Cytidine deaminase axis modulated by miR-484 differentially regulates cell proliferation and chemoresistance in breast cancer

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No potential conflicts of interest were disclosed.
Abstract:

There has been little study of how the evolution of chemoresistance in cancer affects other aspects of disease pathogenesis. Here, we show that an important chemoresistance axis driven by cytidine deaminase (CDA) also acts to suppress cell cycle progression by regulating cyclin E-CDK2 signaling. We found that CDA was regulated by miR-484 in a gemcitabine-resistant model of breast cancer. Elevating miR-484 expression reversed the CDA effects, thereby enhancing gemcitabine sensitivity, accelerating cell proliferation and redistributing cell cycle progression. Conversely, elevating CDA to restore its expression counteracted the chemosensitization and cell proliferative effects of miR-484. In clinical specimens of breast cancer, CDA expression was frequently downregulated and inversely correlated with miR-484 expression. Moreover, high expression of CDA was associated with prolonged disease-free survival in studied cohorts. Collectively, our findings established that miR-484-modulated CDA has a dual impact in promoting chemoresistance and suppressing cell proliferation in breast cancer, illustrating the pathogenic tradeoffs associated with the evolution of chemoresistance in this malignant disease.
Introduction

Breast cancer is the most commonly diagnosed cancer among women worldwide (1). Current breast cancer therapy combines surgery and various adjuvant therapeutic options, including cytotoxic chemotherapy, hormonal therapy, molecular-targeted therapy or a combination of these approaches. However, acquired resistance to chemotherapy represents a major clinical obstacle in successful treatment of breast cancer. It is estimated that one out of two breast cancer patients fails to respond to initial treatment or rapidly acquires resistance to chemotherapeutic agents (2). Moreover, the majority of cancer patients will develop aggressive malignancies that exhibit up to 90% resistance towards one or more drugs, even if they show an initial response to treatment (3). Thus, the development of cancer therapeutics requires an in-depth understanding of drug resistance, drug metabolism and personalized drugs to optimize therapeutic decisions for each patient.

Gemcitabine (2'-2'-difluorodeoxycytidine, dFdC), which is a pyrimidine nucleoside analogue of deoxycytidine, is widely utilized in chemotherapy for non-small cell lung cancer (4), pancreatic cancer (5) and ovarian cancer (6). Since 2004, gemcitabine has been approved by the US Food and Drug Administration as a first-line treatment for patients with recurrent or metastatic breast cancer and for use after the failure of prior anthracycline-containing adjuvant chemotherapy (7). Gemcitabine enters the cell via a nucleoside transporter and undergoes stepwise phosphorylation to be converted into active drug metabolites. Inside the cell, gemcitabine is phosphorylated by various deoxycytidine kinases and incorporated into DNA strands to inhibit DNA replication and cell proliferation while promoting apoptosis in cancer cells (8). More than 90% of administered gemcitabine is inactivated by cytidine deaminase (CDA), which converts gemcitabine into 2'-deoxy-2',2'-difluorouridine (dFdU) (9, 10). Recent studies have suggested that genes in the gemcitabine metabolic pathway are associated with...
gemcitabine resistance (11, 12). MicroRNAs (miRNAs) are noncoding RNA molecules of approximately 21 to 25 nucleotides that modulate gene expression by directly interacting with an mRNA target, thus leading to either degradation of the mRNA transcript or inhibition of the translation process (13). Evidence indicates that miRNAs play pivotal roles in chemotherapy resistance (14). Several studies have revealed that miR-328 is associated with mitoxantrone resistance (15) and that increased miR-221 and miR-222 expression is associated with tamoxifen resistance in breast cancer cells through targeting p27kip (16). However, the gemcitabine resistance-relevant network in breast cancer and their underlying molecular mechanisms have not yet been elucidated.

The primary aim of this study was to explore the molecules involved in gemcitabine resistance and the corresponding underlying regulatory mechanisms in breast cancer. Here, we found that CDA functions as a major mediator of gemcitabine resistance and, more importantly, that CDA acts as a proliferation suppressor and is associated with good outcome in breast cancer. Moreover, an integrated mRNA-miRNA network suggests that CDA is a direct target of miR-484, which can reverse CDA-mediated chemoresistance and proliferation inhibition. Therefore, our results suggested that miR-484-modulated CDA is an important chemoresistance regulator with potent tumor-suppressing activity.
Materials and Methods

Cell lines and cell culture

The MCF7, MDA-MB-231 (MDA-231), MDA-MB-436 (MDA-436), MDA-MB-468 (MDA-468), ZR-75-30 and Hs-578T human breast cancer cell lines and the HEK293T cell line were obtained from the Shanghai Cell Bank Type Culture Collection Committee (CBTCCC, Shanghai, China) in 2012 and cultured in complete growth medium as recommended by the distributor. The identities of cell lines were confirmed by CBTCCC using DNA profiling (short tandem repeat, STR). These cell lines were conserved in our laboratory and subjected to routine cell line quality examinations (e.g. morphology, mycoplasma) by HD Biosciences (Shanghai, China) every 3 months. The cells for experiments were passaged for less than 6 months. The MDA-231 gemcitabine-resistant subline MDA-231-Gem was established according to a previous study (17) by stepwise selection with increasing concentrations of gemcitabine (Eli Lilly, Indianapolis, IN) with 12 cycles at a range of 12-720 nM in the culture medium (Supplementary Table S1). During each cycle, the cells were cultured with different gemcitabine concentrations for one week until new clones were present, followed by culture with normal medium for approximately six weeks to reach 85% confluence.

miRNA and mRNA microarrays

The brief details of the miRNA and mRNA microarrays are described in the Supplementary Methods. These microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database under the accession number GSE63140.

Quantitative PCR (qPCR) and Western blotting

Total RNA was isolated from cells and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). qPCR was performed to detect selected genes using a SYBR Premix Ex Taq system (TaKaRa, Dalian).
The primers are listed in **Supplementary Table S2**. Mature has-miR-345, has-miR-484 and has-miR-339-5p were detected with specific stem-loop primers and normalized to U6 small RNA using TaqMan miRNA assays (Applied Biosystems, CA). All samples were run in triplicate. Western blotting was performed using a general method, with the following antibodies: anti-β-actin (Sigma-Aldrich); anti-CDA, anti-phospho-Rb, anti-Rb, anti-E2F1, anti-cyclin A, anti-cyclin E and anti-CDK2 (Cell Signaling Technology, CA). The densitometry analysis was performed using Image J software (NIH, Bethesda, MD).

**Expression constructs and shRNA constructs**

The retroviral expression constructs for HA-FLAG-CDA were made using MSCV vectors carrying an HA-FLAG tag at the N terminus as previously described (18). The miR-339-5p, miR-345 and miR-484 precursor sequences were constructed in lentivirus-based pEZX constructs by GeneCopoeia, Inc. (Guangzhou, China). The sequences for shCDA-1, shCDA-2 and shCDA-3 were 5′-TGAGAGAGTTTGGCACCAA-3′, 5′-CAGTGACATGCAAGATGAT-3′ and 5′-GTGCTGAACGGACCGCTAT-3′, respectively. The lentivirus-based shRNA constructs were supplied by GeneChem Inc (Shanghai, China).

**Patients and specimens**

In this study, we retrospectively obtained 193 pathologically confirmed primary breast cancer samples and 36 non-cancerous mammary controls to examine the CDA protein level via immunohistochemical (IHC) analysis. In this cohort, breast cancer patients were regularly followed, and the clinical outcomes of 177 cases were obtained, with the last update occurring in September 2013. We also obtained 30 paired primary breast tumors and corresponding adjacent non-cancer tissues to examine the relevant mRNAs and microRNAs profiles by qPCR analysis. The detailed enrollment
criteria for these cohorts are described in the Supplementary Methods. In addition, a large public clinical database (Kaplan-Meier Plotter) of breast cancer was used to explore the association between CDA expression and clinical outcomes (19). This study was approved by the institutional review board (IRB) of Fudan University Shanghai Cancer Center (FDSCC), and all participants provided informed consent to participate in this research.

**Tissue microarray (TMA), IHC staining and IHC variable evaluation**

TMAs were constructed using above paraffin-embedded blocks of breast tumors and blocks of non-cancerous mammary controls. TMAs were composed of duplicate cores from different areas of the same tumor to compare staining patterns. IHC analysis was done essentially as described previously (20). A total of 193 cancerous and 36 non-cancerous mammary cases were included in IHC analyses. IHC variables were scored semi-quantitatively according to the approach of a prior study (21). The brief details of TMAs construction, IHC staining and IHC variable evaluation are described in the Supplementary Methods.

**Cytotoxicity, cell proliferation, and colony formation assays and cell cycle distribution analysis**

Cells in the logarithmic growth phase were used for these assays. The brief details of these assays are described in the Supplementary Methods.

**Luciferase reporter assay targeting CDA 3’-UTR**

The brief details of the luciferase reporter assay are described in the Supplementary Methods.

**Statistical analyses**

Statistical analyses were performed using SPSS Software version 17.0 (SPSS, Chicago, USA). Analysis of variance and Student’s t-test were used to determine the statistical significance between experimental groups. The correlations between the clinical-pathological parameters and CDA
expression levels were tested using the Chi-square test. Survival outcomes were estimated using the Kaplan-Meier method and compared between groups using log-rank test. Univariate Cox proportional hazard model was used to determine the associations of the clinical-pathological parameters with survival outcomes. Multivariate Cox proportional hazard model was applied using a stepwise forward (Wald) method to detect independent prognostic factors of disease-free survival. Two-tailed $P$ values <0.05 were considered significant, and the confidence intervals were quoted at the 95% level. The results were presented as the mean and standard error of the mean (SEM).
Results

Characteristics of established gemcitabine-resistant cells

To uncover the molecular evolution of gemcitabine resistance in breast cancer, we generated a gemcitabine-resistant cell subline of MDA-231 cells via 12 cycles of exposure to gradually increasing concentrations of gemcitabine, as described in the Materials and Methods. The gemcitabine-resistant subline was termed MDA-231-Gem (Fig. 1A). To evaluate the chemosensitivity of the MDA-231-Gem subline, dose-response curves were generated to examine cell viability in response to gemcitabine. As shown in Fig. 1B, the gemcitabine half-maximal inhibitory concentration (IC50) value for MDA-231-Gem cells was 14.48 nM, which was 9-fold higher than that for the parental line (MDA-231, IC50=1.63 nM). Additionally, the clonogenicity of MDA-231-Gem cells was significantly higher than that of MDA-231 cells in response to gemcitabine treatment (Fig. 1C).

To explore the genes involved in gemcitabine resistance, we performed microarrays to obtain the mRNA expression profiles of the parental and resistant cells. And we conducted Gene Ontology (GO) analyses for the intersecting genes, which were generated by combining the Gene Set Enrichment Analysis (GSEA) and mRNA microarray results described in the Materials and Methods. The GO terms revealed that the activated pathways were primarily related to nucleotide and drug metabolism processes (Fig. 1D). The most significant genes in relevant pathways, which are listed in Supplementary Table S3, were validated by qPCR (Supplementary Fig. S1). Collectively, these data showed that we successfully established gemcitabine-resistant MDA-231-Gem cells that exhibited higher viability in the presence of gemcitabine.

Elevated CDA expression was attributed to gemcitabine resistance in breast cancer cells
Encouraged by the GO analysis mentioned above and by the results of previous studies, which have suggested that gemcitabine metabolism is related to gemcitabine’s cytotoxic effect and/or chemoresistant processes, we investigated the pharmacogenomics of the gemcitabine metabolic pathway. On the basis of the mRNA microarrays, the transcriptional levels of key metabolic enzymes related to gemcitabine were included in a metabolic diagram (Fig. 2A) and confirmed by qPCR (Supplementary Fig. S2). We noticed that CDA, which inactivates gemcitabine through deamination, was upregulated in MDA-231-Gem cells compared with other metabolic enzymes.

Next, we examined CDA expression at both the mRNA and protein levels in a panel of breast cancer cell lines (Fig. 2B and 2C). The results demonstrated that CDA was expressed in MDA-231 and MDA-436 human breast cancer cells, whereas CDA was expressed at substantially lower levels in MCF7, ZR-75-30, MDA-468 and Hs-578T cells. Consistent with the trend from microarray results, the mRNA and protein expression levels of CDA in MDA-231-Gem cells were relatively higher than those in other breast cancer cell lines.

To examine how CDA was involved in gemcitabine resistance in breast cancers, we established stable HA-Flag-tagged CDA-overexpressing MDA-231 and MCF7 cells with normal and lower endogenous CDA expression levels, respectively (Fig. 2D and 2E). The cytotoxicity assay showed that the gemcitabine IC50 for MDA-231 cells with exogenous CDA (MDA-231/HF-CDA) was more than 11-fold higher than that for MDA-231 control cells (MDA-231/HF-con; Fig. 2F). Furthermore, similar results were found in MCF7 transfectants; the gemcitabine IC50 for MCF7 cells stably expressing HA-Flag-tagged CDA (MCF7/HF-CDA) was more than 2.3-fold higher than that for control cells (MCF7/HF-con; Fig. 2G).

To assess whether the knockdown of CDA expression could reverse chemoresistance to
gemcitabine, we silenced CDA in MDA-231-Gem cells via infection with a lentivirus containing CDA-targeting shRNAs (Fig. 2H). We found that the depletion of CDA expression reduced breast cancer cell chemoresistance to gemcitabine compared with the controls (Fig. 2I). These results showed that CDA expression tightly correlated with the chemosensitivity of breast cancer cells with regard to gemcitabine treatment.

CDA suppresses cell proliferation and causes cell cycle redistribution in breast cancer cells

Previously, CDA was shown to participate in the metabolism of cytidine/deoxycytidine, accounting for the conversion of cytidine/deoxycytidine into uridine/deoxyuridine in cells (Fig. 3A) (22). Thus, it is plausible that CDA could exert effects on DNA replication in the absence of genotoxic stress. Taking the physiological functions of CDA into consideration, we conducted cell proliferation assays and found that MDA-231-Gem cells with acquired high CDA expression exhibited a lower proliferation rate than parental MDA-231 cells (Fig. 3B). Furthermore, CDA overexpression decreased the cell proliferation rate of MDA-231 and MCF-7 cells (Fig. 3C and 3D), whereas CDA knockdown enhanced the proliferation of MDA-231-Gem cells (Fig. 3E). These data support the notion that CDA can inhibit breast cancer cell proliferation.

In general, DNA replication requires a number of factors that can regulate replication forks, including replication fork components, the nucleotide pool, histones and histone chaperones (23). As CDA serves as a key modulator in the conversion of cytidine/deoxycytidine to uridine/deoxyuridine, this enzyme might affect the balance of the nucleotide pools and slow down the replication fork speed. Therefore, we examined the cell cycle distribution in the indicated cell lines. We found that the percentage of cells in S phase substantially increased in the MDA-231-Gem cells compared to the
parental line (Fig. 3F). In addition, exogenous expression of CDA in MDA-231 and MCF-7 cells led to an increased proportion of cells in S phase (Fig. 3G and 3H). When a lentivirus containing a CDA-targeting shRNA was introduced into MDA-231-Gem cells, the previously observed S phase enrichment was reversed (Fig. 3I). Taken together, these data suggest that CDA plays a key role in modulating cell cycle redistribution and the replication process and, more importantly, that this effect is evident in the absence of cytotoxic stress.

Cyclin E-CDK2 complex and its associated molecules are involved in CDA-mediated cell cycle redistribution

The marked effects of CDA on cell proliferation and cell cycle redistribution led us to examine the mechanisms involved by characterizing its effects on the activity, modification and expression of important regulators of the G1/S phase transition. During G1/S progression, cyclin E associates with cyclin-dependent kinase 2 (CDK2) to form a complex that is required for entry into S phase (24-26). Given the central role of cyclin E-CDK2 in the control of G1/S phase progression, we performed Western blot analyses of the cyclin E-CDK2 complex in the indicated cell lines. We found that cyclin E and CDK2 were downregulated when CDA was highly expressed in MDA-231-Gem cells. Although the trend was weaker, the cyclin E-CDK2 complex expression also decreased in MDA-231/HF-CDA cells (Fig. 4A); whereas silenced CDA increased the expression of cyclin E-CDK2 complex (Fig. 4B). During the cell cycle transition from G1 into S phase, cyclin A associates with CDK2, replacing cyclin E. When the amount of the cyclin A-CDK2 complex reaches a threshold, it terminates the assembly of the cyclin E-CDK2 complex, thus facilitating DNA replication in S phase (27). Herein, we examined cyclin A protein in the indicated cells and observed a similar trend for the cyclin E-CDK2 complex,
which inversely correlated with CDA expression (Fig. 4A and 4B). Similar to all cyclin-CDK complex family members, the cyclin E-CDK2 complex regulates multiple cellular processes by phosphorylating numerous downstream proteins. The cyclin E-CDK2 complex phosphorylates the retinoblastoma (Rb) protein to promote the G1-S phase transition. Phosphorylated Rb protein no longer interacts with the E2F transcription factor, thus promoting the transcription of downstream genes that drive cells to overcome the G1/S checkpoint (28). As shown in Fig. 4, the trends for phospho-Rb status, Rb expression and E2F expression were similar to the expression of the cyclin E-CDK2 complex, suggesting that high CDA levels might reduce cyclin E-CDK2 complex expression and suppress the activities of its downstream pathway. These data indicate that the cyclin E-CDK2 complex and its associated signaling are involved in CDA-induced cell cycle redistribution.

miR-484 serves as a post-transcriptional regulator to downregulate CDA expression by directly targeting its 3′-UTR

Accumulating evidence has shown that miRNA deregulation is involved in multiple cellular and biological processes, including cell proliferation and chemoresistance (14-16, 29, 30). In an attempt to understand the underlying regulatory network of CDA-modulated gemcitabine chemoresistance, cell proliferation and cell cycle redistribution, we hypothesized that several miRNAs might function by directly targeting CDA, thus regulating CDA-induced cellular responses. We predicted potential miRNAs that might target the 3′-UTR sequence of CDA using four prediction algorithms: PicTar5, TargetScan, miRanda and miRWalk. Thirty-four candidate miRNAs were predicted to regulate CDA expression by all four algorithms (Fig. 5A). We compared the miRNA expression profile of MDA-231-Gem cells to that of parental cells using Agilent miRNA microarrays. By intersecting the
results of in silico predictions with discrepant miRNAs from miRNA microarrays, only three candidate miRNAs, miR-339-5p, miR-345-5p and miR-484, were shown to be expressed at substantially lower levels among the 34 predicted CDA-associated miRNAs (Fig. 5B). qPCR analysis confirmed that the expression levels of miR-339-5p, miR-345-5p and miR-484 were reduced in MDA-231-Gem cells compared with parental cells (Supplementary Fig. S3). We then constructed a gemcitabine-metabolic mRNA-miRNA network and a putative direct-target sub-network (Supplementary Fig. S4). As illustrated in this network, CDA was one of the remarkable genes in the gemcitabine metabolic pathway, and miR-339-5p, miR-345-5p and miR-484 displayed stronger correlations with CDA than other miRNAs. Analysis of the 3'-UTR sequence of CDA using TargetScan demonstrated possible binding sites for miR-339-5p, miR-345-5p and miR-484 (Fig. 5C). According to the above analyses, we hypothesized that these three miRNAs might play crucial roles in regulating CDA expression.

To verify whether CDA was regulated by miR-339-5p, miR-345-5p and miR-484 through direct binding to its 3'-UTR, a series of 3'-UTR fragments including the full-length 3'-UTR binding site (either wild-type or mutant) were inserted into the region immediately downstream of the luciferase reporter constructs (Fig. 5D). For luciferase activity assay, miR-339-5p, miR-345-5p or miR-484 was co-transfected with different luciferase-3'-UTR constructs into HEK-293T cells. The relative luciferase activity was remarkably reduced by miR-339-5p, miR-345-5p or miR-484, when the full-length wild-type 3'-UTR of CDA was presented (Fig. 5E, 5F and 5G). These reductions were sequence-specific because the relative luciferase activity did not drop as sharply in UTRs that contained mutant binding sites as in those that contained wild-type binding sites. In addition, Western blot analyses indicated that CDA protein levels decreased slightly in the presence of exogenous miR-339-5p and miR-345-5p; in contrast, the forced expression of miR-484 efficiently inhibited
endogenous CDA protein expression in MDA-231-Gem cells (Fig. 5H). The above observation prompted us to further explore the correlations between CDA expression and miR-339-5p, miR-345-5p and miR-484 in 30 primary breast cancer cases (Fig. 5I, 5J and 5K). Notably, among these three miRNAs, only miR-484 significantly negatively correlated with CDA expression ($P=0.0065$, Pearson’s $R=-0.486$). According to the above analyses, we concluded that miR-484 was the most efficient miRNA that could downregulate CDA expression by directly targeting its 3’-UTR.

miR-484/CDA axis modulates gemcitabine resistance, cell proliferation regulation and cell cycle redistribution in breast cancer cells

Because we showed that miR-484 could post-transcriptionally reduce CDA expression by directly targeting its 3’-UTR, we hypothesized that miR-484 could attenuate CDA-mediated functions. To verify this hypothesis, we constructed lentivirus-based vectors harboring miR-484 and control and established two stable cell lines through lentivirus transduction, which were denoted MDA-231-Gem/miR-484 and MDA-231-Gem/miR-con. As indicated in Fig. 6A, CDA expression levels were substantially reduced by miR-484 compared with the corresponding controls. Subsequently, the stable cell lines were subjected to cell cytotoxicity and proliferation assays and cell cycle analysis. We found that a reduction in CDA through miR-484 overexpression affected the cells in a manner similar to that induced by shRNA interference (Fig. 6B, 6C and 6D).

If CDA indeed acts as a direct target of miR-484, reintroduction of CDA into miR-484–expressing cells should antagonize the effects of miR-484. To test this hypothesis, we introduced a retrovirus-based construct expressing the CDA sequence without its 3’-UTR into MDA-231-Gem/miR-484 cells (Fig. 6E). As expected, we found that the reintroduction of CDA
enhanced gemcitabine resistance in miR-484–expressing cells (Fig. 6F). Furthermore, miR-484-induced cell proliferation was also antagonized by the forced expression of CDA (Fig. 6G), and forced CDA expression counteracted the S phase reduction induced by miR-484 (Fig. 6H). Thus, CDA reintroduction could abrogate multiple miR-484–induced phenotypes. Together, our results revealed that miR-484 could comprehensively attenuate the functions of CDA, suggesting that CDA is a downstream functional target for miR-484.

Expression of CDA is frequently downregulated, inversely correlated with miR-484 and linked to improved disease-free survival (DFS) in breast cancer patients receiving non-gemcitabine-based treatments

Considering the multiple roles of the miR484/CDA axis, we sought to evaluate the significance of this pathway in primary breast tumors in the absence of gemcitabine treatment. CDA expression was examined in 30 pairs of primary breast tumors and their corresponding adjacent non-cancerous mammary tissues using qPCR. As shown in Fig. 7A, the levels of CDA mRNA were frequently downregulated in breast tumors compared with paired non-cancerous mammary tissues (23 of 30). This downregulation was documented using paired t-test with a P value of 0.0034 (Fig. 7B). CDA is the functional target of miR-484; therefore, the expression level of mature miR-484 was also detected in paired mammary tissues by qPCR. In contrast to CDA, miR-484 levels were significantly upregulated in primary breast cancer tissues (P=0.0002, Fig. 7C). Next, we examined the protein expression level of CDA using IHC analyses on TMAs including 193 primary breast tumors and 36 non-cancerous mammary controls (Fig. 7D). We found that the CDA protein exhibited a strong signal in a large fraction of the non-cancerous mammary tissues (34/36, 94.44%), whereas the CDA protein was
expressed at reduced levels in nearly 40% (75/193, 38.86%) of the primary breast tumors (Fig. 7E, top).

The clinicopathological characteristics of the studied cohort are summarized in Supplementary Table S4. We noticed that CDA expression exhibited a tight association with estrogen receptor (ER) status in breast cancer ($P=0.017$; Fig. 7E, bottom); however, no correlation between CDA level and other clinicopathological features was found (Supplementary Table S4).

To assess the clinical significance of CDA in breast cancer, we analyzed the relationship between CDA and DFS in the above cohort. Among 193 breast cancer cases, the clinical outcomes of 177 cases were obtained. Kaplan-Meier analysis suggested that higher CDA expression correlated with prolonged DFS in this cohort ($P=0.023$; Fig. 7F). Consistent with our results, the analysis derived from a large public clinical database of breast cancer (Kaplan-Meier Plotter) provided additional support that high levels of CDA expression correlated with a better DFS in patients with breast cancer ($P=6.1\times10^{-8}$, Fig. 7G) (19). Moreover, both univariate and adjusted multivariate survival analyses suggested a significant difference between CDA-high and CDA-low groups in our cohort. The univariate analysis demonstrated that elevated CDA expression indicated a lower risk for disease relapse (HR=0.485, 95% CI 0.257-0.918; $P=0.026$; Supplementary Table S5), and the adjusted multivariate analysis using Cox regression model also showed that high CDA expression was an independent favorable prognostic factor of DFS in patients with breast cancer (HR=0.375, 95% CI 0.193-0.732; $P=0.004$; Supplementary Table S5). Taken together, our findings suggested that the level of CDA expression might serve as a valuable prognostic marker for breast cancer patients.
Discussion

Gemcitabine chemoresistance is a considerable clinical obstacle in chemotherapy for breast cancer patients. Here, we explored the potential regulatory mechanism responsible for gemcitabine resistance, by utilizing the combination of bioinformatic analysis and functional screening. We demonstrated that CDA overexpression reduced cell sensitivity to gemcitabine, and this finding is consistent with previous studies showing that CDA deaminates deoxycytidine analogue-type cytotoxic drugs, such as cytarabine, gemcitabine and 5-aza-2’-deoxycytidine, thus preventing the intracellular accumulation of their active metabolites (10, 31-33). Taken together, these results indicate that CDA is a regulator that contributes to gemcitabine resistance in breast cancer. These findings may stimulate research into the development of superior CDA inhibitors as potential agents to potentiate gemcitabine chemotherapy.

Unexpectedly, in this study, we found that CDA inhibited cell proliferation in breast cancer cells. Moreover, CDA downregulation was a frequent event in human mammary cancers, and CDA positivity correlated with prolonged DFS in breast cancer patients. These results suggest that CDA might function as a tumor suppressor in mammary tumorigenesis. Apparently, the biological characteristics of CDA in breast cancer development conflict with those of acquired gemcitabine resistance in cancer chemotherapy. Indeed, unlike other chemoresistance genes, which are primarily identified as oncogenes, we showed that CDA acts as a suppressor of cell proliferation and as a prognostic marker of favorable outcome in breast cancer. Thus, our findings indicate that CDA, which could differentially induce gemcitabine resistance and suppress cell proliferation, might play various biological roles during breast cancer development.

The eukaryotic cell cycle is regulated by the coordinated activity of protein kinase complexes, consisting of cyclin-dependent kinases and cyclins (34). For instance, progression through G1 and entry into S phase are regulated by the cyclin E-CDK2 and cyclin A-CDK2 complexes, respectively.
Downstream signaling molecules of the cyclin E-CDK2 pathway, such as E2F1 and Rb, are also involved in cell cycle regulation. The phosphorylation of Rb by cyclin-dependent kinases at the G1/S phase transition leads to the dissociation of Rb from E2F, allowing cells to resume cell cycle progression (36). We found that high CDA expression could suppress the cyclin E-CDK2 complex and its downstream signaling proteins, including the E2F1, phospho-Rb and Rb proteins. It appears that decreased cyclin E-CDK2 complex expression could partly explain why breast cancer cells undergo CDA-induced cell cycle arrest. However, whether the effects of CDA on these proteins are direct or indirect remains to be elucidated and some other mechanisms might be responsible for the CDA-mediated decrease in cell proliferation. Additional experiments are required for a more thorough understanding of these effects.

Notably, there is an emerging concept of cell cycle-mediated drug resistance, that cells may be relatively insensitive to a chemotherapeutic agent due to its current state in the cell cycle (37). Previous reports have demonstrated that fluorouracil, which is an antimetabolite agent similar to gemcitabine, became less effective in colon cancer cells when cell cycle progression was simultaneously arrested by other replication stress agents (38, 39). Considering that elevating CDA expression slows down the replication fork and causes the cell cycle redistribution, the CDA-retarded replication fork may reduce the efficacy of gemcitabine. However, at this point, this hypothesis is highly speculative. In our study, the gemcitabine IC50 value for MDA-231-Gem/sh-CDA-1 cells was 8.42×10^{-4} nM, which was more than 10,000-fold lower than that for the control cells (MDA-231-Gem/sh-con, IC50=13.2 nM). These data suggested that CDA might be a specific regulator of gemcitabine resistance. The results of previous studies have provided evidence that CDA deaminates gemcitabine to prevent the intracellular accumulation of its active metabolite as a chemoresistance-conferring gene (31-33). Thus,
CDA-induced chemoresistance is more likely due to its deamination effect.

One important finding in our study was the discovery of the relationship between CDA and breast cancer patient prognosis, in which CDA highly correlated with DFS and independently contributed to better survival in the absence of gemcitabine treatment. Conversely, Schroder JK et al. reported that CDA was associated with poor prognosis in acute myeloid leukemia patients (40). Regarding this controversial point, we speculated that the specific evaluation criteria for breast cancer in this study differed from those used for leukemia in previous papers. In this study, we observed that CDA expression was associated with prolonged DFS in patients treated with non-gemcitabine-based adjuvant chemotherapy. The criteria for included cases in our cohort must be interpreted with caution. At present, gemcitabine is recommended for the first-line treatment of patients with breast cancer after the failure of prior anthracycline-containing adjuvant chemotherapy, according to Chinese Anti-Cancer Association guidelines or National Comprehensive Cancer Network guidelines (41). Because gemcitabine has not been recommended for adjuvant chemotherapy of patients with primary breast cancer according to current guidelines, most patients with primary breast cancer did not receive gemcitabine during adjuvant chemotherapy in our cohorts. Meanwhile, the patients who received gemcitabine-based treatment in our hospital were confirmed to have recurrent or metastatic breast cancer for which operative treatment was not suitable. Due to these limitations, we could not obtain sufficient breast cancer samples to evaluate the CDA expression in patients receiving gemcitabine treatment. Therefore, we drew conclusions using our current cohorts of primary breast cancer patients receiving non-gemcitabine-based adjuvant chemotherapy. Of course, an additional cohort of patients receiving gemcitabine-based chemotherapy may be needed to form a general understanding of the role of CDA in breast cancer.
As indicated in previous studies, miR-484 exhibits multi-functional characteristics and participates in many physical and pathological processes. MiR-484 can modulate chemosensitivity to carboplatin- and taxol-based regimens by targeting the VEGF protein in neoplastic cells or by interfering with the receptor VEGFR2 in serous ovarian carcinomas (42). In addition, circulating miR-484 in sera may be an endogenous control miRNA and may be used to distinguish pancreatic cancer patients from healthy people (43). Here, we found that miR-484 could sensitize breast cancer cells to gemcitabine treatment and attenuate CDA-mediated growth inhibition by directly targeting the CDA 3′-UTR. Our results provide the first line of evidence that miR-484 is an effective CDA antagonist in breast cancer.

In conclusion, this study elucidates a new molecular mechanism for gemcitabine resistance and cell cycle regulation in breast cancer cells through the miR-484/CDA axis. Our results indicate that this miR-484-modulated CDA has a dual impact in regulating gemcitabine resistance as a chemoresistance indicator and in modulating cell cycle and cell proliferation, further serving as a favorable prognostic marker in breast cancer. Importantly, our findings regarding the miR-484/CDA axis provide a new avenue for understanding of the pathogenic tradeoffs associated with the evolution of chemoresistance in breast cancer.
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Authors’ Contributions

Conception and design: C. Song and X. Hu

Development of methodology: F. Ye, C. Song, X. Hu, S. Li, and D. Chen

Acquisition of data (provided animals, acquired and managed patients, and provided facilities): F. Ye, C. Song, S. Li, F. Qiao, L. Chen, H. Ling, C. Xia, and D. Chen

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, and computational analysis): F. Ye, C. Song, X. Hu, and Z. Cao

Writing, reviewing and/or revising the manuscript: F. Ye, C. Song, and X. Hu

Administrative, technical, or material support (i.e., reporting or organizing data and constructing databases): F. Ye, C. Song, and L. Yao

Study supervision: C. Song, X. Hu, and Z. Shao
References

2001;7:2168-81.
Figure Legends

Figure 1. Establishment of gemcitabine-resistant cells.

A, The gemcitabine-resistant subline MDA-231-Gem was established after 12 cycles of exposure to different doses of gemcitabine. B, The responses of MDA-231-Gem and parental MDA-231 cells to different doses of gemcitabine. The IC50 for each cell line is presented. The assays were performed in triplicate. C, MDA-231-Gem and MDA-231 cells were used for colony formation assays in the presence of 1 µM gemcitabine for 2 weeks (representative images, left; statistical analysis, right; **, P<0.01). D, GO analysis was performed on the basis of mRNA microarrays conducted in MDA-231-Gem and MDA-231 cells.

Figure 2. CDA expression related to gemcitabine metabolism was associated with gemcitabine chemoresistance in breast cancer cells.

A, A diagram showing the metabolic pathway of gemcitabine in cells. Genes involved in the pathway are illustrated with their mRNA fold changes from mRNA microarrays. B, The mRNA levels of CDA were determined by qPCR in a panel of breast cancer cell lines; GAPDH was used as an internal control. C, The protein levels of endogenous CDA were detected by Western blotting in a panel of breast cancer cell lines. D and E, After being infected with a CDA-expressing retrovirus or control retrovirus, the cells were subjected to Western blotting. F and G, Forced expression of CDA promoted gemcitabine resistance in MDA-231 and MCF-7 cells; the IC50 for each cell line is presented. H, After being infected with sh-CDA-expressing or negative lentiviruses, the cells were subjected to Western blotting. I, Depletion of CDA sensitized the cellular response to gemcitabine treatment; the IC50 for each cell line is presented.
Figure 3. Elevated CDA expression led to cell proliferation inhibition and S phase enrichment in breast cancer cells.

A, A diagram showing how CDA is involved in the cytidine/deoxyctydine metabolic pathway in cells.

B, C, D and E, Cell proliferation assays were conducted in the indicated cell lines. High expression of CDA suppresses cell proliferation, whereas the knockdown of CDA expression shows the opposite effect. F, G, H and I, Flow cytometry was performed to detect the cell cycle distribution of the indicated cell lines. CDA overexpression caused S-phase accumulation compared with control cells, whereas the knockdown of CDA expression reversed this phenomenon. The assays were performed in triplicate; representative results are shown. These data represent the mean values, with error bars indicating the SEM (*, P<0.05).

Figure 4. Cyclin E-CDK2 complex and its associated molecules were associated with CDA-induced cell cycle redistribution.

A, CDA suppressed the activation of the cyclin E-CDK2 complex and its downstream molecules. Western blotting was conducted to detect the cell cycle checkpoint-associated proteins using antibodies against cyclin E, CDK2, cyclin A, phospho-Rb, Rb and E2F1. The amounts of CDA, HA and β-actin were detected as controls. B, CDA depletion upregulated the expression of the above proteins. The cell extracts were analyzed by Western blotting using the indicated antibodies. All bands were quantified by densitometry analysis.

Figure 5. CDA was a direct target of miR-484 and inversely correlated with miR-484 expression in breast cancer.
The candidate miRNAs targeting CDA were predicted using TargetScan, PICTAR5, miRanda and miRWalk. The numbers listed in overlapping circles were simultaneously predicted by different algorithms. Scatter plot of miRNA expression fold changes derived from the miRNA microarray dataset. Each dot represents the expression fold change of an individual miRNA in MDA-231-Gem cells compared with MDA-231 cells. The threshold with –log(fold change)≤–1 was set for investigating miRNAs that were significantly decreased following gemcitabine-induced molecular evolution. The candidate miRNAs at the intersection of the in silico predictions and miRNA microarrays are labeled as triangles. Schematic model of the miR-339-5p, miR-345-5p, and miR-484 seed matches in the human CDA 3’-UTR. The human CDA 3’-UTR and corresponding mutant binding site fragments were introduced into the luciferase 3’-UTR reporter constructs. The miRNA seed sequences are illustrated in gray, and the mutant bases in the CDA 3’-UTR are underlined. The relative luciferase activities of luciferase reporters with wild-type or mutant CDA 3’-UTRs were determined in HEK293T cells, which were co-transfected with the miR-339-5p, miR-345-5p, and miR-484 mimics or control oligonucleotide. Renilla luciferase activity was normalized to firefly luciferase activity (*, P<0.05; ns, no significance). CDA protein levels were determined by Western blotting in MDA-231-Gem cells stably infected with lentivirus containing miR-339-5p, miR-345-5p, miR-484 or control. Pearson’s correlation of CDA and miR-339-5p, miR-345-5p and miR-484 at the RNA level in 30 breast cancer tissues.

Figure 6. miR-484 enhanced gemcitabine chemosensitivity, promoted cell proliferation and redistributed the cell cycle by targeting CDA in breast cancer cells.

After being infected with miR-484-expressing lentivirus or control lentivirus, the MDA-231-Gem
cells were subjected to Western blotting. B, The IC50 values for cells stably expressing miR-484 and control cells are shown. C, Cell viability assays were performed in the indicated cells. D, Flow cytometric analyses were performed in the indicated cells. E, Retrovirus containing an HA-Flag-tagged CDA sequence without its 3′-UTR was introduced into MDA-231-Gem/miR-484 cells. CDA protein levels were analyzed by Western blotting. F, G and H, The effects of miR-484 could be reversed by reintroduction of CDA. Cytotoxicity assays, cell viability assays and cell cycle analyses were conducted in the indicated cell lines. The data represent the mean values, with error bars indicating the SEM (*, P<0.05).

**Figure 7.** CDA is often decreased and inversely correlated with miR-484 expression in breast tumors and is associated with favorable clinical outcomes in breast cancer.

A, Relative expression levels of CDA mRNA were measured by qPCR in 30 paired human breast cancer tissues and corresponding adjacent non-cancerous tissues and normalized by GAPDH. B, CDA expression was analyzed using a paired t-test. C, Expression of mature miR-484 was detected by qPCR in 30 paired cancerous and non-cancerous tissues and normalized against an endogenous U6 RNA control. D, Representative images of CDA IHC staining in invasive ductal breast tumors and non-cancerous mammary tissues (original magnification, 100 or 200×). E, Statistical analysis of CDA expression according to the IHC score (top) and the correlation between CDA expression and ER status (bottom). F, Kaplan-Meier analysis of DFS in our cohort. G, Kaplan-Meier analysis of DFS using the Kaplan-Meier Plotter database of breast cancer.
Figure 2
Figure 4

A

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(endo-CDA)
Figure 5

A

PICTAR5

53

Targets

miRanda

107

34

miRWalk

miRanda

miRwalk

B

log fold changes

1982 hsa-miRs

C

CDA 3'UTR (365 bp)

miR-339-5p

miR-484

ORF

miR-345-5p

D

CDA 3'UTR (wild-type)

5' cc CCAGCCCTACAGGGA tc 3'

miR-339-5p

3' GGACCUCUCGUCCUCU 5'

CDA 3'UTR (339-5p mut)

5' cc CCAGCCCTACACCUCU tc 3'

E

Fold change

Control

miR-339-5p

+ - + -

3'UTR wild-type

miR-345-5p

+ - + -

3'UTR 339-5p mutant

F

Fold change

Control

miR-339-5p

+ - + -

3'UTR wild-type

miR-345-5p

+ - + -

3'UTR 345-5p mutant

G

CDA 3'UTR (wild-type)

5' at TACACTCCAGCCTGA gt 3'

miR-484

3' CCCCCUGACUCCGACU 5'

CDA 3'UTR (484 mut)

5' at TACACTCCAGGAGA gt 3'

CDA 3'UTR (wild-type)

5' ac TCCAGCCTGAGTCAG ca 3'

miR-345-5p

3' ACCUGAUCCAGUC 5'

CDA 3'UTR (345-5p mut)

5' ac TCCAGCCTGACAGUG ca 3'

H

MDA-231-Gem

Control

miR-339-5p

miR-345-5p

miR-484

anti-CDA

1.00 0.36 0.33 0.14

anti-β-actin

1.00 1.04 1.11 1.21

I

ΔCt Value CDA/GAPDH

ΔCt Value miR-339-5p/U6

r = -0.304

p = 0.1029

n = 30

J

ΔCt Value CDA/GAPDH

ΔCt Value miR-345-5p/U6

r = -0.231

p = 0.2201

n = 30

K

ΔCt Value CDA/GAPDH

ΔCt Value miR-484/U6

r = -0.486

p = 0.0065

n = 30

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Figure 6
Figure 7

A. CDA mRNA expression in 30 paired mammary tissues

B. CDA/GAPDH

C. miR-484/U6

D. Breast cancer tissues and Non-cancerous tissues

E. The expression levels of CDA in human mammary tissues

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Cytidine deaminase axis modulated by miR-484 differentially regulates cell proliferation and chemoresistance in breast cancer

Fu-Gui Ye, Chuangui Song, Zhi-Gang Cao, et al.

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