The Mechanism of DAB2IP in Chemoresistance of Prostate Cancer Cells

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Abstract

Purpose: The docetaxel-based chemotherapy is the standard of care for castration-resistant prostate cancer (CRPC), inevitably, patients develop resistance and decease. Until now, the mechanism and predictive marker for chemoresistance are poorly understood.

Experimental Design: Immortalized normal prostate and cancer cell lines stably manipulated with different DAB2IP expression levels were used and treated with chemotherapeutic drugs commonly used in prostate cancer therapy. Cell proliferation was measured using MTT assay; Western blot, quantitative PCR, and luciferase reporter assays were used to analyze Clusterin gene regulation by DAB2IP. Immunohistochemical analysis was conducted for evaluating DAB2IP, Clusterin and Egr-1 expression in human prostate cancer tissue.

Results: DAB2IP Knockdown (KD) cells exhibited resistance to several chemotherapeutic drugs, whereas increased DAB2IP in C4-2 cells restored the drug sensitivity. Parallel, DAB2IP KD cells exhibited higher expression of Clusterin, an antiapoptotic factor, whereas elevated DAB2IP in C4-2 cells decreased Clusterin expression. Functionally, knocking down Clusterin by short-hairpin RNA or antisense oligonucleotide OGX-011 decreased drug resistance, whereas overexpressing Clusterin in C4-2 D2 enhanced drug resistance. Mechanistically, DAB2IP blocked the cross-talk between Wnt/β-catenin and IGF-I signaling, leading to the suppression of Egr-1 that is responsible for Clusterin expression. A similar result was observed in the prostate of DAB2IP knockout animals. In addition, we observed a significantly inverse correlation between DAB2IP and Egr-1 or Clusterin expression from clinical tissue microarray.

Conclusions: This study unveils a new regulation of the Egr-1/Clusterin signaling network by DAB2IP. Loss of DAB2IP expression in CRPC cells signifies their chemoresistance. Clusterin is a key target for developing more effective CRPC therapy. Clin Cancer Res; 19(17): 4740–9. ©2013 AACR.

Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related death among men in the United States (1). Although early prostate cancer is generally treatable, most cases eventually progress to an advanced stage, castration-resistant prostate cancer (CRPC; ref. 2). Docetaxel-based chemotherapy is the standard-of-care regimen for CRPC; however, this regimen only prolongs survival, and many patients develop resistance and eventually succumb to their disease (3, 4). The molecular mechanisms underlying acquisition of chemoresistance by advanced prostate cancer are not well defined.

DOC-2/DAB2 interactive protein or ASK1 interacting protein (DAB2IP/AIP1) was previously identified as a member of the RAS–GTPase activating protein family (5, 6), and functions as a tumor suppressor in cancer development (7–11). A recent study using genome-wide association analyses revealed a single-nucleotide polymorphism of the DAB2IP gene associated with risk of aggressive prostate cancer (12). Indeed, downregulation of DAB2IP in prostate cancer is mainly due to epigenetic regulation, which inversely correlates with tumor grade and predicts prostate cancer progression (7–11, 13, 14). DAB2IP functions as a scaffold protein to modulate a variety of biologic activities: cell growth, apoptosis, survival, and epithelial–mesenchymal transition (EMT) leading to metastasis of prostate cancer (15–17). Mechanistically, DAB2IP can inhibit the Wnt-elicited EMT pathway by recruiting PP2A to active GSK-3β. In addition, DAB2IP is able to regulate Ras-MAPK,
**Translational Relevance**

Docetaxel is the first-line chemotherapy for men with metastatic castration-resistant prostate cancer (CRPC), and prolongs survival of most patients; however, the acquisition of chemoresistance is a significant barrier to cure this disease. It is clear that the DOC-2/DA22 interactive protein (DAB2IP) has distinct cellular functions, modulating different signal cascades associated with cell proliferation, survival, apoptosis, and metastasis. In this study, we show how loss of DAB2IP in prostate cancer cells becomes chemoresistance and unveil the underlying mechanism of DAB2IP in modulating Clusterin (CLU) gene expression via cross-talk between Wnt/β-catenin and insulin-like growth factor I (IGF-I)/IGF receptor (IGFR) signaling. These findings are of clinical relevance because this is a new mechanism for CLU gene regulation by DAB2IP, which can lead to develop a targeted therapy by aiming CLU along with DAB2IP as a biomarker for patient selection.

**Materials and Methods**

**Cell lines**

Stable DAB2IP-short hairpin RNA (shRNA)-knockdown (KD) and control (Con) shRNA-scrambled cells were derived from PZ-HPV-7, RWPE-1, and LAPC-4 cell lines. Stable DAB2IP-transfected C4-2 sublines (D1 and D2) and its control (Neo) cells were generated as described previously (16).

**Prostate tumor specimen**

This study was conducted on a total of 194 prostate cancer specimens obtained from the Vancouver Prostate Centre Tissue Bank. Seventy-six of those cases were subjected to neoadjuvant hormone therapy (NHT). The hematoxylin and eosin (H&E) slides were reviewed and the desired areas were marked. Three tissue microarrays (TMA) were manually constructed (Beecher Instruments) by punching duplicate cores of 1 mm for each sample. All the specimens were from radical prostatectomy, except 12 CRPC samples that were obtained from transurethreal resection of prostate (TURP). Tissue samples were arrayed according to Gleason score, primary or CRPC status, and with or without NHT, respectively. The Institutional Review Board of UT Southwestern approved the tissue procurement protocol for this study, and appropriate informed consent was obtained from all patients.

**Chemotherapeutic drugs, antibodies, chemicals, and conditioned medium**

Chemotherapeutic drugs used for prostate cancer therapy, included docetaxel (Aventis Pharmaceuticals); epothilone B (Sigma-Aldrich); gemcitabine (Gemzar, Eli Lilly); and Istandax (FK228, Fujisawa Pharmaceutical). Primary antibodies used were: anti-sCLU-α (B-5, Santa Cruz Biotechnology); anti-Egr1 (C-19, Santa Cruz Biotechnology) for Western blot; anti-Egr1 (BD) for immunohistochemical (IHC) staining; anti-β-catenin (BD); anti-p-IGF1/R (Tyr1131, Cell Signaling Technology); anti-Actin (Sigma); and anti-DAB2IP, generated and used as described previously (5).

Chemical inhibitors, including tyrphostin AG1024 (IGF-IR kinase inhibitor) and lithium chloride (LiCl, GSK-3β inhibitor), were purchased from Sigma and Calbiochem (Darmstadt, Germany), respectively. Second-generation antisense oligonucleotides (ASO) specific for sCLU (OGX-011) and comparable scrambled oligonucleotides (MM) were provided by OncoGenex Pharmaceuticals. Prostate cancer cells were treated with siRNA or oligonucleotides using previously described protocols (20). Recombinant human IGF-I was purchased from R&D. Wnt3A- and control-conditioned media (i.e., Wnt-CM and L-CM, respectively) have been previously described (16) and used to treat cells for 48 hours.

**Plasmid constructs**

Various expression plasmids for DAB2IP, sCLU, Egr1, and β-catenin have been previously described (15, 21). The 4250 bp human CLU promoter (pCLU-luc) have been previously described (21). An Egr1 mutant (Egr1-mut) at –89 bp of pCLU-luc was made using site-directed mutagenesis and primers: Egr1-mut sense: 5′-GATGCCGCCCGCCGAGACCCGCGGCTGGTGC-3′; Egr1-mut antisense: 5′-GTGCCAGCCGGCCGCTGGTGC-3′. pEgr1-luc and its deleted constructs were obtained from Dr. Eileen D. Adamson (The Burnham Institute for Medical Research, La Jolla, CA; ref. 22). The pIGF1-luc reporter construct was obtained from Dr. Peter S. Rotwein (Oregon Health & Science University, Portland, Oregon) as previously described (23, 24).
Cell culture, siRNA oligonucleotides and transfection, luciferase reporter gene assay, quantitative RT-PCR (qRT-PCR), Western blot assay, MTT assay, and IHC staining

For details, see Supplementary Materials and Methods.

Statistical analyses

Data are presented as means ± SEM of at least three independent experiments conducted at least in triplicate. All data analyses were conducted by software of SPSS13.0 for Windows. P values of 0.05 or less were regarded as the threshold value for statistical significance.

Results

DAB2IP modulates the chemosensitivity of prostate cancer cells

To determine the possible role of DAB2IP in modulating chemosensitivities of prostate cancer cells, we knocked down the endogenous DAB2IP expression in two different prostatic epithelial cell lines determined by Western blot (Fig. 1A and B, left panel). For employing FDA-approved chemotherapeutic agents commonly used in prostate cancer therapy, we selected four drugs (i.e., docetaxel, epothilone B, gemcitabine, and Istodax) with different mechanisms of action. In general, DAB2IP-knockdown (KD) cells showed significantly higher resistance to all four drugs (Fig. 1A and B), with increases in IC50 values compared with control (i.e., Con) cells expressing DAB2IP protein.

On the other hand, we generated a stable DAB2IP-expressing subline (i.e., D2) from a CRPC cell line (i.e., C4-2) without detectable endogenous DAB2IP and its control subline (i.e., Neo). D2 cells became more sensitive to chemotherapeutic agents compared with Neo cells (Fig. 1C). These data strongly indicated the impact of DAB2IP on the chemosensitivities of CRPC cells.

DAB2IP regulates CLU gene expression in prostate cancer cells

To understand the underlying mechanism of DAB2IP in this event, we explored possible downstream signaling pathways. We noticed that DAB2IP-deficient cells express elevated levels of clusterin (CLU) protein, including both intracellular full-length pre-secretory clusterin (psCLU, ~60 kDa), and its mature secretory (sCLU, ~40 kDa) proteins in all DAB2IP-KD cells from PZ-HPV-7, RWPE-1, and LAPC-4 compared with their Con cells (Fig. 2A and Supplementary Fig. S1A). Similar changes were also observed in prostatic epithelia derived from DAB2IP knockout (DAB2IP−/−) mice as well as MDA-MB-468 breast cancer...
cells (Supplementary Fig. S1B and S1C). In addition, ectopic DAB2IP expression dramatically suppressed sCLU protein expression in C4-2 (i.e., D1 and D2) cells (Fig. 2A).

Consistently, both CLU mRNA expression and gene promoter activities inversely correlated with DAB2IP expression levels in these cells (Fig. 2B and C, \( P < 0.05 \)).

Figure 2. DAB2IP regulates CLU gene expression in prostate cancer cells. A, Western blot analyses of DAB2IP and CLU expression were carried out in PZ-HPV-7, RWPE-1, and C4-2 sublines. Actin was used as an internal loading control. B, levels of DAB2IP and CLU mRNA expression in PZ-HPV-7, RWPE-1, and C4-2 sublines were determined using qRT-PCR. Relative mRNA levels of each gene were determined by normalizing to 18S rRNA. Results (means ± SEM) were obtained from three independent experiments. *: \( P < 0.05 \) versus Con or Neo cells. C, cells were transiently cotransfected with the human pCLU-luc reporter, DAB2IP siRNA, or a DAB2IP expression vector, together with RSV-\( \beta \)-gal as indicated for 36 hours as described previously. Transfectants were then subjected to luciferase and \( \beta \)-gal assays. After normalizing with the \( \beta \)-gal activity, relative CLU promoter activities were calculated. All experiments were conducted in triplicate. *: \( P < 0.05 \) versus vector control.
sCLU mediates the chemoresistant phenotypes of DAB2IP-deficient prostate cancer cells

sCLU is a cytoprotective protein that, when endogenously or exogenously overexpressed, can afford chemoresistance to various cancer cells (25). To determine a role for sCLU as a downstream determinant for DAB2IP loss in the chemoresistance of PZ-HPV-7 and RWPE-1 KD cells, sCLU levels were knocked down using shRNA specific for the exon I/III boundary region of the secreted form of the protein. Silencing sCLU expression significantly restored the chemosensitivity of KD cells to docetaxel (Fig. 3A, \( P < 0.05 \)). In addition, OGX011, an ASO specifically targeting sCLU, has been shown as a promising therapeutic agent against several cancers (26). Our data clearly indicated that OGX-011 ASO, and not MM, could specifically inhibit sCLU expression and synergistically enhance the cytotoxic effects of docetaxel in RWPE-1 KD cells in a dose-dependent manner (Fig. 3B, \( P < 0.05 \)). In contrast, increased expression of sCLU protein using transient cDNA transfection in C4-2-D2 cells can diminish docetaxel cytotoxicity and prolong cell survival (Fig. 3C, \( P \leq 0.05 \)).

DAB2IP suppresses CLU expression by inhibiting Egr1 gene transcription

Previously, we reported that the IGF-I-mediated sCLU induction was regulated by IGF-IR/Src/mitogen-activated protein kinase (MAPK)/ERK signaling, in which Egr1 is a key transcription factor controlling CLU gene promoter activity (21, 24). In DAB2IP-KD cells, elevated Egr1 protein expression was detected (Fig. 4A, left and Supplementary Fig. S1). In contrast, reduced Egr1 protein expression was observed in both D1 and D2 sublines of C4-2 cells compared with control C4-2-Neo cells (Fig. 4A, right). Data from qRT-PCR (Fig. 4B) and CLU gene promoter reporter assays (Fig. 4C) clearly indicated the inhibitory effect of DAB2IP on Egr1 gene transcription. To elucidate the role of Egr1 in sCLU gene transcription in the presence of DAB2IP, the activity of wild-type (wt) CLU
gene promoter versus the Egr1 mutant form (i.e., Egr1-mut) containing a single mutation at −89 bp for Egr1 binding in CLU gene promoter was determined in PZ-HPV-7 sublines (i.e., Con and KD). A dramatic loss of the reporter gene activity of Egr1-mut compared with that of wt CLU was seen in KD cells (Fig. 4D, left). On the other hand, the wt CLU promoter activity increased in C4-2-D2 cells in a dose-dependent manner with an incrementally increasing Egr1 protein level by transient cDNA transfection (Fig. 4D, right panel). Thus, DAB2IP-inhibited sCLU gene expression appears to be mediated by Egr1 transcription factor.

**DAB2IP inhibits Egr1 and CLU expression via suppressing Wnt/β-catenin and IGF-I/IGF-IR signaling**

To dissect the signaling cascade mediated by DAB2IP in regulating Egr1/sCLU expression, we explored the involvement of the Wnt/β-catenin pathway in sCLU expression. Wnt is able to induce sCLU protein accumulation in several prostate cell lines (Fig. 5A and C, and Supplementary Fig. S2A). In addition, using LiCl (a GSK-3β kinase inhibitor) or ectopic expression of β-catenin restored the expression of Egr1 and sCLU in a dose-dependent manner in C4-2-D2 cells (Fig. 5B and D). DAB2IP is able to recruit PP2A to active GSK-3β leading to the inhibition of Wnt/β-catenin signaling (16); in this study, we further showed that DAB2IP could block the Wnt-elicited Egr1/sCLU signaling axis in C4-2 and PZ-HPV-7 cells (Fig. 5A and C).

In addition to known regulatory mechanism for sCLU gene expression (27–29), we unveiled that IGF-I–induced sCLU gene expression is mediated by β-catenin/T-cell factor (TCF). Here, we examined the potential effect of DAB2IP on the cross-talk between Wnt and IGF-I signaling pathways via Egr1–mediated sCLU gene expression. Significant decreases in IGF-I mRNA and gene promoter activity were noted in the presence of DAB2IP (P < 0.05; Fig. 5E), and this inhibition could be diminished by Wnt (P < 0.05; Supplementary Fig. S2B). Similar results were observed in A549 lung carcinoma cells (Supplementary Fig. S3). Consistent activation of IGF-IR phosphorylation was observed in DAB2IP-deficient cells, which is correlated with both Egr1 and sCLU protein expression in C4-2-Neo or D2 cells (Fig. 5F). NF-κB is known as a potent inducer for Egr1 promoter via binding of p65/RelA in normal human keratinocytes cells upon UVB irradiation and, moreover, DAB2IP has been shown to be able to inhibit the NF–κB-elicited pathway (17, 22). However, using several constructs containing deleted NF-κB...
binding sites in the Egr1 gene promoter, the fold of inhibition of Egr1 gene promoter activities by the presence of DAB2IP remained the same (Supplementary Fig. S4), implying that the inhibitory effect of DAB2IP on Egr1 gene transcription is not mediated through NF-κB.

Taken together, DAB2IP acts as an upstream inhibitor for Wnt–elicited IGF-I/Egr1 signaling cascade leading to sCLU gene transcription.

Inverse correlation between DAB2IP and Egr1 or sCLU in progression of prostate cancer

Loss of DAB2IP expression is often detected in human prostate cancer cell lines and tissues, for example, 70% high-grade prostate cancer tissues were noted with significantly decreased DAB2IP expression compared with associated normal prostate tissue (7, 16, 17). The correlation of DAB2IP with Egr1 or sCLU expression in prostate cancer specimens remained undetermined; thus, the expression profile of DAB2IP, Egr1, and sCLU levels using TMAs was conducted. Consistent with prior studies that showed positive but weak DAB2IP staining in naive prostate cancer tissues (14, 17), the majority of CRPC tissues showed no expression of DAB2IP (representative staining shown in Fig. 6A, left). After quantification, DAB2IP loss became more evident in CRPC than naive specimens (P < 0.05; Fig. 6B left). Importantly, stronger nuclear Egr1 and cytosolic sCLU staining were detected in the same CRPC specimens that correlated well with low DAB2IP staining (representative staining shown in Fig. 6A, middle and right). Quantitative analyses showed that nuclear Egr1 and cytosolic sCLU expression levels were significantly elevated in specimens obtained from castration recurrence or different months of NHT treatment (Fig. 6B, middle and right; Supplementary Fig. S5, P < 0.05). Indeed, Egr1 and DAB2IP expression levels were inversely correlated (Pearson correlation coefficient, −0.14; P = 0.01). Furthermore, sCLU and DAB2IP
expression levels were also inversely correlated (Pearson correlation coefficient, \( r = -0.37; P = 0.02 \)), whereas Egr1 expression was positively correlated with expression of sCLU (Pearson correlation coefficient, \( r = 0.18; P = 0.003 \)). Thus, these data strengthen the notion that loss of DAB2IP in prostate cancer could unleash Egr1/sCLU gene expression, which is associated with chemoresistance.

Discussion

Understanding the mechanisms for chemoresistance of cancer is pivotal, because cancer cells eventually develop chemoresistance (3). For prostate cancer, chemotherapy is commonly used in late stages of disease, such as patients with CRPC which has metastasized to multiple organs. In general, these cancer cells are highly heterogeneous and, likely, have acquired a resistant phenotype or develop chemoresistance very quickly. Thus, current chemotherapeutic regimens only prolong patient survival and are not curative. By identifying early molecular mechanisms that promote survival and metastasis, we may develop new therapeutic strategies to enhance the efficacy of chemotherapy to achieve ultimate curative therapies. We and other groups identified DAB2IP as a unique metastatic suppressor that acts as a signalosome to modulate multiple signaling pathways leading to cell growth, survival, and apoptosis (15–17). In addition, we have shown that DAB2IP-deficient prostate cancer cells exhibit radio-resistance (19). In this study, we have extended these observations and shown that DAB2IP-deficient cells show chemoresistance to a wide-spectrum of chemotherapeutic agents, a resistance mechanism that appears to be mediated through sCLU. Therefore, one significance of this study has provided another novel mechanism underlying the failure of docetaxel, commonly used in CRPC chemotherapy, because two recent publications discovered the effect of this microtubule-targeting chemotherapy in androgen receptor (AR) cellular translocation/trafficking as a critical new insight into mechanisms of resistance of CRPC to taxane (30, 31); meanwhile, our very recent study reported that DAB2IP loss facilitated AR activation in CRPC cells (14).

Indeed, sCLU is known as a key contributor to chemoresistance of many cancer cells (26). The CLU gene codes two isoforms (CLU-I and CLU-II), which gives rise to at least two different functions mediated by two separate protein species: (i) a secreted heterodimeric isoform (i.e., sCLU) and (ii) a prenuclear cytoplasmic (pnCLU) splice variant that lacks the endoplasmic reticulum-targeting domain that can be activated to a proapoptotic form which can localize to the nucleus (i.e., nCLU; refs. 32, 33). sCLU, a stress-activated cytoprotective chaperone that is upregulated by chemotherapeutic agents, can protect cells from apoptosis (34, 35). However, the regulatory processes that control sCLU expression are not fully elucidated.

Figure 6. The relationship of DAB2IP, Egr1, and sCLU expression in prostate cancer tissues. A, representative IHC staining of DAB2IP, Egr1, and sCLU levels in clinical specimens. Case-1, detected DAB2IP expression, but lower expression of Egr1 and sCLU in naive prostate cancer specimen; Case-2, no DAB2IP expression with concomitant higher expression of Egr1 and sCLU in CRPC specimen. B, histogram of DAB2IP, Egr1, and sCLU protein expression in CRPC compared with naive tissues (CRPC vs. naive tissues; \( \chi^2 \), \( P < 0.05 \)). C, schematic representation of the mechanism of DAB2IP in clusterin gene expression associated with chemoresistance in prostate cancer.
Some studies have unveiled several transcriptional regulators for sCLU gene promoter including AP1, heat shock factor 1/2, b-MYB, c-MYC, and signal transducer and activator of transcription 1 (STAT1; refs. 33, 36, 37). Recently, YB-1 was shown to regulate stress-induced sCLU transcription and expression, with sCLU playing a dominant downstream role in YB-1-induced cytoprotection and paclitaxel resistance in prostate cancer cells (38). In addition, we showed that IGF-IR/Src/MEK/Erk signaling is involved in the regulation of sCLU expression via the transactivation of Egr1, a known stress-inducible transcription factor (21). In this study, we show evidence indicating that Wnt/β-catenin signaling is upstream of IGF-1 gene expression. Our data indicate that DAB2IP is a potent inhibitor that modulates multiple signaling pathways, such as Wnt/β-catenin and IGF-I, leading to sCLU gene expression. These data are consistent with our previous study (16) in which DAB2IP is critical for regulating EMT by recruiting PP2A and blocking the Wnt/β-catenin pathway. Although DAB2IP is known to inactivate NF-kB signaling (17, 22) and NF-kB can be a transactivator of Egr1 promoter (17, 22), our data indicated that NF-kB was not involved in Egr1 regulation and downstream sCLU expression.

The paradox of Egr1 function is commonly seen in cancer. Egr1 mediates apoptosis in response to stress and DNA damage by regulating a tumor suppressor network, but it also promotes proliferation of prostate cancer cells by an unknown mechanism (39). Nevertheless, levels of Egr1 mRNA and protein expression correlate with Gleason scores and are inversely correlated with prostate cancer grade (40). However, the status of Egr1 protein in CRPC remained unexplored. Our study provides the first evidence for elevated nuclear Egr1 staining in recurrent prostate cancer or from NHT treatment. An inverse correlation between DAB2IP and Egr1 or sCLU expression was noted not only in prostate cancer but also in cells of DAB2IP−/− mice (Supplementary Fig. S1C). Most importantly, such inverse correlation was especially evident in human CRPC samples (Fig. 6). DAB2IP is frequently lost in advanced prostate cancer, which underlies the oncogenic function of Egr1 in this disease.

Knowing the prosurvival and antiapoptotic activities of sCLU, we report the involvement of sCLU in the chemoresistance of prostate cancer cells to various clinically relevant therapeutic agents. Inhibiting the prosurvival function of sCLU using specific ASOs such as OGX-011 under current clinical trials for prostate and lung cancers can be combined with various cytotoxic agents in order to enhance the therapeutic efficacy (25, 26, 41). Based on the data presented in this work, DAB2IP appears to be another potential factor for boosting the therapeutic efficacy of these agents as well.

In summary, this study at least has shown sCLU and its complex signaling interaction with DAB2IP as a mechanistic basis driving therapeutic resistance in prostate cancer (Fig. 6C). For its significance, understanding such a cross-talk could not only provide a new strategy to improve early prediction of chemoresistance in patients with CRPC through combined detection of multiple molecular markers (i.e., DAB2IP, Egr1, and sCLU), but also indicate DAB2IP as a potential target for developing more effective therapy based on epigenetic modification or peptide strategy in future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Development of methodology: K. Wu, Y. Zou
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Wu, D. Xie, D.A. Boothman
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