ID1 Enhances Docetaxel Cytotoxicity in Prostate Cancer Cells through Inhibition of p21

Hao Geng1, Brooks L. Rademacher1, Janet Pittsenbarger1, Chung-Ying Huang3, Christopher T. Harvey1, Marie C. Lafortune1, Anne Myrthue1, Mark Garzotto2, Peter S. Nelson3,4, Tomasz M. Beer1, and David Z. Qian1

Abstract

To identify potential mechanisms underlying prostate cancer chemotherapy response and resistance, we compared the gene expression profiles in high-risk human prostate cancer specimens before and after neoadjuvant chemotherapy and radical prostatectomy. Among the molecular signatures associated with chemotherapy, transcripts encoding inhibitor of DNA binding 1 (ID1) were significantly upregulated. The patient biochemical relapse status was monitored in a long-term follow-up. Patients with ID1 upregulation were found to be associated with longer relapse-free survival than patients without ID1 increase. This in vivo clinical association was mechanistically investigated. The chemotherapy-induced ID1 upregulation was recapitulated in the prostate cancer cell line LNCaP. Docetaxel dose-dependently induced ID1 transcription, which was mediated by ID1 promoter E-box chromatin modification and c-Myc binding. Stable ID1 overexpression in LNCaP increased cell proliferation, promoted G1 cell cycle progression, and enhanced docetaxel-induced cytotoxicity. These changes were accompanied by a decrease in cellular mitochondria content, an increase in BCL2 phosphorylation at serine 70, caspase-3 activation, and poly(ADP-ribose) polymerase cleavage. In contrast, ID1 siRNA in the LNCaP and C42B cell lines reduced cell proliferation and decreased docetaxel-induced cytotoxicity by inhibiting cell death. ID1-mediated chemosensitivity enhancement was in part due to ID1 suppression of p21. Overexpression of p21 in LNCaP-ID1–overexpressing cells restored the p21 level and reversed ID1-enhanced chemosensitivity. These molecular data provide a mechanistic rationale for the observed in vivo clinical association between ID1 upregulation and relapse-free survival. Taken together, it shows that ID1 expression has a novel therapeutic role in prostate cancer chemotherapy and prognosis. Cancer Res; 70(8); 3239–48. ©2010 AACR.

Introduction

In the treatment of advanced, castration-resistant prostate cancer, docetaxel-based chemotherapy is empirically deployed as no markers are available to select patients who do and do not benefit from treatment (1–3). When used preoperatively in the treatment of high-risk and localized disease, docetaxel-based chemotherapy can reduce serum prostate-specific antigen (PSA), but has not resulted in complete pathologic responses; the long-term benefit is a subject of ongoing randomized clinical trials (4–6). These results emphasize the importance of understanding the molecular mechanisms underlying the prostate cancer chemotherapy response and resistance.

Inhibitor of DNA binding 1 (ID1) is a negative regulator of basic helix-loop-helix transcription factors (7). It has been implicated in regulating a variety of cellular processes, including growth, senescence, differentiation, apoptosis, angio genesis, and neoplastic transformation (8). In various mammalian cell culture models, cellular differentiation is associated with the downregulation of ID1, whereas ID1 overexpression inhibits the ability to differentiate (9). The inhibition of differentiation can also be accompanied by cell proliferation (10). Several lines of evidence have suggested that ID1 transcriptionally inhibits the expression of cyclin-dependent kinase inhibitors p16, p21, and p27 (11–13). Cells that overexpress ID1 progress through the G1-S cell cycle transition much faster than those that do not (7, 14). ID1 has been shown to promote apoptosis in a variety of settings (7). Transgenic mice engineered to overexpress ID1 in T cells show massive apoptosis in thymocytes (15). ID1 overexpression has also been reported to induce apoptosis in dense mammary epithelial cells and cardiac myocytes through a redox mechanism (16, 17).
The role of ID1 in cancer development has also been intensely investigated. ID1 knockout mice exhibit an impaired angiogenic response to tumor xenografts due to reduced endothelial cell differentiation, mobilization, and recruitment (18), but are also more susceptible to tumor formation in a chemically induced skin carcinogenesis model (19). In human breast cancer, overexpression of ID1 is associated with lung metastasis (20, 21). In prostate cancer cell lines, ID1 is linked with increased androgen independence (22), oncogenic signaling (23, 24), and resistance to cytotoxic therapy (25–28). Although ID1 protein overexpression was initially reported in many tumor types, more recent recognition of the nonspecific nature of the antibodies used have brought into question the validity of these observations, including those made in clinical samples of prostate cancer (29). Recently, we observed that ID1 mRNA transcripts were significantly downregulated in patient-matched human prostate cancer epithelium compared with its adjacent benign epithelium derived from laser-capture microdissection (LCM) of frozen needle biopsy samples (30). Overall, the role of ID1 has been well defined in the endothelial compartment and tumor angiogenesis, but not in epithelial cancer cells. The impact of cancer therapies on ID1 expression is not well understood.

Materials and Methods

Cell lines. The human prostate cell line LNCaP and human embryonic kidney cell line 293T/17 (HEK293T/17) were purchased from the American Type Culture Collection. Prostate cancer C42B cell line was a gift from Dr. John Isaacs (Johns Hopkins School of Medicine). Cells were cultured as previously described (31).

Plasmid construction and transfection. Lentiviral-ID1–expressing plasmid was kindly provided by Dr. Rhoda Alani (Johns Hopkins University School of Medicine). Lentivirus vector iDuet 101 and packing plasmids pCMV-Δ8.92 and pMD.G were kind gifts of Dr. Linzhao Cheng (Johns Hopkins University School of Medicine). Full-length p21 was amplified by PCR from cDNA of LNCaP cell line and inserted to a lentiviral vector iDuet 101.

ID1 siRNA. An oligo-based ID1 siRNA cocktail (SMART-pool siRNA) and transfection reagent Dharmafect 3 were purchased from Thermo Fisher Scientific Dharmacon. The transfection reaction was based on the manufacturer’s protocol as previously described (32).

Chromatin immunoprecipitation. LNCaP cells were treated with either solvent or 10 nmol/L docetaxel for 24 hours. The chromatin immunoprecipitation (ChIP) assay was done as previously described (33) using antibodies specific for c-Myc (Santa Cruz Biotechnology), acetyl-histone 3 lysine 9 (Ace-H3K9; Millipore Inc), or isotype rabbit IgG control. DNA recovered from ChIP or input controls were subject to real-time quantitative PCR using primers flanking the E-box in ID1 promoter region (forward 5′-TGGAGATTGG-GACTGTTGGGCA-3′ and reverse 5′-CAGTTCTCGTCCAGGACTGTTCTC-3′). Promoter occupancy was calculated based on the ratio of ChIP to input control.

Cell proliferation and viability. As previously described (34), cancer cells were seeded at a density of 5 × 10^4 per well in 24- or 12-well culture plates. Twenty-four hours after seeding, the attached cells were treated. Viable cells at specific time points were counted by a hemocytometer based on the trypan blue exclusion principle.

Flowcytometry analysis was carried out in BD FACSCalibur analyzer as previously described (31). Results were analyzed by software for sub-G1 (dead cells), G1, S, and G2 phases.

Real-time PCR. Real-time PCR was performed on ABI PRISM 7500 Fast instrument using the SYBR Green PCR Universal Master Mix (Applied Biosystems). β-Actin was used as an internal control. Primer sequences are available on request. The ΔΔCt method was used to represent mRNA fold change.

Statistical analysis. Differences between the means of unpaired samples were evaluated by the Student’s t test and ANOVA using the SigmaPlot and SigmaStats program. P < 0.05 was considered statistically significant. All statistical tests were two-sided. Progression-free survival was calculated using the Kaplan-Meier method and χ2 test.

Results

In prostate cancer cells microdissected from patients, ID1 mRNA was upregulated by chemotherapy and associated with longer relapse-free survival. To identify the molecular signatures associated with chemotherapy, we compared the gene expression patterns of patients with high-risk localized prostate cancer before and after chemotherapy with docetaxel and mitoxantrone (35). To avoid possible bias due to different stages of cellular differentiation, we used LCM to specifically isolate tumor epithelium with the same Gleason grade from both pretreatment and posttreatment tissues (35). We performed cDNA microarray analyses using a head-to-head comparison of specimens from the same patients before and after treatment. We then subtracted gene expression alterations that could be attributed to surgical procedures and different methods of tissue acquisition (30, 35, 36). We found that the gene encoding ID1 was significantly upregulated in the postchemotherapy specimens (S1). Subsequently, we confirmed the upregulation by using quantitative reverse transcriptase-PCR (qRT-PCR) in 35 patient samples (Fig. 1A). In pretreatment- and posttreatment-matched LCM cancer cells with the same Gleason score, we found that ID1 was significantly upregulated by at least 1-fold in 15 of 35 patients (S2). After chemotherapy and radical prostatectomy surgery, all patients were followed by quarterly blood PSA test. Patients with confirmed PSA values higher than 0.4 were considered to have a PSA biochemical relapse (BCR). After a median of 56 months of post-chemotherapy–radical prostatectomy follow-up, we found that 60% (12 of 20) of ID1 stable and 40% (6 of 15) of ID1 upregulation patients developed BCR. The median relapse-free survival was 36.7 months for ID1 stable group, but 43.8 months for ID1 upregulation group, which were significantly different (Fig. 1B). The median time to develop BCR was 10.9 months for the ID1 stable group, but 43.8 months for ID1 upregulation group, which were significantly different (Fig. 1B). The relapse-free survival analysis
stratified by the change of ID1 suggested that chemotherapy-induced ID1 upregulation was associated with the delay of BCR (Fig. 1C). Importantly, Fisher’s exact test analysis showed that the frequencies of relapse were significantly lower in the ID1 upregulation group compared with the ID1 stable group 3 years after the chemotherapy–radical prostatectomy treatment. Specifically, the relapse frequencies for ID1 upregulation versus stable groups were 0% versus 35% after 12 months (P = 0.012), 6.7% versus 50% after 24 months (P = 0.009), and 13.7% versus 50% after 36 months (P = 0.034). These data suggested that ID1 upregulation possibly plays a role in mediating the chemotherapy efficacy or disease behavior and delaying the onset of BCR.

**Docetaxel upregulates ID1 expression through c-Myc in prostate cancer cells.** We confirmed that ID1 can be upregulated by chemotherapy. We treated the LNCaP prostate cancer cell line with increasing doses of either docetaxel or mitoxantrone, and measured the mRNA and protein levels by qRT-PCR and Western blots, respectively. Docetaxel, but not mitoxantrone (data not shown), was able to induce ID1 mRNA and protein expression at concentrations of 10 nmol/L and higher (Fig. 2A and B). In breast cancer cells, ID1 expression has been associated with c-Myc regulation (37). In B-cell lymphoma, global ChIP analysis revealed an E-box c-Myc binding site in the ID1 promoter (38). To investigate the molecular pathways involved in docetaxel-induced ID1 expression, we measured both c-Myc occupancy and local chromatin structure modifications surrounding the ID1 promoter E-box by quantitative ChIP in LNCaP cells. We found that c-Myc protein levels were not significantly altered following docetaxel (10 nmol/L) treatment (S3) and ID1 overexpression (S4). However, c-Myc occupancy in the ID1 promoter E-box was significantly higher in docetaxel-treated cells than in solvent controls (Fig. 2C). This increase was accompanied by the increase of histone acetylation that is commonly associated with transcriptionally active chromatin (Fig. 2C). Further, transient siRNA inhibiting c-Myc reduced both the basal and docetaxel-induced ID1 mRNA levels in LNCaP (Fig. 2D).
Stable ID1 expression enhances docetaxel cytotoxicity in LNCaP cells. We used the LNCaP cell line with low basal ID1 levels to investigate whether ID1 overexpression mediates prostate cancer cell response to docetaxel chemotherapy. We transduced LNCaP cells with pseudo-lentivirus carrying an ID1 expression cassette. The transduced cell line (LNCaP-ID1) had stable and robust ID1 expression compared with the empty-vector control cell line (LNCaP-Ev; Fig. 3A, left). Using cell proliferation assay, we observed that LNCaP-ID1 cells grew much faster than LNCaP-Ev controls (Fig. 3A, right). This increase in proliferation was accompanied by a decrease in G1 phase and an increase in S- and G2-M–phase cell populations measured by flow cytometry [fluorescence-activated cell sorting (FACS); Fig. 3B, left], whereas no significant change in viability or cell death was observed (data not shown).

Next, we tested the sensitivity of LNCaP-Ev and LNCaP-ID1 cell lines to chemotherapy. We treated cells with 0, 1, 5, and 15 nmol/L of docetaxel for 48 hours. By counting viable cells based on trypan blue exclusion, we observed that LNCaP-ID1 cells were significantly less viable than empty vector cells at both 5 and 15 nmol/L compared with their respective solvent-treated controls (Fig. 3B, right). FACS analysis confirmed the enhanced sensitivity. Docetaxel decreased cell cycle progression and increased cell death in both cell lines. Compared with LNCaP-Ev cells, ID1-expressing cells had a significantly larger decrease in the G1 phase (S5) and a larger increase in the sub-G1 (dead cell) population after docetaxel treatment (Fig. 3C, left). The increased cytotoxic effect was also observed by using Western blot analysis of proteins involved in the apoptotic pathway associated with docetaxel.

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BCL2 phosphorylation at serine 70, which inactivates BCL2 and mediates taxane-based apoptosis (39), was more robust in ID1-expressing cells than in -Ev controls after docetaxel treatment. The activation (cleavage) of caspase-3 and poly(ADP-ribose) polymerase (PARP), processes associated with
apoptosis, were more active in docetaxel-treated LNCaP-ID1 than LNCaP-Ev (Fig. 3C, right). In addition, ID1 overexpression in the LNCaP-ID1 cell line caused reductions in mitochondrial DNA and mitochondrial mass (S6). Therefore, we speculate that ID1 overexpression can cause mitochondrial damage, which in turn sensitzes cells to docetaxel-induced cytotoxicity.

**ID1 siRNA reduces docetaxel cytotoxicity in prostate cancer cells.** We next evaluated ID1 loss-of-function to further elucidate the role of ID1 in mediating chemotherapy. In the LNCaP cell line, siRNA against ID1 (si-ID1) reduced docetaxel-induced ID1 expression (Fig. 4A, top). Cells treated with ID1 siRNA grew significantly slower than the nontargeting siRNA controls (si-Control; Fig. 4A, bottom). Forty-eight hours after transfection of the siRNA oligos, cells were treated with increasing concentrations of docetaxel for 2 days. Cells transfected with si-ID1 were less sensitive to docetaxel than cells transfected with si-Control (Fig. 4B), which was associated with reduced cell death (Fig. 4C) and increased numbers of cell arrested in G1 (*, Fig. 4D). We also tested the effect of ID1 siRNA in the C42B prostate cancer cell line, which expresses a robust amount of endogenous ID1 protein (Fig. 5A, top). Unlike in LNCaP, docetaxel did not further induce ID1 expression in C42B (Fig. 5A, bottom). Transient siRNA consistently reduced ID1 protein levels in C42B cells over a period of 2 to 5 days (S7). Consistent with the proliferative effect of ID1 seen in LNCaP, ID1 siRNA slightly but significantly reduced the proliferation of C42B cells (Fig. 5B), whereas cell death remained trivial (data not shown). ID1 siRNA significantly reduced docetaxel efficacy (Fig. 5C). Time course experiments indicated that reduced chemosensitivity was accompanied by a significant reduction of docetaxel-induced cell death in si-ID1 cells compared with the si-Controls (Fig. 5D).

**Figure 3.** ID1 overexpression induces proliferation and enhances chemosensitivity. A, left, a representative Western blot of LNCaP cells transduced by pseudo-lentivirus containing empty vector (Lenti-Ev) or ID1-overexpressing vector (Lenti-ID1). Right, equal numbers of lenti-Ev and lenti-ID1 cells were seeded in 12-well plates for 24 h. Viable cells were counted by a hemocytometer based on trypan blue exclusion at days 1, 3, and 5 after seeding. Proliferation was calculated by dividing the number of viable cells at days 1, 3, and 5 by those at day 1. *, $P<0.01$. ANOVA. B, left, cell cycle distribution of lenti-Ev and lenti-ID1 cells at day 3 of the culture through flow cytometry analysis. *, $P<0.05$. t test. Right, equal numbers of lenti-Ev and lenti-ID1 cells were treated with docetaxel at the indicated doses for 48 h. Viable cells were counted and adjusted to those of 0 nmol/L solvent control. *, $P<0.01$. C, left, cells were treated with solvent or 10 nmol/L docetaxel for 48 h. Flow cytometry was performed. The percentage of sub-G1 cells in each cell line was plotted as cell death. *, $P<0.01$. t test. Right, representative Western blots of cells treated by solvent or 10 nmol/L docetaxel for 48 h. All results are mean and SD of four independent experiments.
**ID1 sensitizes LNCaP cells to docetaxel through p21 inhibition.** To elucidate the potential mechanisms that are responsible for ID1-enhanced docetaxel sensitivity in LNCaP cells, we compared gene and/or protein expression of genes involved in androgen signaling, cytotoxic responses, and cell cycle/cell death regulation in the LNCaP-Ev and LNCaP-ID1 cell lines. There was no significant change in androgen receptor, p16, p27, Bad, Bax, BCL2, and BCL-xl gene expression (S8). On the other hand, AKT and c-jun-NH2-kinase (JNK) signaling in LNCaP-ID1 cells were higher than LNCaP-Ev in solvent-treated conditions (S9). Consistent with a previous report (22), PSA expression was significantly upregulated (S8). However, under both androgen-stimulated (1 nmol/L R1881) and depleted (charcoal-stripped serum) conditions, ID1 overexpression equally enhanced docetaxel sensitivity (S10). Thus, the investigated ID1 pathway seems to be independent of the androgen/androgen receptor pathway. Consistent with the function in repressing cyclin-dependent kinase inhibitors, ID1 overexpression repressed p21 expression in LNCaP-ID1 cells (S9). Because p21 has been shown to attenuate chemotherapy cytotoxicity by inducing cell cycle arrest but not cell death (40–42), we hypothesized that the ID1-initiated reduction of p21 was one of the causes for the enhanced docetaxel sensitivity in LNCaP-ID1 cell line. Indeed, LNCaP-ID1 cells compensated with stable lentiviral p21 overexpression had higher p21 protein levels (Fig. 6A, left), grew significantly slower (Fig. 6B, left), and were significantly less sensitive to docetaxel compared with LNCaP-ID1 cells transduced with a vector control (Fig. 6B, right). Because the rate of cell cycle can also play a role in chemosensitivity (43) and the LNCaP-ID1 cells grew much faster than LNCaP-Ev (Fig. 3A, left), we wanted to determine if the loss of docetaxel sensitivity can be recapitulated by merely inhibiting cell cycle and proliferation without compensating for p21. We cultured LNCaP-ID1 cells under a fetal bovine serum (FBS)–depleted condition for 48 hours. Western blot analyses revealed that p21 levels were not significantly changed (Fig. 6A, right). Cell proliferation experiments showed significant growth retardation due to FBS depletion (Fig. 6C, left). However, docetaxel induced similar levels of cytotoxicity in both fast-proliferating and slow-proliferating LNCaP-ID1 cells (Fig. 6C, right). These data indicate that elevated cell proliferation rates do not account for enhanced chemosensitivity to docetaxel. On the other hand, the ID1-initiated p21 inhibition contributes to both enhanced cell proliferation and docetaxel sensitivity.

![Figure 4](cancerres.aacrjournals.org)
Discussion

In the current study, we identified ID1 as a molecular enhancer of docetaxel cytotoxicity. Prostate cancer cells with stable overexpression of ID1 exhibited higher sensitivity to docetaxel than empty vector controls. This enhanced sensitivity was accompanied by increases in BCL2 phosphorylation, caspase-3 activation, PARP cleavage, and ultimately cell death. Being an important transcriptional regulator of cell differentiation, ID1 was expected to affect multiple pathways, which may in turn influence chemosensitivity. One of these pathways was investigated here. Consistent with previous reports, stable ID1 overexpression significantly decreased the expression of the cyclin-dependent kinase inhibitor p21 in the LNCaP cell line. This led to increased cell proliferation and cell cycle progression, but also enhanced cytotoxicity to docetaxel. We used serum starvation to slow down the LNCaP-ID1 cell cycle progression and proliferation, and found that the slow-proliferating cells were equally sensitive to docetaxel as the fast-proliferating ones. However, stable p21 overexpression reversed the enhanced cytotoxicity. This suggests that ID1 modulation of chemosensitivity is proliferation independent but p21 dependent.

The casual relationship of the ID1-p21 pathway and chemosensitivity enhancement is consistent with the observations made more than a decade ago, in which the p53- and/or p21-negative cancer cell lines were prone to apoptosis in response to cytotoxic chemotherapy (40–42). Numerous studies have shown that p21 is capable of blocking caspase activation induced by both intrinsic and extrinsic cell death signals. The cell cycle regulatory property of p21 was recently separated from its antiapoptotic function, in which investigators observed that a small deletion of the p21 protein disrupted its ability to inactivate the ASK1/JNK pathway but not the ability to initiate cell cycle arrest (44). The JNK pathway has been shown to mediate the cytotoxicity of docetaxel. We speculate that one way for ID1 to chemosensitize cells is to downregulate p21, which may lead to longer and/or more robust activation of JNK signaling as seen in S8 (45, 46). In addition, we showed that docetaxel-induced ID1 expression was associated with the increased...
c-Myc binding at the ID1 promoter E-box. Although c-Myc is usually associated with cancer cell proliferation, Myc can also induce synthetic lethality within specific p53-positive cellular contexts (47, 48). The LNCaP cell line has wild-type p53, and our current observation is consistent with a pathway in which docetaxel induces ID1 expression through c-Myc. ID1 can subsequently inhibit p21, which promotes cell cycle progression, but also commits cells to death pathways in response to treatment. Additional mechanisms leading to ID1 chemo sensitization may also exist due to the pleiotropic effect of ID1. In our study, most of the patients with ID1 upregulation also had ID3 upregulation after chemotherapy (S1).

The results of our current study both confirm and argue against some of the existing knowledge regarding the status and function of ID1 in prostate cancer. Previously, ID1 protein overexpression has been observed in human prostate cancer specimens (49, 50). However, using matched benign and cancerous prostate epithelium obtained through LCM, we observed significantly higher ID1 mRNA expression in benign cells (30), and tumor ID1 mRNA levels were higher in patients who were BCR-free (S11). ONCOMINE analysis also revealed that ID1 expression decreased as the disease progressed (S12). Using a more specific anti-ID1 antibody, we did not detect ID1 protein expression in localized prostate cancer tissues (data not shown), which confirmed a recently published histologic study using the same antibodies (29). These data suggest that ID1 overexpression is not associated with the development of localized prostate cancer. Previous in vitro experiments have shown that ID1 overexpression enhances cell proliferation, activates several oncogenic signaling cascades, and promotes resistance to paclitaxel. In our study, we confirmed some of the proliferative and oncogenic properties of ID1. Further, using more detailed cellular and biochemical analysis, we showed that expression of ID1 increased prostate cancer cell line responses to docetaxel in

Figure 6. ID1 mediates docetaxel sensitivity by downregulating p21. A, left, a representative Western blot showing the p21 levels in LNCaP-ID1 cells stably transduced with lentivirus containing either vector or p21. Right, a representative Western blot showing the p21 level in LNCaP-Ev and LNCaP-ID1 cultured in medium containing 10% or 0% FBS. B, left, cell proliferation was measured in LNCaP-ID1 cells stably transfected with vector or p21 overexpression over a 48-h period. Right, LNCaP-ID1 cells containing stable p21 overexpression (ID1+p21) or vector (ID1+vector) were treated with docetaxel at the indicated doses for 48 h. The viability was calculated based on viable cell numbers at each dose adjusted to the solvent (0 nmol/L) controls. *, P < 0.01, t test. C, left, proliferation was measured in LNCaP-ID1 cells cultured in medium containing either 10% or 0% FBS over a 48-h period. *, P < 0.01, t test. Right, LNCaP-ID1 cells cultured in either 10% or 0% FBS were treated with the docetaxel at the indicated doses for 48 h. Viability was calculated based on viable cell numbers at each dose adjusted to the solvent (0 nmol/L) controls. All results are mean and SD of three independent experiments.
contrast to the published reports. Part of the discrepancies between our data and prior studies may be due to differences in cell lines. Based on our observation that clinical prostate cancers had low ID1 expression levels, we chose LNCaP as the primary \textit{in vitro} model because it has the lowest ID1 mRNA and protein levels among all prostate cancer cell lines tested. In other studies, investigators primarily used DU-145 and PC-3, which have measurable levels of ID1 protein and a loss of wild-type p53 function. These \textit{in vitro} and \textit{in vivo} results suggest a potentially important clinical role for ID1 in cancer therapy. Currently, docetaxel-based chemotherapy is widely used in treating cancer; however, the therapeutic efficacy has been hard to predict. Based on the current results, ID1 mRNA expression may prove useful as a molecular predictor for docetaxel efficacy in cancer patients after chemotherapy or as a pretreatment marker to select cancer patients who may be more responsive to specific cytotoxic agents. Further study using larger independent patient sets would be necessary to confirm this hypothesis.


tables and figures

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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