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Differential Prognostic Relevance of Promoter DNA Methylation of *CDO1* and *HOPX* in Primary Breast Cancer

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Abstract. *Background/Aim: We previously identified that promoter DNA methylation of cysteine dioxygenase type 1 (CDO1) and homeobox only protein homeobox (HOPX) were both cancer specific, and have a clinical potential as prognostic biomarkers in breast cancer (BC). The present study compared the differential prognostic relevance of methylation status of the CDO1 and HOPX genes in BC. Materials and Methods: Methylation levels (TaqMethVs) were quantified in 7 BC cell lines and 133 BC patients by TaqMan methylation-specific PCR and functional traits were explored for CDO1. Results: TaqMethVs were associated between CDO1 and HOPX (r^2=0.072, p=0.002). Multivariate Cox proportional hazards model could identify CDO1 hypermethylation as well as Ki-67 as independent prognostic factors related to disease-specific survival (p=0.016, p<0.001). Overexpression of CDO1 decreased the anchorage-independent growth capacity in BC cell lines. Conclusion: CDO1 is a definite tumor suppressor gene, while its prognostic relevance was more than expected in the context of its functional relevance.*

Breast cancer (BC) is the most common cancer in women worldwide. Recent estimates by the International Agency for Research on Cancer (IARC) show that BC is the most commonly diagnosed cancer in women, making up 24.2% or about 1 in 4 of all new cancer cases diagnosed in women worldwide (1). The incidence of BC is increasing in the world due to increase in life expectancy, increased urbanization and adoption of western lifestyles. It is thought that the incidence of BC will continue to increase. Therefore, early detection and individualized treatment, in order to improve clinical and survival outcomes, remains the cornerstone of BC control. Thus, novel approaches for the diagnosis and prognosis of BC are still needed.

In BC, genomic events are fewer than in other cancers (2). We previously developed pharmacological reversal of epigenetic silencing (3) and uncovered a myriad of transcriptionally repressed genes, such as *PGP9.5* (4), *NMDAR2B* (5), *DFNA5* (6), and *HOPX* (7) in human gastrointestinal cancers. Until now, we have originally reported cysteine dioxygenase type 1 (*CDO1*) and homeobox only protein (*HOP*) homeobox (*HOPX*) as novel methylation genes in primary BC (8, 9). Through clinicopathological analysis including prognostic information, we have already reported a positive relationship of both genes to prognosis of BC (9, 10).

BC treatment is unique, because personalized therapy is robustly progressing after the subtype classification was proposed at the 2011 St. Gallen consensus meeting was based on gene expression data sets (11, 12). The expressions of hormone receptors, HER2 and Ki-67 are important determinants in the context of the selection of therapy at present. On the other hand, reports on aberrant promoter DNA methylation and their relations to BC prognosis are reported in about 700 papers until 2018, which have covered more than 200 genes, while only 31 genes described by the 48 papers were investigated by quantitative methylation specific PCR (Q-MSP). Then, among the 48 papers, only three genes, *CDO1* (10, 13), *PITX2* (14, 15), and *HOPX* (9) were proven to be independent prognostic factors by multivariate prognostic analysis. There have been no reports describing all three genes simultaniously.

From our laboratory, promoter DNA methylation for *CDO1* and *HOPX* was assessed for highly relevant prognostic factors in primary BC, and their prognostic

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relevance were seen, especially for *CDO1* hypermethylation in triple-negative type (10) and *HOPX* hypermethylation in HER2-negative type (9). In Q-MSP, prognostic value is assessed by the most optimized cut-off values instead of presence or absence of methylation. Using such optimized cut-off values, we analyzed the two original tumor suppressor genes in a comparative manner to confirm better prognostic marker utility in primary BC. Furthermore, functional analysis of the *CDO1*, a stronger prognostic factor determined by this study, was performed to deeply understand the molecular insights of the prognostic contribution of the *CDO1* hypermethylation in primary BC.

Materials and Methods

Human primary BC tissues and methylation data. A previous study by our group obtained methylation values of either *CDO1* or *HOPX* in 172 primary BC patients with no prior chemotherapy who underwent surgical resection of the primary tumors at Kitasato University Hospital between January 1, 1995 and December 31, 1999 (9,10), among whom data of the 133 primary BC patients were available commonly for both genes.

TNM classification was made according to the latest 7th edition of the Union for International Cancer Control (UICC). This study was performed in accordance with the clinical research guideline of the ethics committee of Kitasato University School of Medicine (B15-161).

BC cell lines. The seven BC cell lines, SK-BR3, YMB-1, CRL, MDA-MB231, YMB-1E, MDA-MB453, MCF-7 cells were previously described (10). Colorectal cancer cell line, DLD1 was provided from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The hepatocellular carcinoma cell line, HepG2, was also purchased from RIKEN BioResource Centre (Ibaraki, Japan) as control cell lines.

RNA purification and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from cell lines were extracted using Rneasy Mini Kit and RNeasy FFPE Kit (QIAGEN, Hilden, Germany). Total RNA was reverse-transcribed with SuperScriptIII reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed, and the PCR products were separated on 1.5-2.0% agarose gel, then visualized by ethidium bromide (10).

Plasmid and transient transfection. The full-length cDNA sequence of *CDO1* was isolated using PCR and sub-cloned into pcDNA™ 3.1D/V5-His-TOPO vector with confirmation of no mutation of *CDO1* (Invitrogen). The vector with self-ligation was used as a mock control. Plasmid vectors were transiently transfected into 7 BC cell lines using Lipofectamine 2000 reagent (Invitrogen).

Anchorage-independent colony formation assay. Anchorage-independent cell growth was analyzed by plating 0.36% top agarose (Bacto™ Agar, Becton Dickison and Company, Franklin Lakes, NJ, USA) containing 1.0×10^5-5.0×10^5 cells on a surface of 0.72% bottom agarose in 6-well plates. Cells were fed weekly by overlying fresh soft agar solution. Colonies were visualized with ethidium bromide after 2-3 weeks of incubation. Two independent experiments were performed, and each experiment was done in triplicate.

Proliferation assay. Cell proliferation and viability (1.0×10^5 cells/well) were measured using the Premix WST-1 Cell Proliferation Assay System (Takara Bio, Tokyo, Japan) in 96-well plates. Dates are expressed as an absorbance at 450nm. Experiments were performed in triplicate.

Invasion assay. The invasive property of cell was measured using CytoSelect™ 96-well Cell Invasion Assay Kit (CELL BIOLABS, San Diego, CA, USA). Cells were seeded at density of 1.0-5.0×10^5 cells/ml in serum free media in the membrane chamber. Media containing 10% fetal bovine serum was added in the feeder tray and the membrane chamber was placed back into the feeder tray. After incubation for 24 h, the membrane chamber was fixed and stained by 4X Lysis Buffer/CyQuant® GR dye solution. The fluorescence was measured at 480/520nm. Experiments were performed in triplicate.

Statistical analysis. For continuous variables, Student's *t*-test or analysis of variance (ANOVA) was used for comparison between two groups, or among multiple variables, respectively, while for categorical variables, we used χ^2 test or Fisher exact test. Clinicopathologic characteristics and follow-up dates were analysed in terms of disease specific survival (DSS). The follow-up time was calculated from the date of surgery to death or end-point, and patients with other disease deaths were censored. DSS was calculated by Kaplan-Meier methods, and survival differences were assessed in the log-rank test. Variables suggesting potential prognostic factors on univariate analyses ($p<0.05$) were subjected to multivariate analyses using a Cox proportional-hazards model. All statistical analyses were performed using JMP®11 software (SAS Institute Inc., Cary, NC, USA).

Results

Correlation of promoter DNA methylation status of CDO1 and HOPX with prognosis in 133 primary BC patients. We initially compared quantitative methylation values of both *CDO1* and *HOPX* with prognosis in 133 primary BC patients who were informative for methylation data of both genes (9, 10). Clinicopathological characteristics of the 133 BC patients of this study are shown in Table I. For prognostic analysis, we used 3 kinds of cut-off values (high value, median value, and low value).

For *CDO1*, almost all cut-off values showed a statistical significance of prognosis between hypermethylation group and hypomethylation group by log-rank plot analysis, so we herein used a median value of 58 as definitive prognostic cut-off value as in the previous studies (10), where BC patients with *CDO1* hypermethylation (n=62) showed significantly worse prognosis than those with *CDO1* hypomethylation (n=71) ($p=0.009$) (Figure 1A). Additionally we used high cut-off value of 212.9 and low cut-off value of 42.5, leading to change of patient numbers distributed between hypermethylation/hypomethylation (n=13/120, 80/53, respectively). The high and low cut-off

Table I. *Clinicopathological characteristics of the 133 primary breast cancer (BC) patients.*

Variables	Number	%
Age median (range)	50.2 (22-84)	
Gender		
Male	1	0.7
Female	132	99.3
Operation method		
Lumpectomy	36	27
Mastectomy	97	72.9
Histological type		
Invasive ductal carcinoma	126	94.7
Others	7	5.3
pT factor		
T1	61	45.9
T2	59	44.4
T3	10	7.5
T4	3	2.3
pN factor		
N0	60	45.1
N1	36	27.1
N2	19	14.3
N3	18	13.5
pStage		
I	35	26.3
II	59	44.4
III	39	29.3
IV	0	0
Hormone recepter (IHC)		
Positive	89	66.9
Negative	44	33.1
HER2		
Positive	35	26.3
Negative	98	73.7
Ki-67 (IHC)		
Positive	29	21.8
Negative	104	78.2
Subtype		
Luminal	74	55.6
HER2	35	26.3
Triple negative	24	18
Post-operative adjuvant therapy		
Only chemotherapy	63	47.4
Only hormone therapy	24	18
Chemotherapy & hormone therapy	25	18.8
None	21	15.8
CDO1 (58.0)*		
High	71	53.4
Low	62	46.6
HOPX (16.9)*		
High	60	45.1
Low	73	54.9
Reccurence		
Yes	51	38.3
No	82	61.7
Cancer related death		
Yes	34	25.6
No	99	74.4

The median length of follow-up for censored cases was 10.1 years. *Cut-off values of CDO1 and HOPX are previous reported median cut-off values.

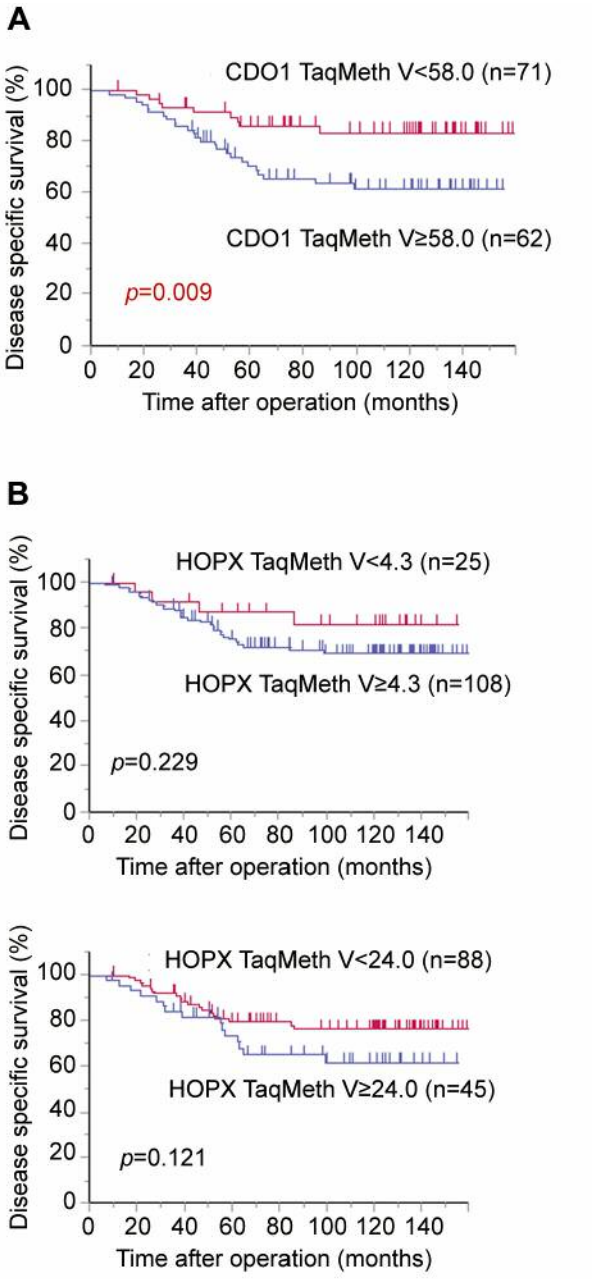

Figure 1. *Kaplan-Meier curves for DSS according to each TaqMeth Vs in CDO1 (A) and HOPX (B). A: The cut-off value was 58.0 which was the median TaqMeth Vs reported by Minatani et al. (p=0.009). B: Two cut-off values were 4.3 and 24.0 which were each low optimized and high optimized TaqMeth vs reported by Kikuchi et al. (p=0.229, p=0.121).*

values were determined by plotted *p*-value and relative risk according to the log-rank plot analysis for TaqMethVs (10).

On the other hand, for *HOPX*, two kinds of very unique cut-off values showed outstanding relevance of prognosis between hypermethylation group and hypomethylation group by log-rank plot analysis, so we also herein used high value of 24 and

Table II. *Univariate and multivariate prognosis analysis for disease specific survival (DSS).*

Clinicopathological parameters		Univariate	Multivariate		
	Number	*p*-Value*	HR	95%CI	*p*-Value#
pT factor					
T1	61	0.002			NS
T2	59				
T3	10				
T4	3				
pN factor					
N0	60	<0.001			NS
N1	36				
N2	19				
N3	18				
Pathological type					
Invasive ductal carcinoma	126	1			
Others	7				
Hormone recepter (IHC)					
Positive	89	0.006			NS
Negative	44				
HER2					
Positive	35	0.372			
Negative	98				
Ki-67 (IHC)					
Positive	29	<0.001	7	2.4-22.1	<0.001
Negative	104				
Subtype					
Luminal	74	0.002			NS
HER2	35				
Triple negative	24				
Post-operative adjuvant therapy					
Only chemotherapy	63	0.003			NS
Only hormone therapy	24				
Chemotherapy & hormone therapy	25				
None	21				
Authracycline chemotherapy					
Yes	11	0.006			NS
No	122				
Hormone therapy					
Yes	49	0.002			NS
No	84				
CDO1 (58.0)					
High	71	0.009	3.6	1.3-11.6	0.016
Low	62				
HOPX (16.9)					
High	60	0.322			
Low	73				

*Log-rank test. #Cox-proportional hazard model.

low value of 4.3 as definitive prognostic cut-off value as in the previous press (9). BC patients with *HOPX* hypermethylation (n=45) showed worse prognosis than those with *HOPX* hypomethylation (n=88) (p=0.121) based on the cut-off value of 24, while *HOPX* hypermethylation (n=25) showed worse prognosis than *HOPX* hypomethylation (n=108) (p=0.229) based on the cut-off value of 4.2 (Figure 1B). Furthermore, as analyzed by the median cut-off value in *CDO1*, we used median value as a cut-off value of 16.9 in *HOPX*, leading to change of patient numbers distributed between hypermethylation/hypomethylation (n=60/73) (9). Prognostic relevance of *HOPX* methylation status was eliminated in primary BC, putatively due to smaller numbers tested in the present study as compared to the early reported one (9).

A Cox proportional hazards model was employed to conduct a multivariate prognostic analysis (Table II). In

univariate analysis, high pT (p=0.002), pN factor (p<0.001), positive hormone receptor (p=0.006), Ki-67 (p<0.001), subtype (p=0.002), post-operative adjuvant therapy (p=0.003), anthracycline chemotherapy (p=0.006), hormone therapy (p=0.002) and *CDO1* methylation (cut-off value was 58, p=0.009) showed significant prognostic factors. In multivariate analysis, Ki-67 positivity (p<0.001) and high TaqMeth V of *CDO1* (p=0.016) were independent prognostic factors related to DSS in primary BC.

The association of methylation values between CDO1 and HOPX in 133 primary BC patients. We then investigated the association of methylation values between *CDO1* and *HOPX* in 133 primary BC tissues, because previous studies pointed out CpG island methylator phenotypes (CIMP) (16). The cases were arranged in descending order for the TaqMeth V of *CDO1* (upper panel of Figure 2A) and those of HOPX were shown in the cases which are same with *CDO1* (lower panel of Figure 2A). There was a significant association of the TaqMeth Vs between *CDO1* and *HOPX* (p=0.002, r2=0.072), however the association was unexpectedly weak (Figure 2B). If the promoter DNA methylation status was delineated by the median values, the methylation status of both *CDO1* and *HOPX* was highly associated (p<0.001) (Figure 2C). In conclusion, methylation status of both genes was sure to be associated with each other, but the association was not so strongly paralleled. Therefore, prognostic relevance of both genes should be separately debated.

The promoter DNA methylation of CDO1 predicts poor prognosis in primary BC. We further analyzed prognosis in 133 BC patients by differential combination of the TaqMeth Vs of *CDO1* and *HOPX*. The BC patients were classified into 3 patterns based on the differential cut-off values (low, median, and high). The 133 BC patients were classified into 4 groups (high/high, low/high, high/low, and low/low methylation status for *CDO1*/*HOPX* methylation status, respectively) by each cut-off value. Group I was defined as high/high methylation for *CDO1*/*HOPX*. Group II was defined as low/high methylation for *CDO1*/*HOPX*. Group III was defined as high/low methylation for *CDO1*/*HOPX*. Group IV was defined as low/low methylation for *CDO1*/*HOPX*. According to the Kaplan-Meier curves of the 3 patterns, groups I and III reproducibly showed poorer prognosis than otherwise groups (groups II and IV) (Figure 3). These 2 groups (I and III) were always represented by *CDO1* hypermethylation. This finding indicated that the promoter DNA methylation of *CDO1* is the prognostic marker irrespective of HOPX methylation status.

Hypermethylation of CDO1 predicts worse prognosis even in primary BC with Ki-67 positive, an alternate independent prognostic factor. As mentioned above, positive Ki-67 was a significant prognostic factor independent of *CDO1* hypermethylation (median cut-off of 58) in the multivariate prognostic analysis (Table II). We thus examined the survival curve separately in primary BC patients with K-67 negative (n=104) and Ki-67 positive (n=29) according to the median TaqMeth V of *CDO1* (Figure 4A). Hypermethylation group of *CDO1* showed poorer prognosis than hypomethylation group in both Ki-67 positive cases and negative cases (p=0.159, and p=0.024, respectively) (Figure 4A). We further classified the 133 BC patients into 4 groups by the most important prognostic factors of *CDO1* methylation and Ki-67 by differential cut-off values (low, median, and high) of *CDO1*. Group I was defined as Yes/Yes for *CDO1* hypermethylation/Ki-67 positivity. Group II was defined as Yes/No for *CDO1* hypermethylation/Ki-67 positivity. Group III was defined as No/Yes for *CDO1* hypermethylation/Ki-67 positivity. Group IV was defined as No/No for *CDO1* hypermethylation/Ki-67 positivity. Group I was showed the poorest prognosis, while group IV exhibited the best prognosis (Figure 4B). Group I was showed extremely poor prognosis in all patterns according to the low, median, and high cut-off values (p<0.0001 in all cases) (Figure 4B). The promoter DNA methylation of *CDO1* could therefore be designated as potent indicator of prognosis in combination with Ki-67 positivity in primary BC.

Tumor suppressive functions of CDO1in human BC cell lines. From the clinical data, hypermethylation of *CDO1* was strongly associated with poor prognosis in primary BC. Since such outstanding clinical relevance must be represented by the functional involvement of the *CDO1* in aggressive BC phenotype, we examined BC cell lines to assess the expression and function of the *CDO1*.

CDO1 expression was never observed in 7 BC cell lines by RT-PCR (left panel of Figure 5A, a positive control was HepG2, a hepatocellular cancer cell line, and a negative control was DLD1, a colorectal cancer cell line). First, transient transfection of the plasmid vector with the full-length *CDO1* into the 7 BC cell lines such as MCF-7, SK-BR3, YMB-1, CRL, YMB-1E, MDA-MB231 and MDA-MB453 confirmed *CDO1* expression at mRNA level by RT-PCR (right panel of Figure 5A).

Overexpression of *CDO1* into YMB-1, YMB-1E and CRL cells showed a significantly decreased colony formation compared to the counterparts (mock) in anchorage-independent colony formation assay (left panel of Figure 5B). The reduction rate of the colonies was shown, compared to the mock cells as 100% (right panel of Figure 5B). The reduction of the colonies was confirmed in almost BC cell lines except MDA-MB231 cells. MDA-MB453 cells were not formed a colony in nature.

In addition, overexpression of *CDO1* also suppressed the log-phase growth in proliferation assay, especially in YMB-1E cells (p=0.021) (left panel of Figure 5C). On the other

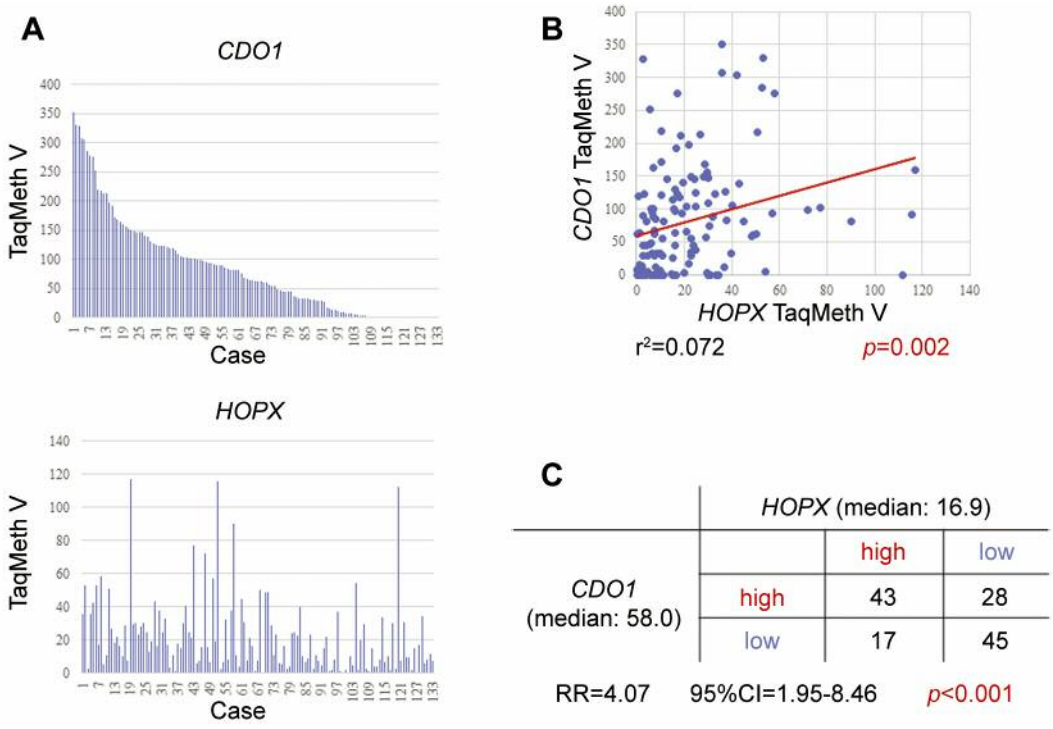

Figure 2. *The association of TaqMethVs of CDO1 to HOPX in primary BC patients. A: Cases were arranged from the left in order of hypermethylation CDO1, and HOPX methylation and the same case was graphed. B: In methylation of CDO1 and HOPX, there is no strong correlation, but a significant relationship is recognized (r^2=0.072, p=0.002). C: Strong correlation between TaqMethVs of CDO1 and HOPX in 133 primary BC patients (p<0.001).*

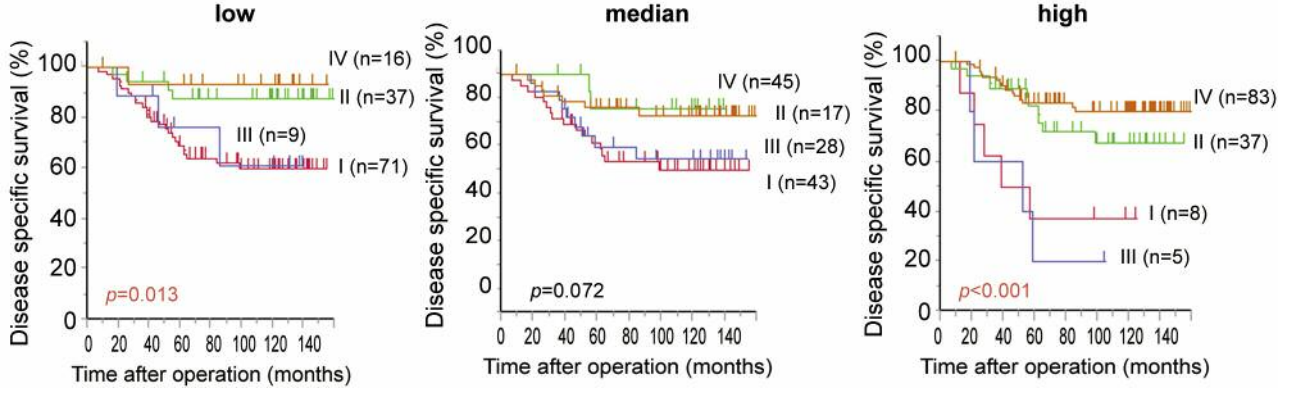

Figure 3. *Kaplan-Meier curves for DSS according to separately defined cut-off values. Groups I and III which showed a CDO1 hypermethylation showed a poorer prognosis than the other groups with CDO1 hypomethylation in all patterns (p=0.013, p=0.072, p<0.001).*

hand, overexpression of *CDO1* did not show significantly suppressed activity in matrigel invasion assay (right panel of Figure 5C). These results indicated that *CDO1* has a definite tumor suppressive activity, however the strength of the tumor suppressive function was weaker than expected from the prognostic relevance in primary BC.

Discussion

There have been numerous reports describing epigenetic prognostic factors of BC (17, 18), however a final validation has never been performed, and definitive prognostic factors are still highly demanded in addition to hormone receptor,

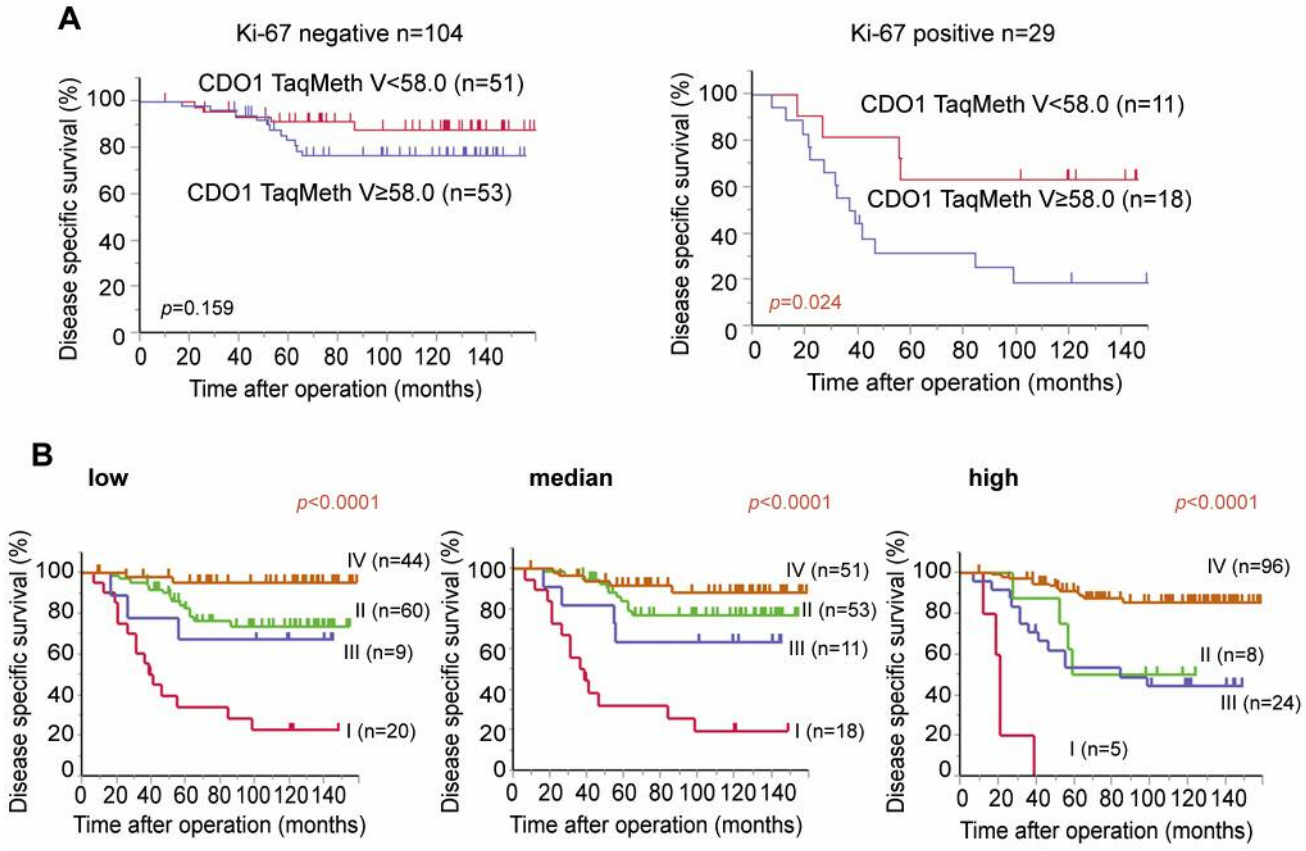

Figure 4. *Kaplan-Meier curves for DSS according to CDO1 methylation and Ki-67. A: Hypermethylation CDO1 showed a poor prognosis regardless of Ki-67 value. B: Hypermethylation CDO1 and Ki-67 positivity (group I) showed the poorest prognosis at three kinds of separately defined cut-off values, and hypomethylation CDO1 and Ki-67 negativity (group IV) showed the best prognosis (p<0.0001).*

HER2, and Ki-67. In the present study, we compared the 2 potential epigenetic prognostic markers of *CDO1* hypermethylation and *HOPX* hypermethylation using the same BC samples, and the final focus was given on *CDO1* hypermethylation. Surely, methylation status of both genes are closely associated to each other, however complete matching was not confirmed, so their prognostic relevance should be separately debated. In any combination of the cut-off values, *HOPX* methylation could not stratify BC patient prognosis instead of *CDO1* methylation.

Ki-67 is a well-known prognostic factor in BC, and in our study, it was again proved to be the most potent independent prognostic factor as well as *CDO1* hypermethylation in BC. This result is consistent with world-wide consensus of prognosis of BC (19), and recapitulated our previous finding in terms of Ki-67 (20). In our early report, *CDO1* hypermethylation was a potent prognostic indicator in triple-negative BC, while in this study, *CDO1* hypermethylation was for the first time proven to be a potent prognostic indicator in BC even with positive Ki-67. Triple-negative BC included more patients with Ki-67-positive than otherwise patients (20), so *CDO1* hypermethylation may represent excellent prognostic factor even in aggressive BC.

Promoter DNA of *CDO1* has been frequently found methylated in various human cancers, and prognostic relevance of its hypermethylation has been extensively reported in breast cancer (10), esophageal SCC (21), esophageal adenocarcinoma (22), gallbladder cancer (23), colorectal cancer (24) from our laboratory, and breast cancer (13, 25), prostate cancer (26), renal cancer (27), and lung cancer (28) from other research groups. These indicated that *CDO1* hypermethylation is a common landmark explaining dismal prognosis of human cancer.

We wanted to investigate how much *CDO1* is involved in metastatic phenotypes of BC, and *CDO1* was transfected in seven kinds of BC cell lines. It actually suppressed anchorage-independent growth in several BC cell lines, suggesting a definite involvement in cancer metastatic ability. On the other hand, it did not affect the invasive capacity of BC cells. These functionally modest findings were not as expected, based on

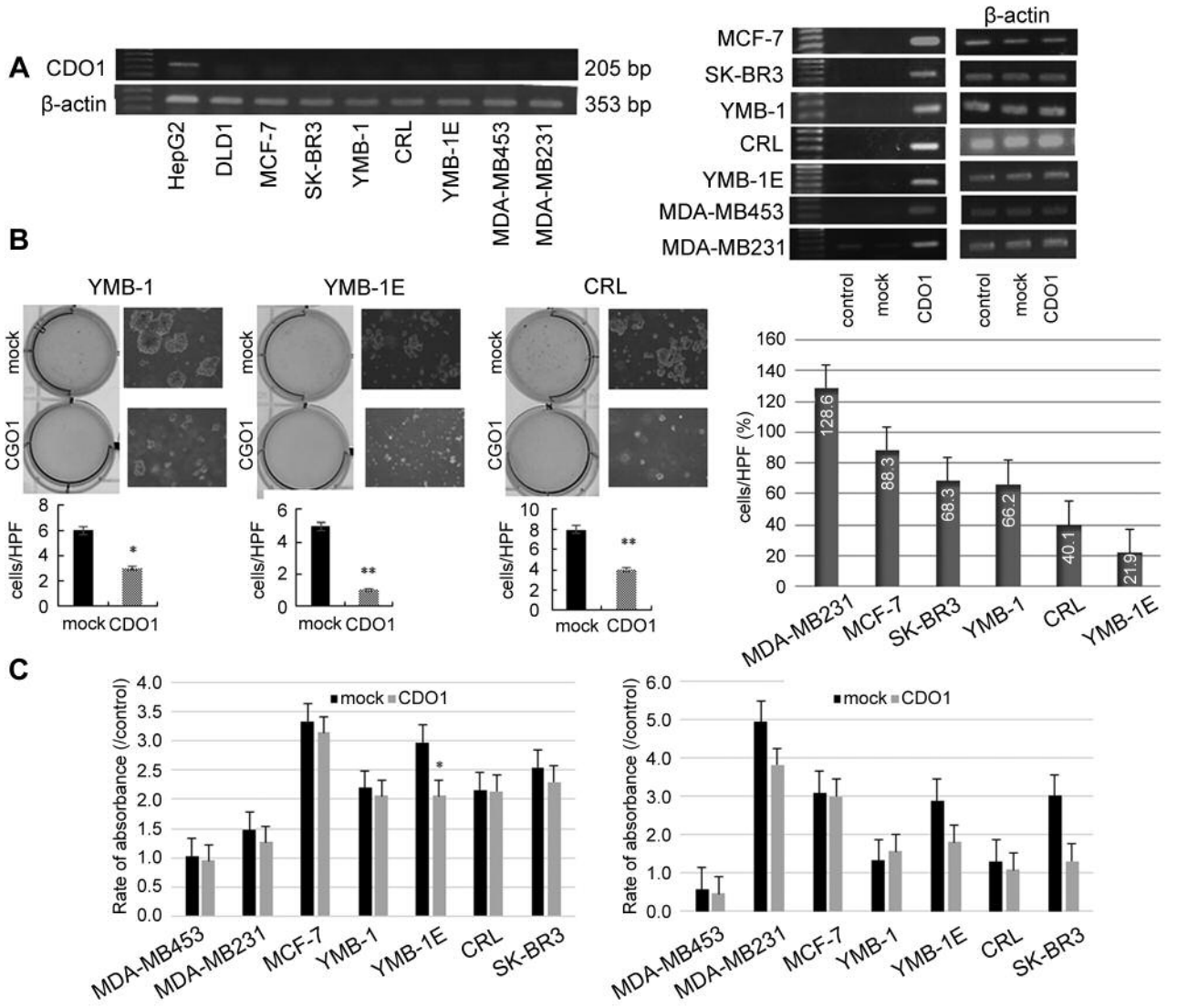

Figure 5. *Forced expression of CDO1 in BC cell lines. A: CDO1 mRNA expression was assessed by RT-PCR in seven BC cell lines (left panel). CDO1 plasmid was transiently transfected, and CDO1 expression was recognized at mRNA in seven BC cell lines (right panel). β-actin was used as a loading control. B: Anchorage-independent colony formation assay in BC cell lines with enforced expression of CDO1. Forced expression of CDO1 significantly reduced colonies in YMB-1, YMB-1E and CRL cells under a phase-contrast microscope (left panel). Almost all BC cell lines showed colony reduction (right panel). C: WST-1 assay (left panel) and matrigel invasion assay (right panel) in forced expression of CDO1.*

the prognostic relevance of *CDO1* methylation in BC, since they were considered ideal prognostic biomarkers with perfect prognostic profiles correlated with *CDO1* methylation status (10). The higher *CDO1* methylation the tumors harbored, the poorer prognosis the patients exhibited, so almost all cut-off values exhibited statistically significant difference between *CDO1* hypermethylation and *CDO1* hypomethylation (Figure 3), and risk of death increased as methylation values of *CDO1* became higher (10). Allowing for such discrepancy between robust prognostic relevance and modest functional involvement, *CDO1* hypermethylation may represent the time from cancer initiation, which could be designated as cancer clock, rather than its functional involvement.

Clinical utility of the *CDO1* hypermethylation can be approved as a prognostic marker of BC, just after it could be validated in specific clinical (homogenous) conditions. *CDO1* hypermethylation was actually demonstrated to be a significant prognostic factor in BC with lymph node metastases (13), with anthracycline treatment (25), with triple-negative (10), or with Ki-67-positive. Current therapeutic strategies against BC are based on subtype, so prognostic relevance of *CDO1* methylation must be validated

according to the subtype of BC such as luminal type, HER2 type, and triple-negative type. For luminal type BC, prognosis is excellent in terms of the current clinical therapy, however its incidence is the largest among BC and BC patients who have recurrent disease and finally died largely included luminal type. So, prognostic factors are still highly demanded. In the present study, BC patients even with negative Ki-67 showed marginal difference according to *CDO1* methylation status. Future validation is still anticipated regarding prognostic relevance for luminal type BC.

In conclusion, *CDO1* hypermethylation is a definite tumor suppressor gene, while its prognostic relevance was more than expected in the context of its functional relevance. The prognostic relevance of *CDO1* hypermethylation in the context of other cancers may indicate time since cancer initiation.

Conflict of Interests

The Authors declare that they don't have any conflicts of interest.

Authors' Contributions

Conception and design: YT, K. Yamashita, MW; Acquisition of data: YT, MW, K. Yokota, HH, TK, MK, NM, HN, HK; Analysis and interpretation of data: YT, YK, K. Yamashita; Drafting the article: YT, K. Yamashita; Critically revising the article for important intellectual content: YT, K. Yamashita, MW; Final approval of the version to be published: YT, K. Yamashita, MW; All authors read and approved the final manuscript.

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