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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Epigenetic Status of *CDO1* Gene May Reflect Chemosensitivity in Colon Cancer with Postoperative Adjuvant Chemotherapy

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ABSTRACT

Background. *Cysteine dioxygenase type 1* (*CDO1*) acts as a tumor suppressor gene, and its expression is regulated by promoter DNA methylation in human cancer. The metabolic product mediated by CDO1 enzyme increases mitochondrial membrane potential (MMP), putatively representing chemoresistance. The aim of this study is to investigate the functional relevance of *CDO1* gene in colon cancer with chemotherapy.

Patients and Methods. We investigated 170 stage III colon cancer patients for *CDO1* methylation by using quantitative methylation-specific polymerase chain reaction (PCR). To elucidate the functional role of *CDO1* gene in colorectal cancer (CRC) biology, we established cell lines that stably express *CDO1* gene and evaluated chemosensitivity, MMP, and tolerability assay including anaerobic environment.

Results. Hypermethylation of *CDO1* gene was an independent prognostic factor for stage III colon cancer on multivariate prognostic analysis. Surprisingly, patients with *CDO1* hypermethylation exhibited better prognosis than those with *CDO1* hypomethylation in stage III colon cancer with postoperative chemotherapy ($P = 0.03$); however, a similar finding was not seen in those without postoperative chemotherapy. In some CRC cell lines, forced expression of *CDO1* gene increased MMP accompanied by chemoresistance and/or tolerance under hypoxia.

Conclusion. *CDO1* methylation may be a useful biomarker to increase the number of stage III colon cancer patients who can be saved by adjuvant therapy. Such clinical relevance may represent the functionally oncogenic property of *CDO1* gene through MMP activity.

DNA hypermethylation of tumor suppressor genes (TSGs) is a major epigenetic change in human cancer. Using the pharmacological unmasking microarray, we have identified many TSG candidates with promoter DNA methylation specific to primary human cancer tissues.[1–4] Among these genes, *cysteine dioxygenase type 1* (*CDO1*) was frequently silenced in various human cancers by hypermethylation of its promoter DNA.[5]

DNA methylation of *CDO1* gene is highly cancer specific with area under the curve of 0.96 in the receiver operating characteristic curve to discriminate colorectal cancer (CRC) from normal-appearing mucosa (NAM).[6] We further investigated clinicopathological features of *CDO1* promoter DNA methylation in primary CRC including early tumorigenesis.[6] As a result, the methylation level was confirmed to steadily increase during the adenoma–carcinoma sequence. Significant increases in *CDO1* methylation were seen between NAM and low-grade adenoma, and between low-grade adenoma and high-grade adenoma. A significant increase was also seen in CRC tissues with liver metastasis. Furthermore, we investigated the inverse

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correlation of *CDO1* gene promoter methylation and its expression in CRC tissues. Taken together, these findings indicated that *CDO1* gene plays a critical role in tumor development as a TSG during CRC carcinogenesis and cancer progression.

Conversely, in glioma, *CDO1* gene expression is augmented as tumors progress, and its forced expression can affect the mitochondrial membrane potential (MMP) through pyruvate dehydrogenase activity, which may explain the oncogenic potential of *CDO1* gene in glioma cells.[7] Such function is contradictory to the notion that *CDO1* is a TSG from an epigenetic point of view in human cancers. CDO1 may also affect chemosensitivity as well as cancer progression.

The efficacy of postoperative adjuvant chemotherapy has been reported in stage III colon cancer.[8–10] However, only some stage III colon cancer patients survive following adjuvant postoperative chemotherapy. This phenomenon suggests differential chemosensitivity among stage III colon cancer patients. However, to the best of the authors' knowledge, no biomarker to predict which patients will exhibit good response to adjuvant chemotherapy has been identified.

In this study, we quantified the methylation status of *CDO1* gene by using quantitative methylation-specific PCR (qMSP) and analyzed the prognosis of stage III colon cancer patients. As a result, *CDO1* methylation status could enrich the patients who exhibit good prognosis in stage III colon cancer with postoperative chemotherapy. We further investigated the functional mechanism of *CDO1* to explain such counterintuitive clinical outcomes in specific clinical situations.

PATIENTS AND METHODS

Cell Lines

DLD-1, HCT15, and HCT116 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Carlsbad, CA, USA). HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco). Cells were cultured in medium containing 10% fetal bovine serum as described elsewhere.[11]

Registration of Colon Cancer Patients

We chose 170 colon cancer patients with stage III to analyze the correlation between *CDO1* methylation status and prognosis. All patients underwent colectomy at Kitasato University East Hospital between 2007 and 2012 and were histopathologically diagnosed with colon adenocarcinoma as defined in the 7th edition of the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum, and Anus.[12] This study was conducted in accordance with the Declaration of Helsinki, and all patients signed a consent form approved by the Research Ethics Committee of Kitasato University School of Medicine. All patients underwent curative resection. For adjuvant postoperative chemotherapy, 75 patients received oral uracil–tegafur (UFT) plus leucovorin, 16 patients received capecitabine, 9 patients received capecitabine/oxaliplatin, 21 patients received modified folinic acid–fluorouracil–oxaliplatin (mFOLFOX)6, and 14 patients received other regimens.

Extraction of Genomic DNA, Total RNA, and Protein and Their Assessments

Genomic DNA, total RNA, and protein extraction were performed as described in our previous report.[11] We used paraffin-embedded tissues to obtain genomic DNA from cancer tissues. Genomic DNA was bisulfite converted using the EZ DNA Methylation-Gold™ kit (Zymo Research, Irvine, CA, USA). Reverse-transcription (RT)-PCR and Western blotting were conducted as described in our previous report.[11] The primers used for qMSP and RT-PCR are presented in Table S1.

Quantitative Methylation-Specific PCR

Quantitative TaqMan methylation-specific PCR (qMSP) was carried out using iQ Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in triplicate on the C1000 Touch™ thermal cycler CFX96 real-time system (Bio-Rad). PCR conditions and primer sequences are described in our previous report.[13] Serial dilutions of bisulfite-modified DNA from CRC cell line DLD1 were used to construct the calibration curve on each plate as methylation-positive control, and hepatoblastoma cell line HepG2 was used as negative control. The methylation value (designated as TaqMeth V as previously described[13]) was defined by the ratio of the amplified signal value of methylated *CDO1* to the value for β-actin, which was then multiplied by 100. This ratio was used as a measure of the relative level of methylated DNA in samples.

Plasmid Construction of CDO1 Gene for Transfection into Cell Lines

Full-length *CDO1* complementary DNA (cDNA) was synthesized from HepG2 cells as previously described.[11] The primers used for plasmid construction are presented in Table S1. Cells were transfected with the generated plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as per manufacturer instructions. Stable cell lines were selected by using G418. *CDO1* messenger RNA

(mRNA) and protein expression after transfection was confirmed by RT-PCR and Western blotting, respectively.

Tolerability Assay after Exposure to 5-FU

Cells were seeded in a 96-well plate (1×10^4 cells/well) and incubated at 37 °C. The next day, cells were incubated with 5-fluorouracil (5-FU) at various concentrations for 48 h. Cells without 5-FU exposure were incubated concurrently as controls for the WST-1 and JC-1 assays.

Tolerability Assay in Anaerobic Environment

Cells were incubated under hypoxic condition to evaluate tolerability in an anaerobic environment. The hypoxic condition was set using BIONIX®, a commercially available kit consisting of an airtight pouch and oxygen absorber. Cells were cultured in the pouch with the oxygen absorber. WST-1 and JC-1 assays were performed to evaluate cell proliferation and MMP 48 h after incubation under hypoxia. Cells that were concurrently cultured in the pouch but without the oxygen absorber were used as controls for the WST-1 and JC-1 assays.

Cell Proliferation Assay

We used Cytoselect™ WST-1 cell proliferation assay reagent (Cell Biolabs, Inc., San Diego, CA, USA) to assess cell proliferation. Samples were read at absorbance of 450 nm and results are expressed as percentage of control.

Mitochondrial Membrane Potential Assay

To assess the MMP of cell lines, we used the JC-1 assay kit according to manufacturer protocol. The color of JC-1 dye shifts from green to red as the MMP increases. This assay was performed using flow cytometry for quantitative analysis. Samples were measured on FACSVerse™ (BD, Franklin Lakes, NJ, USA) with fluorescein isothiocyanate (FITC, excitation 494 nm/emission 519 nm) and PE-Texas Red (excitation 488 nm/emission 615 nm). The data were analyzed using Kaluza software v1.3 (Beckman Coulter, Brea, CA, USA) as described elsewhere.[14]

Statistical Analysis

The Chi squared test was used for categorical variables, while the Mann–Whitney *U* test or analysis of variance was used for continuous variables. Cumulative relapse-free survival (RFS) was estimated by Kaplan–Meier method and compared using log-rank test. RFS was measured from date of surgery to date of recurrence or last follow-up. Univariate variables ($P < 0.05$) were subjected to analysis by a multivariate Cox proportional hazards regression model. All statistical analyses were conducted using SAS software package JMP, version 11.0 (SAS Institute, Tokyo, Japan).

RESULTS

Prognostic Analysis Using CDO1 TaqMeth V in Stage III Colon Cancer

We initially quantified the methylation status of *CDO1* gene in 170 stage III primary colon cancer tissues by qMSP. The mean TaqMeth V was 42.2 (± 23.8 SD). We divided the patients into two groups according to TaqMeth V, obtaining an optimal value of 31.8, which clearly discriminated the prognosis of these patients (Fig. 1a). The TaqMeth V Low group showed significantly worse prognosis than the TaqMeth V High group ($P = 0.04$), although there were no significant differences in clinicopathological factors between the two groups (Table S2).

Multivariate analysis revealed that venous invasion and TaqMeth V Low were independent factors representing poor prognosis (Table S3). This result is inconsistent with our recent report in total CRC, which suggested that TaqMeth V High is a negative prognostic factor in total CRC.[6] The discrepancy in the results may be influenced by chemosensitivity in stage III colon cancer.

We then analyzed whether TaqMeth V influences prognosis separately in patients who underwent postoperative adjuvant chemotherapy and those who only received operation. In the adjuvant chemotherapy group, the TaqMeth V Low group showed significantly worse prognosis than the TaqMeth V High group ($P = 0.04$) (Fig. 1b), while there was no difference in RFS in the surgery-only group (Fig. 1c). These results suggest that the differential prognosis of stage III colon cancer patients according to *CDO1* TaqMeth V derived from chemosensitivity of the postoperative adjuvant chemotherapy group.

Functional Role of CDO1 Gene in Chemoresistant Phenotype in CRC Cell Lines

To elucidate the reason why the prognosis of stage III patients with TaqMeth V Low who received postoperative adjuvant chemotherapy was poor, we analyzed the functional role of *CDO1* gene in CRC cell lines. We focused on two definitive factors that can affect chemoresistance: resistance to chemotherapeutic drugs and their delivery to tumor sites.

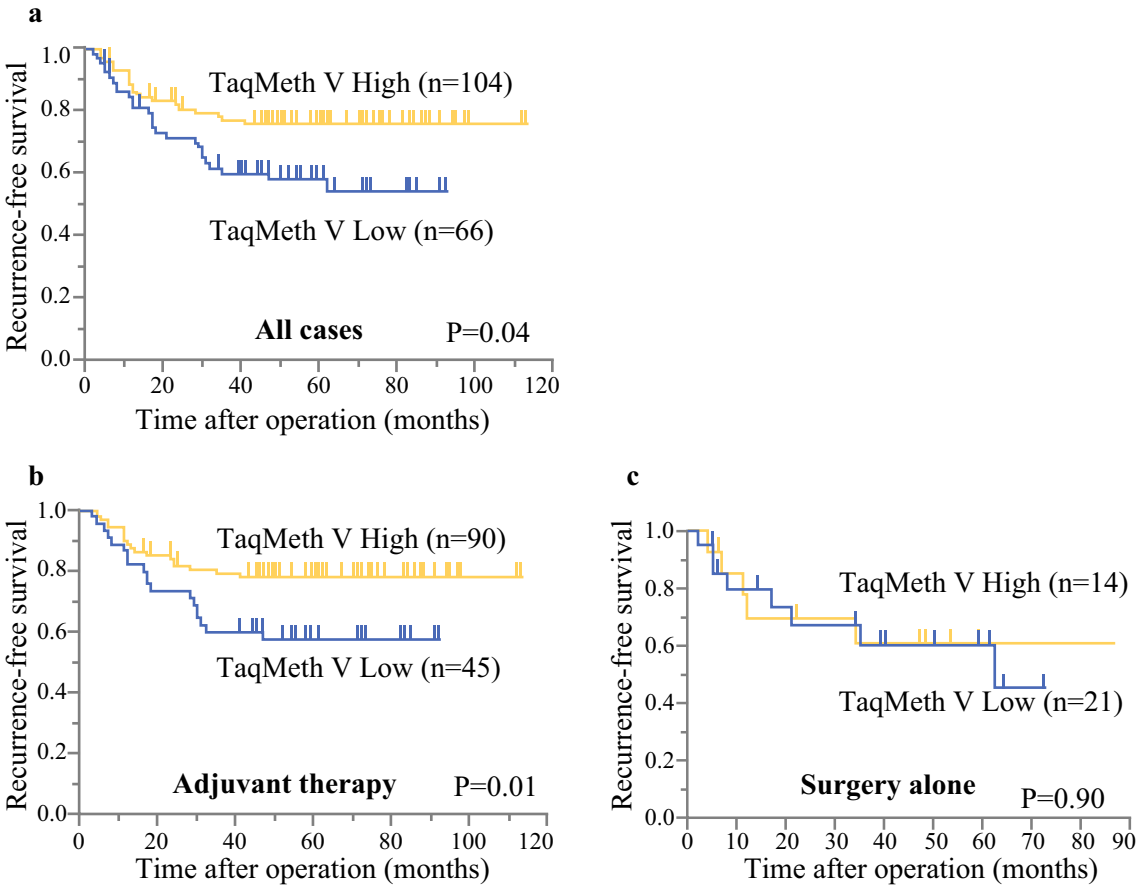

FIG. 1 Kaplan–Meier curves according to *CDO1* TaqMeth value in all cases, adjuvant chemotherapy group, and surgery-alone group. The prognosis of stage III cases with adjuvant chemotherapy was discriminated by *CDO1* TaqMeth value, while the cases without adjuvant therapy were not: **a** patients with higher TaqMeth values showed better prognosis than those with lower TaqMeth values in all stage III patients; **b** patients with higher TaqMeth values also showed better prognosis than those with lower TaqMeth values in the adjuvant chemotherapy group; **c** a difference in prognosis was not seen in the surgery-alone group

Prabhu et al.[7] reported that intratumoral levels of CDO1 and its metabolic product cysteine sulfinic acid (CSA) were increased in glioma. Accumulation of CSA induced attenuation of oxidative phosphorylation by inhibition of pyruvate dehydrogenase (PDH) and was associated with augmented tumorigenesis in glioma cells. We also hypothesized that *CDO1* gene expression and subsequent accumulation of CSA can result in tumor cells that are tolerant to the anaerobic environment through attenuation of oxidative phosphorylation. If this hypothesis is correct, then *CDO1*-expressing cells could survive even in an anaerobic environment so that chemotherapeutic drugs could not reach tumor cells (Fig. 2). We, for the first time, confirmed these two functional aspects of *CDO1* gene in CRC cells.

Forced Expression of CDO1 Increased Resistance to 5-FU Therapy in CRC Cell Lines

First, we used three CRC cell lines (DLD1, HCT15, and HCT116 cells). No cell line expressed *CDO1* gene at mRNA level. We established two clones of stable *CDO1*-expressing cells per cell line (CDO1 #1 and CDO1 #2) (Fig. S1).

To elucidate whether *CDO1*-expressing cell lines develop resistance to chemotherapy, we performed cell proliferation assay after 5-FU treatment. We selected 5-FU because colon cancer patients were most frequently treated with 5-FU-containing drug regimens. The rate of absorbance was increased only in *CDO1*-expressing DLD1 cells, but not in *CDO1*-expressing HCT15 and HCT116 cells (Fig. 3a). We further performed the JC-1 assay after 5-FU exposure in DLD1 cells to elucidate whether the MMP of surviving cells increased after 5-FU treatment. JC-1 dye is a fluorescent dye that shifts from green to red as the MMP increases. Higher red/green ratio was observed in *CDO1*-

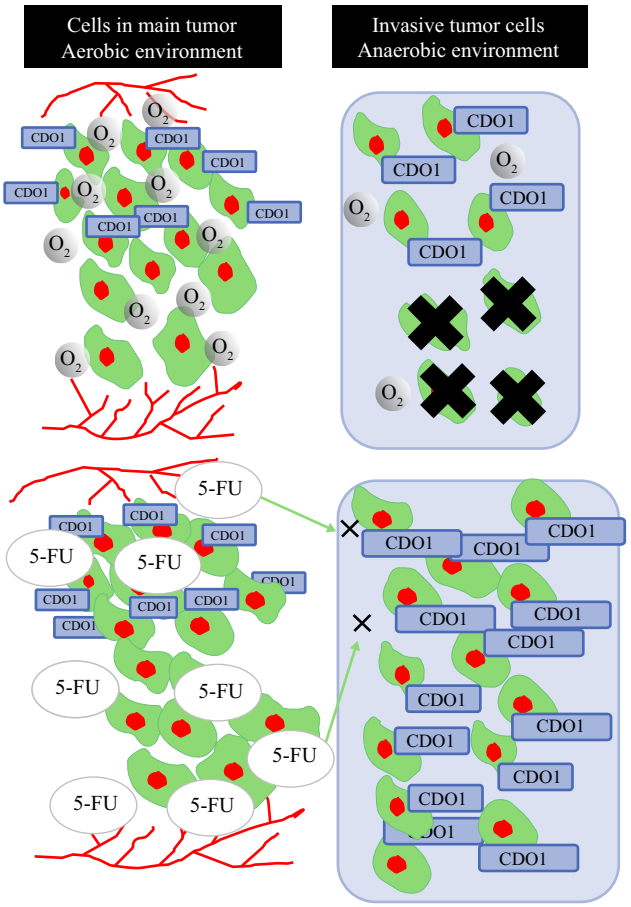

FIG. 2 Schematic illustration of the mechanism of chemotherapy resistance in relation to the survival ability of tumor cells in an anaerobic environment

expressing DLD1 cells (Fig. 3b). Among DLD1 cells, *CDO1*-expressing cells were more tolerant to 5-FU chemotherapy and the MMP of cells resistant to 5-FU chemotherapy in *CDO1*-expressing cells was higher than that in mock-treated cells. Therefore, some cells that have higher MMP were tolerant to chemotherapy, although this tendency was not observed in all cell lines. These results suggest that other factors may exist that can explain the chemoresistant phenotype of *CDO1*-expressing cells.

Forced Expression of CDO1 Gene Raised MMP, Increasing Cellular Tolerance to an Anaerobic Environment

Attenuation of cellular respiration could translate to increased MMP, which could be measured by the JC-1 assay. We initially performed this assay by using fluorescent microscopy. Red-colored cells were more frequently seen among *CDO1*-expressing cells than mock-treated cells in all three cell lines (Fig. 4a). We also performed this assay using other cell lines that were not subjected to any specific conditions including transfection. The cells showed green fluorescence to a similar level as mock-transfected cells (data not shown). Next, we performed this assay by using flow cytometry for quantitative analysis. The ratio of red- to green-colored cells was calculated. *CDO1*-expressing cells demonstrated increased red/green ratios in all three cell lines (Fig. 4b).

We finally assessed tolerability to an anaerobic environment. *CDO1*-expressing cells and mock-treated cells were cultured under hypoxic condition (0.1% O_2). The rate of absorbance and red/green ratio were increased in *CDO1*-expressing DLD1 and HCT15 cells but not in HCT116 cells (Fig. 5a, b). These results suggest that *CDO1*-expressing DLD1 and HCT15 cells, which had high MMPs, were tolerant to hypoxia and could readily survive in an anaerobic environment.

DISCUSSION

CDO1 gene has attracted attention with regard to its extraordinarily frequent cancer-specific methylation in head and neck, esophageal,[5,15] lung,[5,16] breast,[5] gastric,[5] colorectal,[5] gallbladder,[17] biliary tract,[18] prostate,[19] bladder,[5] and endometrial[20] cancers. Such unique characteristics usually represent tumor-suppressive functions, and *CDO1* gene has been shown to harbor such function through generation of reactive oxygen species (ROS).[21,22] This may be an indirect effect because glutathione (GSH), a potent ROS inhibitor, can be formed from cysteine, glutamate, and glycine (Fig. S2). Namely, *CDO1* expression induces reduction of GSH through cysteine consumption, resulting in increased ROS generation, which causes apoptosis of cancer cells. Supporting this concept, *CDO1* hypermethylation steadily increases during tumor progression in gastrointestinal cancers such as esophageal, gastric, colorectal, and gallbladder cancers.[5,15,17] In particular, during the CRC adenoma–carcinoma sequence, *CDO1* methylation gradually increases from normal mucosa to adenoma and more advanced carcinoma.[6]

In this study, we discovered contradictory findings in stage III colon cancer from a prognostic point of view. First, TaqMeth V Low was associated with worse prognosis than TaqMeth V High in total stage III colon cancer. Contrary to stage I/II colon cancer, postoperative adjuvant chemotherapy is usually recommended for stage III colon cancer; thus, we hypothesized that this counterintuitive clinical outcome may be modified by this interventional treatment. Actually, subgroup analysis also revealed that the group of patients with TaqMeth V High showed better prognosis than those with TaqMeth V Low only in the adjuvant chemotherapy group (Fig. 1b, c). This result is

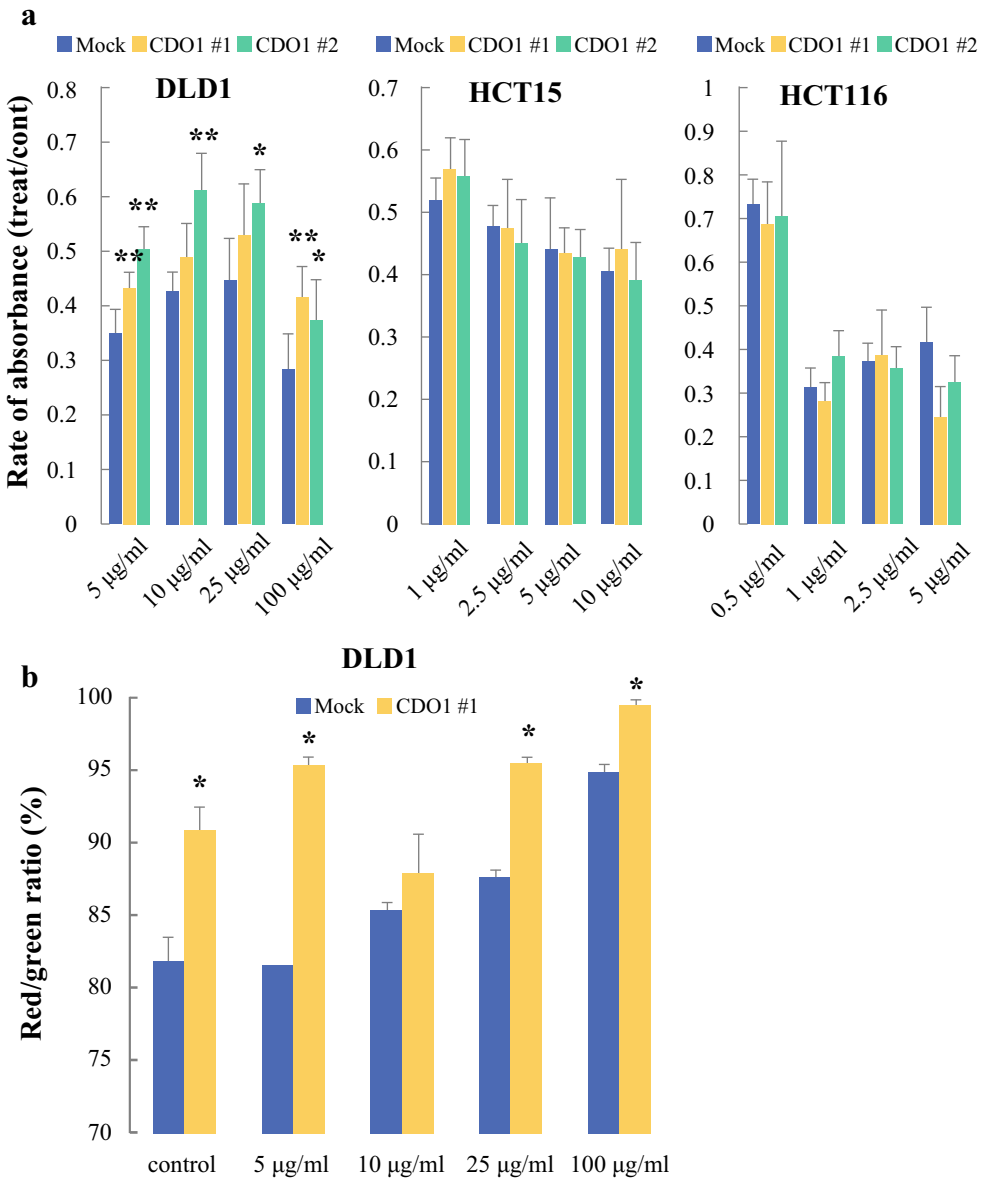

FIG. 3 Results of WST-1 and JC-1 assays after 5-FU treatment in mock-treated cells and *CDO1*-expressing cells. $*P < 0.05$. $**P < 0.01$. **a** The effect of 5-FU treatment was weakened only in DLD1 cells after forced expression of *CDO1* gene. **b** *CDO1*-expressing DLD1 cells showed higher red/green ratio than mock-treated cells after exposure to 5-FU at most concentrations tested

interesting because classical oncology notes that aggressive cancers are paradoxically sensitive to chemotherapy. Nevertheless, such molecular mechanisms have not yet been elucidated. We have also shown similar results in gastric and esophageal cancers (unpublished observations).

To confirm our hypothesis, a functional assessment was made by generating CDO1 stable transfectants in three CRC cell lines, which were tested for tolerance to 5-FU chemotherapy and hypoxic condition together with change in MMP activity. In terms of tolerance to 5-FU, only *CDO1*-expressing DLD1 cells developed resistance. However, this modest tumor effect suggested that the change in drug resistance can be accounted for by an alternative mechanism to affect the differential prognosis of stage III colon cancer with postoperative chemotherapy.

One limitation of the present study is the different responses observed between the three *CDO1*-expressing cells to chemotherapy or hypoxia. This discrepancy in response between cell lines may be partially explained by their ability to generate GSH. GSH generation is limited by the expression of CD44v and xCT unit,[21,22] and the three cell lines may have various CD44v and xCT unit expression levels. CD44v and xCT expressions were investigated, and reduced expression of CD44v was found in HCT15 cells (Fig. S3), suggesting that cysteine metabolism is not deemed effective in HCT15. Additionally, in our study, HCT116 cells showed lower tolerance to both 5-FU treatment and hypoxia than DLD1 and HCT15 cells. Our recent report describing anticancer treatment sensitivity in HCT116 cells also showed silenced expression of *CRBP1*

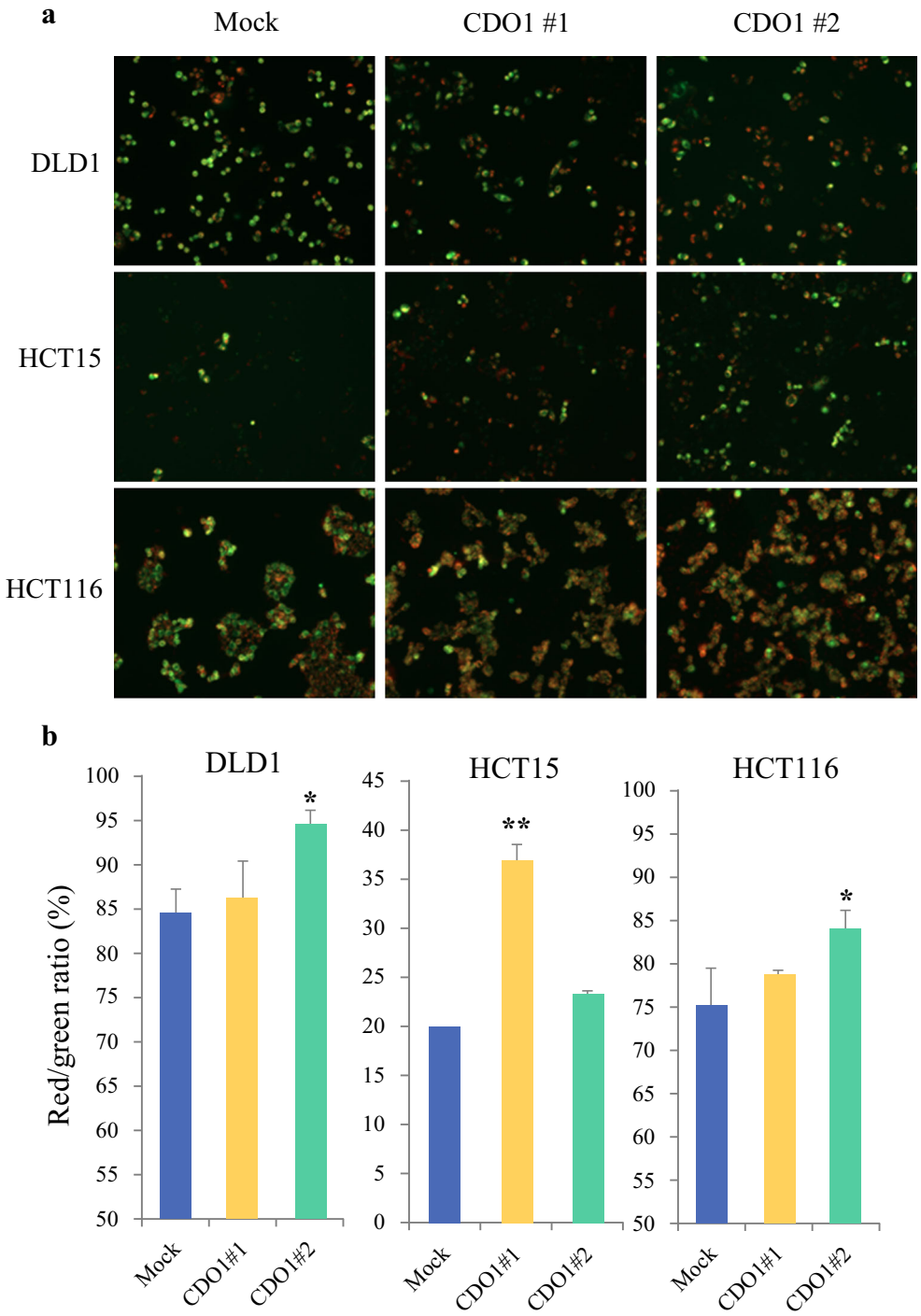

FIG. 4 Results of JC-1 assay in CRC cell lines: **a** representative image of cells stained with JC-1 dye. All cell lines turned red by forced expression of *CDO1* gene. **b** Flow cytometry results of JC-1 assay. Some *CDO1*-expressing cells had higher red/green ratio than mock-treated cells

gene, which is involved in cell viability.[11] We speculate that HCT116 cells are naturally more susceptible to these stressful conditions; thus, changes in phenotype do not occur clearly in CDO1-expressing cells.

CDO1 hypermethylation is characteristic of advanced colon cancers,[6] and advanced cancer patients with *CDO1* hypermethylation are likely to be the most sensitive to adjuvant chemotherapy. The former phenotype is explained by tumor suppressor aspects of *CDO1* gene through ROS generation, while the latter may be accounted for by

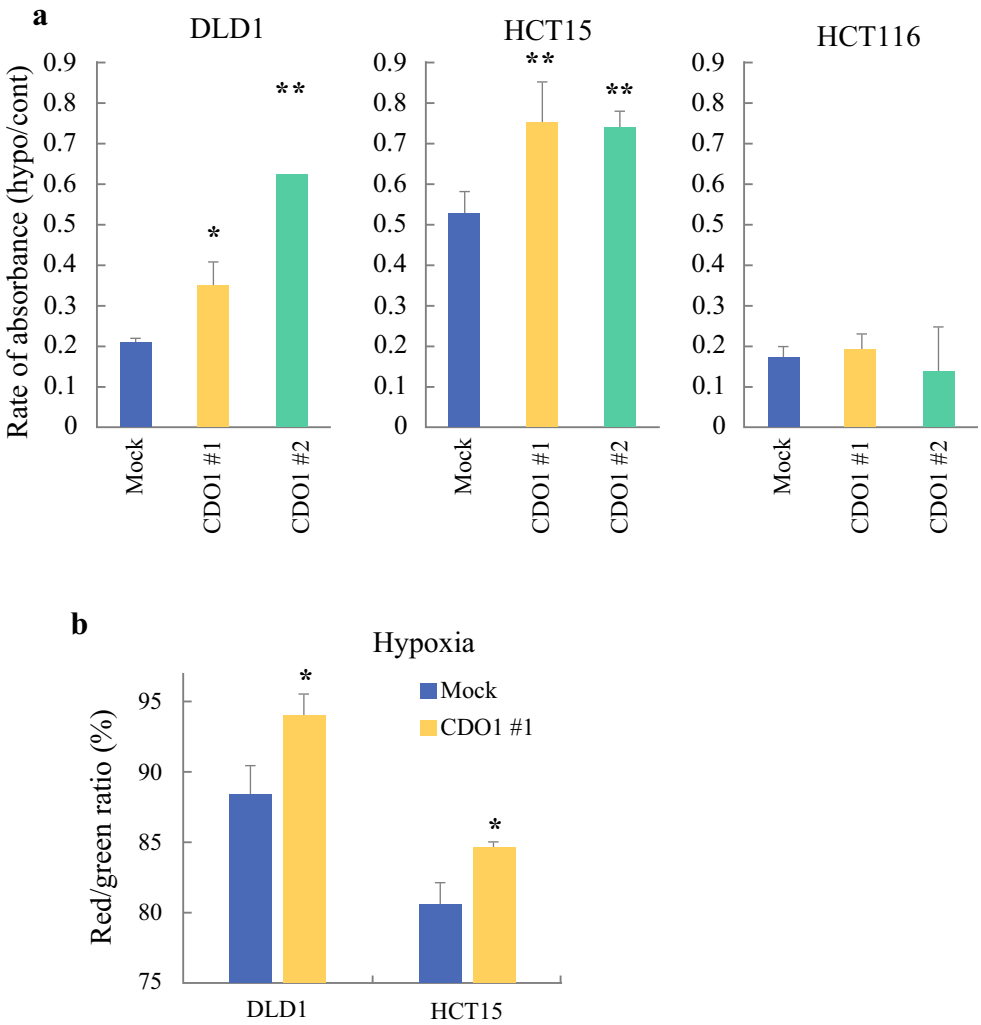

FIG. 5 Results of WST-1 and JC-1 assays after incubation in 0.1% O_2 condition in mock-treated and *CDO1*-expressing cells. $*P < 0.05$. **a** DLD1 and HCT15 cells showed increased tolerance to hypoxia by forced expression of *CDO1* gene, **b** *CDO1*-expressing cells showed higher red/green ratio than mock-treated cells in hypoxia

oncogenic traits. Prabhu et al.[7] demonstrated that the increase of MMP through the CDO/CSA axis was accompanied by tumorigenic phenotypes in glioma cells, and we also observed activated MMP in all CRC cell lines tested in the current study (Fig. 4). The increase in MMP could be represented by inhibition of oxidative phosphorylation, which contributes to the Warburg effect. Moreover, tolerability assay to hypoxic condition demonstrated, for the first time, that *CDO1* gene enables DLD1 and HCT15 cells to survive in an anaerobic environment. As described above, cytotoxic drugs would not be delivered to such an environment, so that *CDO1*-expressing cells in anaerobic areas are far from chemotherapeutic drugs. This could derive from the combination of at least these separate factors (sensitivity to 5-FU treatment and drug delivery) to explain the scientific cause of the differential prognosis of stage III colon cancer with postoperative adjuvant chemotherapy.

In conclusion, hypermethylation of *CDO1* gene was associated with good prognosis in stage III colon cancer patients with postoperative adjuvant chemotherapy. Expression of *CDO1* gene in tumor cells will change their phenotype to be clinically chemoresistant. Reduced sensitivity to 5-FU and insufficient drug delivery can affect the phenotype. The role of hypermethylation of *CDO1* promoter as a prognostic marker in chemotherapy should be assessed in a prospective study and/or other cancers.

DISCLOSURE None of the authors has any conflicts of interest to declare regarding this study.

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