Frequent Inactivation of Cysteine Dioxygenase Type 1 Contributes to Survival of Breast Cancer Cells and Resistance to Anthracyclines

Jana Jeschke, 1,2,3 Heather M. O’Hagan, 2 Wei Zhang, 2 Rajita Vatapalli, 1,2 Marilia Freitas Calmon, 1,2 Ludmila Danilova, 2 Claudia Nelkenbrecher, 3 Leander Van Neste, 4 Ingrid T.G.W. Bijsmans, 5 Manon Van Engeland, 5 Edward Gabrielson, 6 Kornel E. Schuebel, 2 Andreas Winterpacht, 3 Stephen B. Baylin, 2 James G. Herman 2* and Nita Ahuja 1,2*

Department of 1 Surgery and 2 Oncology, The Johns Hopkins University, Baltimore, MD, USA; 3 Institute of Human Genetics, University Hospital Erlangen, University of Erlangen-Nürnberg, Erlangen, Germany; 4 MDxHealth SA, Liège, Belgium; 5 Department of Pathology, GROW-School for Oncology and Developmental Biology, Maastricht University Medical Center, Maastricht, The Netherlands; 6 Department of Pathology, Johns Hopkins University, Baltimore, MD, USA

Running Title: Inactivation of CDO1 and resistance to anthracyclines

Key Words: Cysteine Dioxygenase Type 1, DNA methylation, mutation, oxidative stress, anthracycline resistance

Financial Support: This study was supported by the Susan G. Komen Foundation, German Academic Exchange Service (DAAD), and Dr. Jost Henkel Stiftung.

*Corresponding authors: Nita Ahuja and James G. Herman; Johns Hopkins University, CRB1, Suite 541, 1650 Orleans Street, Baltimore, MD 21287, USA; phone: 410-955-8506; fax: 410-614-9884; email: nahuja@jhmi.edu and hermaji@jhmi.edu

Disclosure: James G. Herman and Stephen B. Baylin receive research funding and are consultants to MDxHealth. Leander Van Neste is an employee of MDxHealth.

Word Count: 4989
TRANSLATIONAL RELEVANCE

Breast cancer (BC) is a heterogeneous disease driven by molecular changes of genetic and epigenetic nature. By screening for genome-wide DNA methylation changes in BC, we identified Cysteine Dioxygenase Type 1 (CDO1) as a DNA hypermethylated gene. We show that CDO1 is frequently DNA methylated in breast primary tumors and that this event is associated with adverse clinical features and poor prognosis. Based on the suggested role for CDO1 in the oxidative stress response of cancer cells, we examined its role in the resistance to the reactive oxygen species (ROS)-generating chemotherapeutic class of anthracyclines. We found that restoration of CDO1 function in BC cells alters the oxidative stress response in a way that it leads to sensitization to anthracyclines. We further provide potential clinical implications for this finding by showing that priming with 5-azacytidine of BC cells with epigenetically silenced CDO1 increases the sensitivity to anthracycline therapy. This finding provides a potential clinical strategy to overcome resistance to this drug and DNA methylation of CDO1 may be useful as a marker to select patients for priming with 5-azacytidine.

ABSTRACT

Purpose: Genome-wide DNA methylation analyses have identified hundreds of candidate DNA hypermethylated genes in cancer. Comprehensive functional analyses provide an understanding of the biological significance of this vast amount of DNA methylation data which may allow the determination of key epigenetic events associated with tumorigenesis. 

Experimental Design: To study mechanisms of CDO1 inactivation and its functional significance in BC in a comprehensive manner, we screened for DNA methylation and gene mutations in primary BCs and analyzed growth, survival and ROS production in BC cells with restored CDO1 function in the context anthracycline treatment. Results: DNA methylation-
associated silencing of CDO1 in BC is frequent (60%), cancer-specific and correlates with disease progression and outcome. CDO1 function can alternatively be silenced by repressive chromatin, and we describe protein damaging missense mutations in 7% of tumors without DNA methylation. Restoration of CDO1 function in BC cells increases levels of ROS and leads to reduced viability and growth, as well as sensitization to anthracycline treatment. Priming with 5-azacytidine of BC cells with epigenetically silenced CDO1 resulted in restored expression and increased sensitivity to anthracyclines. **Conclusion:** We report that silencing of CDO1 is a critical epigenetic event which contributes to the survival of oxidative stressed BC cells through increased detoxification of ROS and thus leads to resistance to ROS-generating chemotherapeutics including anthracyclines. Our study demonstrates the importance of CDO1 inactivation in BC and its clinical potential as a biomarker and therapeutic target to overcome resistance to anthracyclines.

**INTRODUCTION**

Loss of proper tumor suppressor function leads to the initiation and progression of human cancer (1) and aberrant epigenetic alterations including DNA promoter hypermethylation can be responsible for such functional loss (2). Techniques to analyze genome-wide DNA methylation have become useful tools to identify hundreds of new candidate DNA hypermethylated genes in cancer. Comprehensive functional analyses can provide an understanding of the biological significance of the vast amount of DNA methylation data generated, allowing for the discovery of novel tumor suppressor genes and molecular mechanisms underlying tumor growth control, and biomarkers for early detection, prognosis and response to therapeutic agents in cancer (3-7).

*Cysteine Dioxygenase Type 1 (CDO1)*, recently identified as a candidate hypermethylated gene within the functional BC hypermethylome (7), is a non-heme iron dioxygenase (8). CDO1 determines the flux between cysteine catabolism and glutathione synthesis (9) by catalyzing the oxidation of cysteine to cysteine sulfinic acid in the presence of molecular
oxygen (10). Abnormal or deficient CDO1 activity has been implicated in a variety of neurological and autoimmune diseases such as Parkinson’s, Alzheimer’s, rheumatoid arthritis, systemic lupus erythematosus (11-13), and recently in carcinogenesis. CDO1 is a promising prognostic biomarker in malignancies with loss of CDO1 expression being associated with relapse of Wilms tumor (14) and DNA methylation of the CDO1 promoter is associated with poor prognosis in BC patients (7, 15). CDO1 has also been implicated in several studies to play a role in the oxidative stress response of cancer cells (16, 17).

Many cancer cells, in particular at advanced stage, function with higher basal levels of endogenous oxidative stress than normal cells. Under persistently increased ROS production, cancer cells adapt to such stress to escape oxidative damage and ROS-induced apoptosis by developing an enhanced, endogenous detoxification capacity (18). The mechanisms of ROS stress adaptation involve the activation of ROS-scavenging enzymes and endogenous antioxidants (19, 20) such as glutathione (21). While increased ROS stress promotes initiation and progression of cancer (22, 23), excessive levels of ROS can be toxic (24) and lethal if exceeding a threshold above cellular tolerability (25). This concept is of therapeutic interest, since it is thought that increased ROS production makes cancer cells more vulnerable to damage by further ROS insults induced by exogenous ROS-generating agents including the chemotherapeutic class of anthracyclines (24, 26). The redox adaptation of cancer cells, however, can provide a mechanism for resistance to anthracyclines (24, 27).

We present a comprehensive study that addresses the functional significance of silencing of CDO1 during breast tumorigenesis. We report the frequent inactivation of CDO1 by multiple mechanisms (60% DNA methylation, 20% missense mutations) in BC but also across multiple other types of cancer. Cells with restored CDO1 function show reduced growth, viability and ROS detoxification capacity and increased sensitivity to anthracyclines. Given these findings, we suggest that silencing of CDO1 is a critical event that drives tumorigenesis.
and contributes to survival of oxidative stressed BC cells and their resistance to anthracyclines through reducing cellular ROS levels.

**MATERIAL AND METHODS**

**Cell Culture and Drug Treatment**

Cell lines were purchased from American Type Culture Collection (ATCC) and cultured in appropriate media (Mediatech) supplemented with 10% FBS (Atlanta Biologicals) and 1x Penicillin-Streptomycin (Mediatech) at 37°C in 5% CO₂ atmosphere. The HCT116 derivative cell line lacking the major DNA methyltransferases DNMT1 and 3b (\(DNMT1^{-/-}\) and \(DNMT3b^{-/-}\); Double Knockout or DKO) was maintained as previously described (28). Drug treatment with 5-aza-2’-deoxycytidine (DAC) and Trichostatin A (TSA) were carried out as previously described (7). For glutathione depletion, cells were treated with 0.5 mM buthionine sulfoximine (BSO). Doxorubicin was supplemented in doses ranging from 0.078 µM to 20 µM to determine LD50 dosage and 5-azacytidine in doses ranging from 1 µM to 5 µM, chosen for maximal CDO1 expression.

**Patient Samples**

Primary tumor specimen and normal breast tissues from cancer-free donors were obtained from the archives of the Department of Pathology, Johns Hopkins University and Department of Pathology, GROW-School for Oncology and Developmental Biology, Maastricht University Medical Center, The Netherlands with Institutional Review Board approval and Health Insurance Portability and Accountability Art compliance. Genome-wide methylation and expression data of primary tissues were also utilized from The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov/).

**Gene Expression, Methylation Analysis, and ChIP**

RNA was extracted using RNeasy Mini Kit (Qiagen) or purchased from Stratagene (normal tissues). For RT and real-time RT-PCR, 1 µg RNA was reverse-transcribed into cDNA using
Ready-To-Go You-Prime First-Strands Beads (GE Healthcare) with addition of pd(N)$_6$ Random Hexamers (GE Healthcare) according to the manufacturer’s instructions. Genomic DNA was extracted following a standard phenol-chloroform extraction and bisulfite modified using the EZ DNA Methylation Kit (Zymo Research). Methylation-Specific PCR (MSP) was performed as previously described (29). PCR products for bisulfite sequencing were cloned using the TOPO TA Cloning Kit (Invitrogen), purified from single colonies using QIAprep Spin Miniprep Kit (Qiagen), and sequenced with M13 reverse primer by Johns Hopkins Medical Institutions Synthesis & Sequencing Facility. For ChIP, cells were crosslinked in 1% formaldehyde as previously described (30). Nuclear extraction using CEBN and CEB, and ChIP on $\sim$1 x $10^6$ cells per IP was performed as previously described (31). $\alpha$-H3-K4me2 and $\alpha$-H3-K27me3 antibodies from Millipore were used. IP specific products were amplified using real-time PCR.

**Mutation Analysis**

_CDO1_ coding exons were amplified and purified PCR products were bidirectionally sequenced using DNA Sequencing Kit BigDye-Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Forster City, CA, USA). Sequencing products were separated with the Applied Biosystems ABI3730 Sequencing System and analyzed with Lasergene software (DNASTAR). Protein damaging scores for identified mutations were calculated using PolyPhen-2 software at: [http://genetics.bwh.harvard.edu/pph2/](http://genetics.bwh.harvard.edu/pph2/).

**Immunohistochemistry**

_CDO1_ protein expression was detected on sections of formalin-fixed and paraffin-embedded breast tissue (normal and tumors) using Vectastain blocking serum (Vector Laboratories), $\alpha$-CDO1 primary antibody (Abcam), $\alpha$-rabbit biotinylated secondary antibody (Vector Laboratories), horseradish peroxidase–labeled Vectastain Elite ABC Rabbit IgG kit (Vector Laboratories) and 3,3’-diaminobenzidine (Sigma) as substrate. All slides were counterstained with DAKO hematoxylin and Scotts Blue.
Expression Vectors

Wildtype CDO1 (NM_001801.2) or mutant CDO1 (Y157F (32)) was cloned into pcDNA3.1/V5-His B expression vector (Invitrogen). Tetracycline-inducible CDO1-stable cells were generated using the T-REx System (Invitrogen). Expression of CDO1 was induced with 0.5 μg/ml Doxycycline.

Western Blot

CDO1 protein expression was detected in whole cell protein extracts with either α-V5 (Invitrogen) or α-CDO1 (Abcam).

Colony Formation and Soft Agar Assay

Cells, transiently transfected with CDO1, were harvested 24 hrs post transfection, replated in 10 cm² dishes in triplicates and selected with 0.8 mg/ml Geneticin/G418 (Invitrogen) for 15 days. Colonies were stained with Giemsa and counted. Soft agar assays were started 48 hrs following transfection. 1.5 x 10⁴ cells in complete media containing 0.4% agar were layered on top of 0.6% agar in 24-well plates in duplicates. Colonies were selected with 0.8 mg/ml Geneticin/G418 (Invitrogen) for 28 days and counted after staining with 0.005% crystal violet.

Cell Viability Assay

Cell viability was measured using CellTiter96® kit (Promega). Cells were incubated in MTS reagent for 4 hrs at 37 °C. Absorbance was measured at 490 nm.

Measurement of ROS Production

ROS production was measured using CM-H2DCFDA probe (Invitrogen). Cells were loaded with 5 μM CM-H2DCFDA probe in phenol-red free and serum-free media for 1 hr. Fluorescence was measured at 493 nm excitation and at 523 nm emission.

Statistics
Tumor stage and gene methylation status were correlated using Pearson’s $\chi^2$. Hazard ratio (HR) for prognostic value of gene methylation status was calculated using univariate Cox regression analysis. Student’s t test and trend test were used to perform group comparisons for colony formation, soft agar, ROS and cell viability assays. Gene expression and methylation status of TCGA data were correlated calculating a Spearman correlation coefficient. $P$ values of less than 0.05 were considered significant. All statistical analyses were performed using the STATA 9.2 software package.

**RESULTS**

Silencing of CDO1 is associated with DNA promoter hypermethylation or repressive chromatin structure

CDO1 (Supplementary Figure 1 illustrates the genomic location, structure and CpG island of the CDO1 gene) was identified as a candidate hypermethylated gene within the functional BC hypermethylome (7), where potential DNA hypermethylated genes appear in a zone in which gene expression was not detectable in untreated cells and cells treated with the HDAC inhibitor TSA (< 0.5 log-fold), but increased greater than 0.5 log-fold in cells treated with the DNMT inhibitor DAC. CDO1 appeared in this characteristic spike of potentially DNA hypermethylated genes in three out of four tested invasive BC cell lines (3.49 log-fold in MDA-MB-231, 1.24 log-fold in MCF7, and 1.48 log-fold T-47D) (Figure 1A). Conversely, CDO1 did not appear in the spike of potentially DNA hypermethylated genes in non-transformed human mammary epithelial cells (HMECs), suggesting that CDO1 DNA methylation occurs specifically in cancer. In HMECs, CDO1 was silenced at basal level (untreated), but re-expressed with DAC (0.73 log-fold) and TSA (0.54 log-fold) (Figure 1A).

The mRNA expression status of CDO1 was validated in untreated as well as DAC and TSA treated MDA-MB-231, MCF7 and T-47D BC cells or HMECs (Figure 1B). Additionally, we detected CDO1 baseline transcription in normal breast tissue (NB). Next, we examined the basal DNA methylation status of CDO1 using bisulfite sequencing. The CDO1 promoter is
unmethylated in NB and HMECs, densely methylated in MDA-MB-231 and MCF7 cells and partially methylated in T-47D cells (Figure 1C). To investigate whether the loss of CDO1 expression in HMECs could be due to histone modifications, we next performed ChIP for histone marks at the CDO1 promoter region in HMECs, MDA-MB-231 and 293 cells. CDO1 expressing 293 cells are enriched for the active H3K4me2 mark and have low levels of the repressive H3K27me3 mark, whereas MDA-MB-231 cells, which have CDO1 densely methylated, have low levels of the active and the repressive mark (Figure 1D). In contrast, HMECs, which have CDO1 silenced but not methylated, display a bivalent chromatin pattern with the highest levels of the repressive H3K27me3 mark and relatively high levels of the H3K4me2 mark.

DNA methylation-associated silencing of CDO1 is cancer-specific, frequent and correlates with disease progression and outcome. We studied DNA methylation of CDO1 in 20 NB specimens from cancer-free patients (Supplementary Table 1A) as well as in a cohort of 185 primary BCs including stages 0 (DCIS) to 4 tumors (Supplementary Table 1B). DNA methylation of CDO1 is frequent and cancer-specific, i.e. DNA methylation was detected in 108 out of 185 BCs (~60%), but in none of the tested 20 NB samples (Figure 2A left for selected samples). Next, we examined CDO1 protein expression by IHC on selected tumor samples and in NB tissue. In NB, CDO1 is uniformly expressed in the cytoplasm of ductal termini cells (Figure 2A right, upper panel left). In DCIS, CDO1 expression is lost in hyperproliferative ductal termini (HD) where CDO1 is methylated but not in normal differentiated ductal termini (ND) (Figure 2A right, upper panel right and Figure 2A left, sample BC 013a). In higher stage BCs, we also observed a correlation between CDO1 expression and CDO1 methylation status. A methylated CDO1 promoter was associated with loss of CDO1 expression (BC 060a, Figure 2A left and 2A right, lower panel left) while an unmethylated promoter correlated with retained expression of CDO1 (BC 062a, Figure 2A left and 2A right, lower panel right). We next utilized data of 255
primary BCs from the TCGA database and determined a significant inverse correlation ($\rho = -0.47$, $p = 1.7e-15$) between DNA methylation and expression of CDO1 (Figure 2B).

We next tested whether the presence of CDO1 methylation altered prognosis of women with BC. Common prognostic clinicopathologic variables were compared to CDO1 methylation status in our cohort of 185 BC patients. The frequency of CDO1 methylation significantly increased with tumor stage, i.e. 44% in stage 0 / DCIS, 53% in stage 1 and 2, and 81% in stage 3 and 4 tumors (Figure 2C and Supplementary Table 2). Additionally, CDO1 was significantly more frequently methylated in tumors with lymphovascular invasive (lvi) / perinodal invasive (pni) BCs ($p = 0.011$; 52% in lvi / pni neg BCs and 73% in lvi / pni pos BCs). Furthermore, a methylated CDO1 promoter status was associated with an unfavorable patient outcome (HR 2.13, 95% CI 1.17 - 3.86, $p = 0.013$), but not independently of age and stage (HR 1.10, 95% CI 0.57 - 2.12, $p = 0.771$). A survival curve based on the univariate Cox regression model is shown in figure 2D.

DNA methylation-associated silencing of CDO1 occurs in multiple cancer types

We expanded our efforts to analyze the DNA methylation and expression status of CDO1 in other tumor types. We assayed the CDO1 expression status in normal tissues as well as in three corresponding cancer cell lines (ovary, lung, pancreas and liver). CDO1 was expressed in all tested normal tissues but not expressed in association with DNA promoter methylation in the corresponding cancer cell lines with the exception of HEPG2 liver cancer cells, in which the CDO1 promoter is unmethylated (Supplementary Figure 2A). We further determined CDO1 methylation status in primary tumor specimens of these tumor types. We found CDO1 to be commonly methylated (>60%) in ovary, lung and pancreas cancer (Supplementary Figure 2B), but not in hepatocellular cancer (9%). Again, using data from the TCGA database, analysis of 104 primary lung cancers and 584 primary ovarian cancers confirmed an inverse relationship (lung cancer: $\rho = -0.60$, $p = 1e-63$ and ovarian cancer: $\rho = -0.62$, $p = 1.6e-63$) between DNA methylation and expression of CDO1 (Supplementary
Figure 2C). As in BC, we observed a correlation between tumor stage and CDO1 methylation frequency in TCGA lung and ovarian cancers (Supplementary Figure 2D).

Tumor-specific point mutations within the CDO1 gene have a predicted protein damaging effect.

To test whether CDO1 may be inactivated by ways other than epigenetic mechanisms, we screened for mutations within the CDO1 gene in 60 primary BCs (unmethylated CDO1 promoter status). We found 9 SNPs in 10 patients (17%) (Table 1) leading to amino acid substitutions (missense mutations). The identified mutations did not associate with an unfavorable patient outcome. In order to evaluate the functional significance of these mutations, the PolyPhen-2 software was utilized to calculate a protein damage score. Three mutations (T4I, L62F, and E79K) reached a score of ~1, predicting for a protein damaging effect (values near 1 are predicted to be deleterious) with the highest possible probability. The Y157F mutation was introduced as a control into this assay. This mutation, within the catalytic center of the CDO1 enzyme, has been shown to reduce the enzymatic activity to up to ~95% (32). A calculated protein damaging score of 0.999 reliably predicted for the experimental proven loss of function caused by this mutation. Subsequently, we confirmed the three mutations with the highest damage scores as tumor-specific by screening normal tissue of the four patients that harbored these mutations in their tumor. Supplementary Figure 3 provides chromatograms of the tumor and matching normal tissue. Overall, we identified four out of 60 patients (7%) that harbor tumor-specific and protein-damaging point mutations within the CDO1 gene.

Restoration of CDO1 function reduces growth and viability of cancer cells and their capacity to detoxify ROS.

To test whether CDO1 can alter cancer cell growth, we performed colony formation and soft-agar assays after transient expression of CDO1 in MCF7 and MDA-MB-231 cells, in which...
endogenous expression of \textit{CDO1} is silenced by DNA methylation. Following expression of enzymatic active wild-type \textit{CDO1} (\textit{CDO1-WT}) in MCF7 and MDA-MB-231 cells, cells formed markedly fewer colonies (Figure 3A and Supplementary Figure 4A and 4B) on plastic and in soft-agar than cells transfected with empty vector (pcDNA3.1) or mutant \textit{CDO1} protein (\textit{CDO1-MU}). Notably, expression of enzymatic impaired \textit{CDO1-MU} protein suppressed growth of cells compared to cells expressing empty vector, possibly due to the incomplete catalytic loss of the Y157F mutation (32). We confirmed re-expression of \textit{CDO1} at protein level by western blot (Figure 3A).

Given \textit{CDO1}'s key role in the cysteine and glutathione metabolism (9, 17), we next studied ROS levels and cell viability in MDA-MB-231 cells having inducible expression \textit{CDO1-WT} or \textit{CDO1-MU}. ROS production was 33\% higher in cells having inducible \textit{CDO1-WT} expression as compared to mock cells and these cells were 20\% less viable (Figure 3B). ROS production was slightly, but not significantly, increased between mock cells or those expressing \textit{CDO1-MU} protein without a change in cell viability. These results suggest that expression of enzymatic active \textit{CDO1} reduces viability of MDA-MB-231 cells through decreasing their ROS detoxification capacity.

Next, we treated 293 cells, which endogenously express \textit{CDO1}, with the oxidative damaging and glutathione depleting agent buthionine sulfoximine (BSO) (33). Interestingly, 24 hrs upon treatment with BSO, 293 cells show decreased levels of \textit{CDO1} protein and increased levels of ROS production that return to baseline at 48 hrs post treatment (Figure 3C). This suggests that \textit{CDO1} protein level may decrease in response to increasing ROS production as an antioxidant adaptive mechanism.

\textbf{\textit{CDO1} induced reduction in ROS detoxification sensitizes BC cells to anthracycline treatment}

Anthracyclines, such as Doxorubicin, are cytotoxic ROS-generating chemotherapeutic agents (27) widely used in the treatment of BC. Resistance to these agents is believed to be
conferred by the upregulation of the ROS detoxification capacity in adaptation to intrinsic oxidative stress in cancer cells (27). To test whether inactivation of CDO1, as observed above, might contribute to the resistance of BC cells to Doxorubicin therapy, we treated CDO1 inducible MDA-MB-231 cells with different doses of Doxorubicin. Cells expressing enzymatic active CDO1-WT, but not CDO1-MU, were significantly less viable at Doxorubicin doses of 0.078 µM and 0.3125 µM than mock cells (Figure 4A and 4B). As expected, Doxorubicin treatment increases ROS production as compared to untreated cells. Expression of CDO1-WT, but not CDO1-MU, further increased the Doxorubicin-induced ROS production compared to mock cells (Figure 4B).

To determine whether restoration of CDO1 expression through treatment with 5-azacytidine was a viable strategy to sensitize BC cells to Doxorubicin therapy, we pretreated MDA-MB-231, MCF7 and T-47D cells with 5-azacytidine for 72 hrs. This treatment resulted in re-expression of CDO1 and an up to 40% decreased cell viability when cells were subsequently treated with Doxorubicin compared to cells not pretreated with 5-azacytidine (Figure 5A and 5B). To further implicate the re-expression of CDO1 in this synergistic effect, we pretreated MDA-MB-231 cells with Doxycycline-induced CDO1 expression with 5-azacytidine and subsequently with Doxorubicin. We observed no difference in viability between cells that overexpressed CDO1 and were pretreated with 5-azacytidine and cells that overexpressed CDO1 and were not pretreated with 5-azacytidine (Figure 5C and 5D).

DISCUSSION

In the current study we show that aberrant DNA methylation of CDO1 is a tumor-specific and frequent (~60%) event in BC that is associated with gene silencing. We observed a stage-dependent increase in CDO1 methylation frequency that significantly correlates with disease progression and outcome. Loss of CDO1 expression by DNA methylation is also a frequent event in multiple other cancer types. In addition to other studies that uncovered CDO1 as aberrantly methylated and silenced in colorectal cancer (34) and malignant glioma (35), we
found promoter hypermethylation of *CDO1* in association with gene silencing in ovary, lung, pancreas and hepatocellular cancer. We further demonstrate that *CDO1* function can also be lost by other mechanisms. In HMECs, we correlated silenced expression of *CDO1* with a decrease of the active H3K4me2 histone mark and an increase of the repressive H3K27me3 mark, indicative of bivalent chromatin, suggesting that a repressive chromatin structure at the *CDO1* promoter can adequately suppress the expression of *CDO1*, similar to the poised state of embryonic stem cells (36). In addition to aberrant epigenetic regulation of *CDO1* gene expression, we discovered genetic aberrations that potentially alter *CDO1* function. Screening of primary BCs with an unmethylated *CDO1* status revealed missense mutations in 17% of these tumors and when tested for functional significance half of these mutations predicted for protein damage. Unlike *CDO1* methylation, the identified mutations within the *CDO1* gene did not correlate with disease outcome, potentially due to the small number of samples screened.

Under persistent increased ROS production, cancer cells adapt to such stress to escape oxidative damage and cell death by developing an enhanced, endogenous antioxidant capacity (18, 21). We find that cells that re-express enzymatic active *CDO1* harbor more ROS and are less viable than cells expressing enzymatic impaired *CDO1*. Restoration of *CDO1* function in BC cells may shift the flux from glutathione synthesis towards cysteine catabolism resulting in a decreased antioxidant capacity that is not sufficient to keep ROS levels below a toxic threshold. Similar findings have been made by Dominy *et al.* (17). Overexpression of *CDO1* resulted in reduced levels of cysteine and glutathione and in enhanced sensitivity to a glutathione-dependent stressor, suggesting that glutathione levels and cellular redox capacity change in response to *CDO1* expression through the limitation of cysteine, the substrate for glutathione synthesis. Based on these and our data we suggest that epigenetic silencing of *CDO1* may occur in cancer cells with increased ROS production and that this event may contribute to the survival of these oxidative stressed cancer cells through an increased ROS detoxification.
It is thought that increased ROS production makes cancer cells more vulnerable to damage by further ROS insults induced by exogenous ROS-generating agents such as anthracyclines (24, 26). However, an enhanced antioxidant capacity not only enables cancer cells to survive under increased ROS stress and contributes to cancer cell transformation and metastasis (37-39) but also leads to resistance to ROS-generating agents (24, 27). In this respect, we observed that MDA-MB-231 cells, upon treatment with the anthracycline Doxorubicin, are more sensitive when they re-express enzymatic active CDO1 as compared to cells which express functional impaired CDO1. This finding is particularly interesting when taking into account that hypermethylation of CDO1 is an outcome predictor in anthracycline treated, estrogen receptor-positive and lymph node-positive BC patients (15). Our finding, that BC cells with loss of CDO1 function are less sensitive to Doxorubicin treatment, provides a mechanism for the predictive value of CDO1 methylation in anthracycline treated patients and expands our understanding of how cancer cells escape the damage of ROS-generating chemotherapeutics. Anthracyclines are key components of the treatment of BC patients and loss of CDO1 expression might be a useful marker for prediction of resistance to this therapy and for selection of patients for priming therapy with 5-azacytidine to overcome resistance. Our data support that priming with 5-azacytidine of BC cells with epigenetically silenced CDO1 may sensitize them to anthracycline therapy partly through the re-expression of CDO1.

Given the inactivation of CDO1 by multiple mechanisms across multiple types of cancer, a pattern that has been observed for important tumor growth suppressive genes, the reduced growth, viability and ROS detoxification capacity of cells with restored CDO1 function, we suggest that CDO1 may have tumor suppressive function and that silencing of CDO1 may contribute to the survival of oxidative stressed cancer cells and their resistance to anthracyclines through increased ROS detoxification. Dependence of cancer cell survival has recently been shown to rely on the methylation of CDO1 as one of the driver epigenetic...
events (40). Our findings not only support these results, but also explore in detail the functional significance of epigenetic silencing of CDO1 during breast tumorigenesis.

ACKNOWLEDGEMENTS

We thank Sharon Metzger and Theresa Sanlorenzo-Caswell from the Johns Hopkins Tumor Registry for their help with clinicopathologic patient information as well as Kathy Bender and Rick Moore for administrative support.

REFERENCES


**TABLES**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Patient</th>
<th>PolyPhen-2 Damage Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4I</td>
<td>177a</td>
<td>0.907</td>
</tr>
<tr>
<td>G25S</td>
<td>203a</td>
<td>0</td>
</tr>
<tr>
<td>D26N</td>
<td>085a</td>
<td>0.001</td>
</tr>
<tr>
<td>V28I</td>
<td>166a</td>
<td>0</td>
</tr>
<tr>
<td>E41K</td>
<td>210a</td>
<td>0</td>
</tr>
<tr>
<td>L62F</td>
<td>162a; 048a</td>
<td>1</td>
</tr>
<tr>
<td>M73I</td>
<td>210a</td>
<td>0.09</td>
</tr>
<tr>
<td>E79K</td>
<td>087a</td>
<td>1</td>
</tr>
<tr>
<td>G195D</td>
<td>164a</td>
<td>0.009</td>
</tr>
<tr>
<td>Y157F*</td>
<td>control</td>
<td>0.999</td>
</tr>
</tbody>
</table>

*reduces enzymatic activity of CDO1 to up to 95%

**FIGURE LEGENDS**

Figure 1- Silencing of **CDO1** is associated with DNA promoter hypermethylation or repressive chromatin structure

(A) Appearance of **CDO1** within the BC hypermethylome. Cell lines were treated with either 5 μM DAC for 96 hrs or 300 nM TSA for 18 hrs. Gene expression changes (analyzed on 4x44K Agilent platform) are plotted by fold change (log scale) after DAC (Y-axis) or TSA (X-axis) treatment. (B) Quantitative mRNA expression of **CDO1** in DAC (5 μM 96 hrs) or TSA (300 nM 18 hrs) treated cells is shown in fold change (log2) relative to Mock-treated cells. Expression of **CDO1** in normal breast (NB) is shown in relation to basal expression levels of **CDO1** in other cell lines. Group comparisons were performed using Student's t test. * p < 0.05. (C) Bisulfite sequencing of the **CDO1** promoter region from -192bp to +60bp relative to the TSS. White and black circles represent unmethylated and methylated CpG dinucleotides,
respectively. (D) ChIP at the CDO1 promoter region from -154bp to -29bp relative to TSS for α-H3k4me2 and α-H3k27me3. Data presented are the mean levels of enrichment relative to input obtained by real-time PCR from two independent experiments ± SEM.

Figure 2- DNA methylation-associated silencing of CDO1 is cancer-specific, frequent and correlates with disease progression and outcome

(A) DNA methylation and protein expression status of CDO1. CDO1 promoter region from -168bp to -45bp relative to the TSS was assayed by MSP in a cohort of 20 normal breast tissues from non-cancer patients and 185 primary BCs of stages 0 (DCIS) to 4 with U and M marking unmethylated and methylated bands, respectively. Representative examples are shown for each cohort and each tumor stage. In vitro methylated DNA (IVD), DKO cells, normal lymphocytes (NL), and H2O controls were assayed along with samples. Protein expression status of CDO1 was assayed by immunohistochemistry in NB and selected primary BCs. Note, BC013a, a DCIS sample, displays loss of CDO1 expression in hyperproliferative ductuli (HD) but expression of CDO1 in normal ductuli (ND). (B) Scatter plot depicting correlation between expression log base 2 values (y-axis) (analyzed on Agilent 244K Custom Gene Expression G4502A-07 platform) and DNA methylation ß values (x-axis) (analyzed on Illumina HumanMethylation 27k platform) of CDO1 in 255 primary BCs from TCGA Data Portal. A Spearman correlation coefficient of ρ = -0.47 and a p value of 1.7e-15 were calculated. A p < 0.05 was considered statistically significant. (C) DNA methylation frequency (in %) of CDO1 plotted by tumor stage of 185 primary BCs. CDO1 methylation status and tumor stage were correlated using Pearson’s χ² test. * p < 0.05. (D) Survival curve depicting prognostic value of CDO1 methylation status for outcome prediction in 185 primary BCs. Hazard ratio (HR) for prognostic value was calculated using univariate Cox proportional hazards regression model. * p < 0.05.
Figure 3- Restoration of CDO1 function reduces growth and viability of cancer cells and their capacity to detoxify ROS

(A) Tumor cell clonogenicity was assessed on plastic and in soft-agar. Cells were transiently transfected with pcDNA3.1 (empty vector), pcDNA3.1-CDO1-WT (wild-type CDO1) or pcDNA3.1-CDO1-MU (mutant CDO1), replated 24 hrs post transfection for selection with Geneticin/G418. After 18 days of selection, colonies were stained with Giemsa and counted. Data presented are the mean of two independent experiments ± SEM. Group comparisons were performed using Student's t test and trend test. * p < 0.05. Re-expression of CDO1 was confirmed 48 and 96 hrs post transfection by western blot using α-V5 antibody, targeting the V-5-His tag of recombinant CDO1 protein, and α-GAPDH as a control. (B) ROS production and cell viability were assayed in Tetracycline-inducible CDO1-stable MDA-MB-231 cells before and after treatment with 0.5 μg/mL Doxycycline, and (C) 293 cells before and after the treatment with 0.5 mM BSO (for depletion of glutathione) by fluorescence of the CM-H2DCFDA probe. Obtained values were normalized to untreated or treated empty vector controls and plotted as % relative to untreated MDA-MB-231-CDO1-WT or untreated 293 cells. Data presented are the mean of three independent experiments ± SEM. Group comparisons were performed using Student's t test. * p < 0.05. Re-expression of CDO1 in MDA-MB-231 cells 48 hrs post Doxycycline treatment or downregulation of CDO1 expression in 293 cells 24, 48 and 72 hrs after glutathione depletion with BSO was assessed by western blot using α-CDO1 antibody and α-β-Actin as a control.

Figure 4- CDO1 induced reduction in ROS detoxification sensitizes BC cells to anthracycline treatment

(A) Cell viability of Doxycycline-induced/not Doxycycline-induced CDO1-stable MDA-MB-231 cells before and 48 hrs after treatment with different doses of anthracycline (Doxorubicin) was measured by MTS assay. Data presented are the mean of two independent experiments ± SEM. Group comparisons were performed using Student’s t test. * p < 0.05.
(B) ROS production, using CM-H2DCFDA probe, and cell viability of same cells before and after treatment with 0.078 µM Doxorubicin was measured. Data presented are the mean of two independent experiments ± SEM. Obtained values for (A) and (B) were normalized to anthracycline-untreated or treated Doxycycline-induced/not Doxycycline-induced empty vector control cells and plotted as % relative to anthracycline-untreated and not Doxycycline-induced MDA-MB-231-CDO1-WT cells. Group comparisons were performed using Student’s t test. * p < 0.05.

**Figure 5- Reactivation of epigenetically silenced CDO1 through priming with 5-azacytidine contributes to the sensitization of BC cells to anthracycline treatment**

(A) Cell viability of MDA-MB-231, MCF7 and T47-D cells primed with 5-azacytidine at doses ranging from 1 µM to 5 µM for 72 hrs and subsequently treated with Doxorubicin at doses ranging from 0.078 µM to 20 µM for 48 hrs. Obtained values are plotted as % relative to Doxorubicin-untreated cells. Group comparisons were performed using Student’s t test. * p < 0.05 for 1 µM 5-azacytidine in MCF7 and T47-D cells or 2 µM in MDA-MB-231 cells. + p < 0.05 for 2 µM 5-azacytidine in MCF7 and T47-D cells or 5 µM in MDA-MB-231 cells. (B) Quantitative re-expression of CDO1 in 5-azacytidine (1 µM, 2 µM or 5 µM for 72 hrs) treated cells prior Doxorubicin treatment is shown in fold change (log2) relative to Mock-treated cells. Group comparisons were performed using Student’s t test. * p < 0.05. (C) Cell viability of Doxycycline-induced CDO1-stable MDA-MB-231 cells (Doxycycline was supplemented every 24 hrs throughout the entire experiment) primed with 5-azacytidine at a dose of 2 µM for 72 hrs and subsequently treated with Doxorubicin at a dose of 1.25 µM for 48 hrs. Obtained values are plotted as % relative to Doxorubicin-untreated cells. As a control, cell viability was measured in CDO1-stable MDA-MB-231 cells that were not treated with 5-azacytidine. (D) Restoration of CDO1 protein expression in MDA-MB-231 cells 72 hrs post treatment with Doxycycline and with or without 5-azacytidine was confirmed by western blot using α-CDO1 antibody and α-β-Actin as a control.
Figure 1

A

B

C

D

MDA-MB 231

MCF7

T47D

HMEC

TSA (log base 2)

DAC (log base 2)

MDA-MB 231

MCF7

T47D

HMEC

Mock DAC TSA

Mock DAC TSA

Mock DAC TSA

Mock DAC TSA

Fold Change (log2)

Fold Change (log2)

Fold Change (log2)

Fold Change (log2)

Relative Enrichment (ChIP/Input)

Relative Enrichment (ChIP/Input)

Relative Enrichment (ChIP/Input)

Relative Enrichment (ChIP/Input)

H3k4me2

H3k27me3

IgG

IgG
Figure 2

A

B

C

D

Breast cancer

N = 255

Expression (log base 2)

p = -0.47; p = 1.7e-15

DNA Methylation (β values)

N = 36

N = 107

N = 42

Stage 0

Stage 1+2

Stage 3+4

CDO1 unmethylated

CDO1 methylated

HR = 2.13; p = 0.013

Non-DNA methylation

Normal Breast, CDO1 unmethylated

Normal Breast, CDO1 methylated

BC 013a, Stage 0, CDO1 methylated

BC 013a, Stage 0, CDO1 unmethylated

BC 065a, Stage 4, CDO1 methylated

BC 065a, Stage 4, CDO1 unmethylated

BC 062a, Stage 4, CDO1 unmethylated

Explanations:

- B: DNA methylation analysis for breast cancer patients.
- C: Scatter plot showing correlation between expression and DNA methylation.
- D: Survival analysis with Kaplan-Meier curves for CDO1 unmethylated vs. methylated.
Figure 3
Figure 4

A

```
Relative Cell Viability (%)
0  20  40  60  80  100  120

0uM  0.078uM  0.3125uM
Doxorubicin

WT  WT/+Dox
```

B

```
Relative ROS Production (CM-H2DCFDA/Cell Count)
0  50  100  150  200  250

CDI-1-WT-Mock  CDI-1-WT-AC  CDI-1-Mu-AC
Mock  Anthracycline

-Dox  +Dox

Mock  Anthracycline
```

```
Relative Cell Viability (%)
0  20  40  60  80  100  120

0uM  0.078uM  0.3125uM
Doxorubicin

MU  MU/+Dox
```

```
Relative ROS Production (CM-H2DCFDA/Cell Count)
0  50  100  150  200  250

CDI-1-WT-Mock  CDI-1-WT-AC  CDI-1-Mu-AC
Mock  Anthracycline

-Dox  +Dox
```

N.S.  *
Figure 5

A

MDA-MB-231

MCF7

T47D

Relative Cell Viability (%)

Doxorubicin

Mock — 2μM Aza — 5μM Aza

Mock — 1μM Aza — 2μM Aza

Mock — 1μM Aza — 2μM Aza

C

MDA-MB-231

Relative Cell Viability (%)

Doxorubicin

Mock + CDO1 — 2μM Aza + CDO1

B

Fold Change (log2)

Mock 2μM 5μM

5-Azacytidine

Fold Change (log2)

Mock 1μM 2μM

5-Azacytidine

Fold Change (log2)

Mock 1μM 2μM

5-Azacytidine

D

CDO1-WT

DOX + +

5-Aza — +

β-Actin

CDO-1

Downloaded from clincancerres.aacrjournals.org on April 1, 2017. © 2013 American Association for Cancer Research.
Frequent Inactivation of Cysteine Dioxygenase Type 1 Contributes to Survival of Breast Cancer Cells and Resistance to Anthracyclines

Nita Ahuja, Jana Jeschke, Heather M O'Hagan, et al.

Clin Cancer Res  Published OnlineFirst April 29, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-12-3751

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2013/05/01/1078-0432.CCR-12-3751.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.