to Materials and Methods. 4. As for their reply to my recommendation to analyze the association between REIC/Dkk-3 type-a methylation status and HER-2 amplification status, the authors replied, "we are focused on the relation between REIC/Dkk-3 type-a methylation and its mRNA expression". However, the authors showed "REIC/Dkk-3 methylation and clinicopathological correlation" in Result (Page 13, ll 12-15). Since HER-2 amplification status is one of the important clinicopathological characteristics, the authors should analyze the association between REIC/Dkk-3 type-a methylation status and HER-2 amplification status.

Response;

1. We examined the expression of *REIC/Dkk-3* type-b in breast, lung, malignant pleural mesothelioma, prostate cancer cells and described the results in Table 2 and Fig.2d. The expression of *REIC/Dkk-3* type-a was corresponded with *REIC/Dkk-3* type-b ($p<0.01$) in all types of cell lines but some discrepancies in lung cell lines.

We added the primer information for *REIC/Dkk-3* type-b in page11 L9-11.

We added the result and discussion for the expression of *REIC/Dkk-3* type-a with type-b in page14 L5-8, page16 L3-9.

We also made some modification in abstract.

2. To strengthen the novelty of this study, we added “The qCOBRA assay can provide more reliable results because the conventional methylation-sensitive restriction enzyme assay that Kobayashi et al. (2002) performed was recently known to be prone to false positive results due to spurious incomplete digestion (Xinog Z et al. 1997). We also analyzed the correlation between *REIC/Dkk-3* type-a and type-b expression in various cancer cell lines.” in page 6 L9-13.

3. We added the method for linear regression analysis in page10 L2-6. We also discussed the validity of 10% threshold in page15 L7-13.

4. We added HER2 status in primary breast cancers by immunohistochemistry. We add HER2 status in Table 3A. However, there was no correlation between HER2 status and *REIC/Dkk-3* methylation. We added the methodology in page12 L3-6.