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ORIGINAL ARTICLE

MUC1 induces drug resistance in pancreatic cancer cells via upregulation of multidrug resistance genes

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MUC1 (CD227), a membrane tethered mucin glycoprotein, is overexpressed in >60% of human pancreatic cancers (PCs), and is associated with poor prognosis, enhanced metastasis and chemoresistance. The objective of this study was to delineate the mechanism by which MUC1 induces drug resistance in human (BxPC3 and Capan-1) and mouse (KCKO, KCM) PC cells. We report that PC cells that express high levels of MUC1 exhibit increased resistance to chemotherapeutic drugs (gemcitabine and etoposide) in comparison with cells that express low levels of MUC1. This chemo resistance was attributed to the enhanced expression of multidrug resistance (MDR) genes including *ABCC1, ABCC3, ABCC5* and *ABCB1*. In particular, levels of MRP1 protein encoded by the *ABCC1* gene were significantly higher in the MUC1-high PC cells. In BxPC3 and Capan-1 cells MUC1 upregulates MRP1 via an Akt-dependent pathway, whereas in KCM cells MUC1-mediated MRP1 upregulation is via an Akt-independent mechanism. In KCM, BxPC3 and Capan-1 cells, the cytoplasmic tail motif of MUC1 associates directly with the promoter region of the *Abcc1/ABCC1* gene, indicating a possible role of MUC1 acting as a transcriptional regulator of this gene. This is the first report to show that MUC1 can directly regulate the expression of MDR genes in PC cells, and thus confer drug resistance.

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INTRODUCTION

Pancreatic cancer (PC) is the fourth leading cause of cancer-related deaths in the United States. The 5-year survival rate is ~3% and the median survival rate is <6 months.[1] The current therapeutic interventions include surgical resection, radiation therapy, chemotherapy and immunotherapy.[2] Less than 20% of PC patients are eligible for surgery because the disease is often diagnosed in late stages.[3] However, in most cases where surgery is an option, the tumor recurs within 1–2 years and patients develop hepatic metastasis.[4] In case of patients with inoperable PC, the standard treatment is chemotherapy that includes gemcitabine. In this group of patients the survival is increased by a dismal 5 weeks.[5] The poor outcome of chemotherapy is partly due to the drug-resistant phenotype of PC cells. Thus, failure of effective chemotherapeutic treatment results in high mortality in PC patients.[5] This underscores the importance of understanding the mechanism of drug resistance and developing strategies that would improve the outcome of chemotherapy.

Drug resistance can be classified into two categories: *de novo* resistance or acquired resistance. Cancer patients that exhibit *de novo* resistance do not respond to chemotherapy from the start. However, in acquired resistance, the cancer cells initially respond to a chemotherapeutic drug but eventually acquire resistance to it. The cells might also show cross-resistance to other structurally and mechanistically unrelated drugs—a phenomenon commonly known as multi drug resistance (MDR).[6] Owing to acquisition of MDR, treatment regimens that combine multiple agents with different targets are no longer effective.[5,7]

One of the primary mechanisms by which cancer cells attain drug resistance is via upregulation of a family of ATP-binding cassette (ABC) transporters. These transporters or drug efflux pumps contribute to the MDR phenotype in cancer cells by increasing the efflux of anticancer drugs, thereby reducing their accumulation inside the cancer cells.[8] P-glycoprotein, MRP1-9 and BCRP are some of the ABC transporters that have been positively linked to the MDR phenotype in cancer cells. The *Mdr1* (or *ABCB1/Abcb1*) gene, which encodes for P-gp, is a well-characterized *mdr* gene. The *ABCC/Abcc* (1–9) gene encodes for the MRP family of multidrug transporters that are responsible for the acquired drug resistance. The *ABCC1/Abcc1*gene encodes for MRP1, which is structurally very similar to P-gp.[9] Overexpression of the *mdr* genes in cancer cells is considered to be the primary determinant of the MDR phenotype. Another common mechanism of acquiring drug resistance is through enhanced activation of PI3K/Akt and Erk1/2 pathways. These pro-survival pathways inhibit induction of apoptosis in cancer cells. Interestingly, it has recently been shown that PI3K/Akt activation regulates expression of the *ABCC1* gene in prostate cancer cells.[10] Studies have shown that in MUC1-overexpressing cancer cells both Erk1/2 and PI3K pathways are overstimulated.[11,12] These reports indicate a possible role of these pathways in conferring drug resistance in MUC1-overexpressing PC cells.

MUC1 is a transmembrane mucin glycoprotein that is expressed at the apical surface of epithelial cells.[13] In over 80% of human pancreatic adenocarcinomas (PDA), a differentially glycosylated form of MUC1 is predominantly overexpressed.[14,15] MUC1 is a heterodimer, which consists of a unique N-terminal extracellular domain and a C-terminal intracellular domain. The N-terminal domain consists of variable number tandem repeats of 20 amino acids that are extensively modified by O-glycosylation.

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The C-terminal domain includes a 53-amino-acid-long extracellular region, a 28-amino-acid-long transmembrane domain and a 72-amino-acid-long cytoplasmic tail (CT).[16–18] The transmembrane (TM) and the seven tyrosine residues of MUC1 CT are highly conserved (88% and 100% identical, respectively) among different species, suggesting important functional roles. MUC1 CT serves as an adaptor protein that brings together kinases and other proteins for the propagation of signals, which leads to increased cell proliferation, changes in adhesive state of the cell, invasion into the extracellular matrix and deregulation of apoptosis.[11,19,20] Importantly, studies have shown that MUC1-overexpressing breast, colon and thyroid cancer cells are unresponsive to chemotoxic agents.[11,12]

Thus, the goal of the present study was (1) to determine if MUC1-overexpressing PC cells are resistant to chemotherapeutic drugs and (2) to delineate the mechanism by which MUC1-associated resistance occur. We report that MUC1 regulates the *mdr* gene expression via both Akt-dependent and -independent pathways, which confers the MDR phenotype to PC cells. This is the first report that demonstrates a direct relationship between expression of MUC1 and *mdr* genes, in particular *ABCC1* in PC.

RESULTS

PC cells expressing high levels of MUC1 are less sensitive to chemotherapeutic drugs that are reversed upon MUC1 downregulation

To determine the relative expression of endogenous MUC1 in BxPC3 and Capan-1 cell lines, immunohistochemical analysis of cells grown in chamber slides was performed using an antibody against the tandem repeat of MUC1 (HMFG2). Immunohistochemical staining showed that Capan-1 cells have higher endogenous MUC1 expression as compared with BxPC3 cells (Figure 1a). This was confirmed using western blotting assay using antibodies against the tandem repeat (HMFG2) and CT of MUC1 (CT2). Both antibodies showed that Capan-1 cells have higher endogenous MUC1 compared with BxPC3 cells (Figure 1b). Next, we show MUC1 expression in Capan-1 cells following treatment with control and MUC1-specific siRNA (small interfering RNA) by western blot. Complete knockdown of MUC1 is observed in Capan-1 cells post 48-h treatment with MUC1-specific siRNA (Figure 1c and Supplementary Table 1).

To determine the effect of MUC1 in drug resistance, BxPC3 and Capan-1 cells were treated with etoposide and gemcitabine, and proliferation post treatment was determined using H^3-thymidine

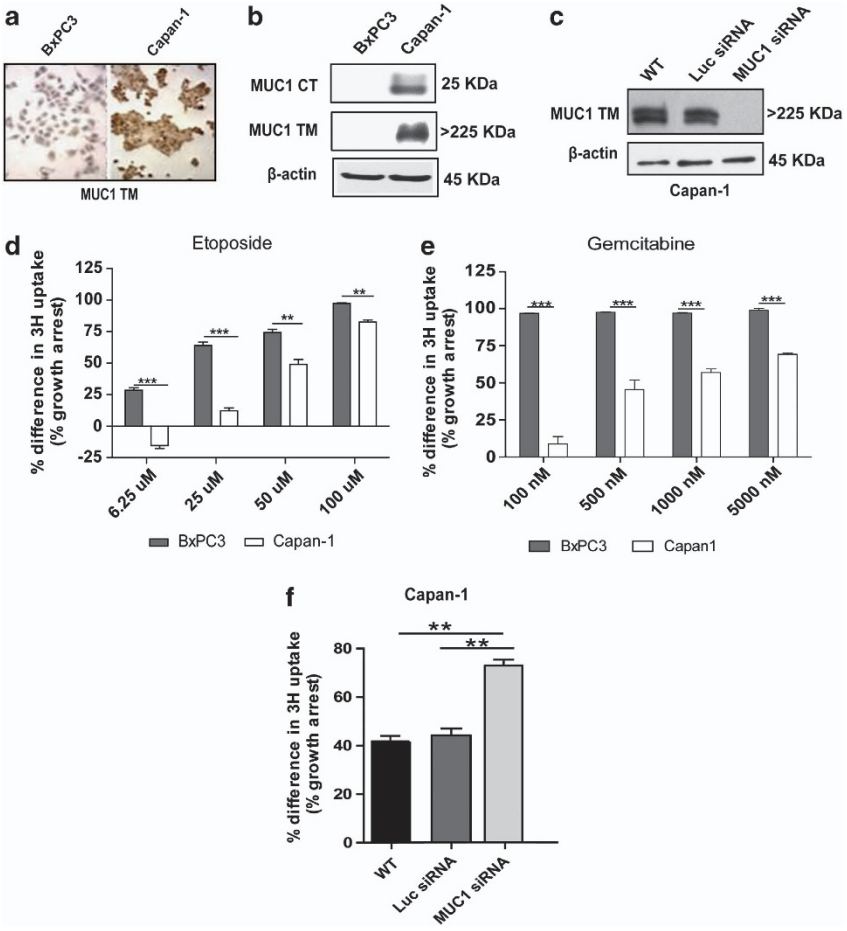

Figure 1. MUC1 expression and drug sensitivity of a panel of cancer cells. (**a**) Staining of endogenous MUC1 expression in BxPC3 and Capan-1 cells using HMFG2 antibody, Capan-1 expresses high levels as depicted by the brown staining whereas BxPC3 cells have negligible levels of MUC1 staining. (**b**) Western blot analysis of MUC1 expression in BxPC3 and Capan-1 cells by western blot using HMFG2 and CT2 antibody. (**c**) Western blot analysis of MUC1 expression in Capan-1 cells following treatment with MUC-1 specific siRNA (48 h). (**d**, **e**) H^3-thymidine incorporation to measure proliferation in PC cells following 24 h treatment with etoposide and gemcitabine ($n=4$). Significantly higher proliferation was observed in Capan-1 cells, which express high levels of MUC1 (***$P<0.001$). (**f**) Percent difference in H^3-thymidine uptake in control siRNA and MUC1 siRNA treated cells as a function of Capan-1 WT cells. Cells were treated for 24 h with 500 nM of gemcitabine ($n=4$). Cells treated with MUC1 siRNA showed significantly reduced proliferation in response to gemcitabine as compared with untreated or control siRNA treated cells (**$P<0.05$).

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incorporation assay. Etoposide is a toposiomerase II inhibitor, whereas gemcitabine is a nucleoside analog. Low MUC1-expressing BxPC3 cells showed greater sensitivity to etoposide and gemcitabine compared with high MUC1-expressing Capan-1 cells. At 25 μM dose of etoposide, we observed a 62.8% growth arrest in BxPC3 cells. In contrast, at the same dose, only 12.14% growth arrest was observed in Capan-1 cells (Figure 1d). Similarly, at 500 nM dose of gemcitabine, ~100% growth arrest was observed in BxPC3 cells, compared with only 50% growth arrest in Capan-1 cells (Figure 1e). Further, when Capan-1 cells treated with MUC1 siRNA were exposed to 500 nM of gemcitabine, a 31% increase in growth arrest was observed compared with untreated cells or cells transfected with control scrambled siRNA (Figure 1f).

For further investigations, mouse PDA primary cells genetically lacking Muc1 (KCKO) and ones expressing human MUC1 (KCM) were included in this study. Upon using the CT2 antibody that recognizes the CT of both mouse and human MUC1, KCM cells showed high expression of MUC1 while KCKO cells showed no detectable levels (Figure 2a and Supplementary Table 2). To further validate the effect of MUC1 in drug resistance, KCKO and KCM cells were treated with etoposide and gemcitabine. We found 76% and 88% of growth arrest upon treatment of KCKO cells with 1.25 μM and 2.5 μM of etoposide, respectively. In contrast, only 52% and 57% of growth arrest was observed in KCM cells at 1.25 and 2.5 μM of etoposide, respectively, indicating that KCM cells were more resistant to etoposide (Figure 2b, left panel). At 5 μM of etoposide, both cell lines irrespective of their MUC1 status were sensitive. Similar resistance of KCM cells to gemcitabine was observed. At 3 nM of gemcitabine, 60% of growth arrest was observed in KCKO cells compared with only 34% of growth arrest in KCM cells. At higher doses, there was no difference in growth arrest between KCKO and KCM cells (Figure 2b, right panel). MTT assay was also performed to validate the cytotoxic effects of these drugs on the same cell lines. At 50 μM of etoposide, 48% of cell death was observed in KCKO cells compared with only 27% cell death in KCM cells (Figure 2c, left panel). Similarly, at 150 nM of gemcitabine, 53.3% of cell death was observed in KCKO cells compared with only 40% cell death in KCM cells (Figure 2c, right panel).

To further confirm that the effect was due to MUC1 expression, we stably expressed full-length MUC1 in BxPC3 cells that have low levels of endogenous MUC1 (BxPC3.MUC1), and as control we transfected BxPC3 cells with empty vector that contains the neomycin resistance gene (BxPC3.Neo). First we show the relative expression of MUC1 in these cells (Figure 3a and Supplementary Table 3). BxPC3 MUC1 cells express high levels of MUC1 while BxPC3 Neo cells have negligible levels. BxPC3 MUC1 cells were significantly resistant to both the genotoxic drugs as compared with the BxPC3 Neo cells. At 25, 50 and 75 μM of etoposide, cells with low MUC1 showed significantly higher growth arrest compared with cells expressing high levels of MUC1 (Figure 3b, left panel). Similar results were observed with 6.25–25 nM of gemcitabine (Figure 3b, right panel). MTT assay was performed to validate the cytotoxic effects of etoposide and gemcitabine on both cell lines. At 75 μM of etoposide, 64% of cell death was observed in BxPC3 Neo cells compared with only 39.6% cell death in BxPC3 MUC1 cells (Figure 3c, left panel). Similarly, at 50 nM of gemcitabine, 42.7% of cell death was observed in BxPC3 Neo cells

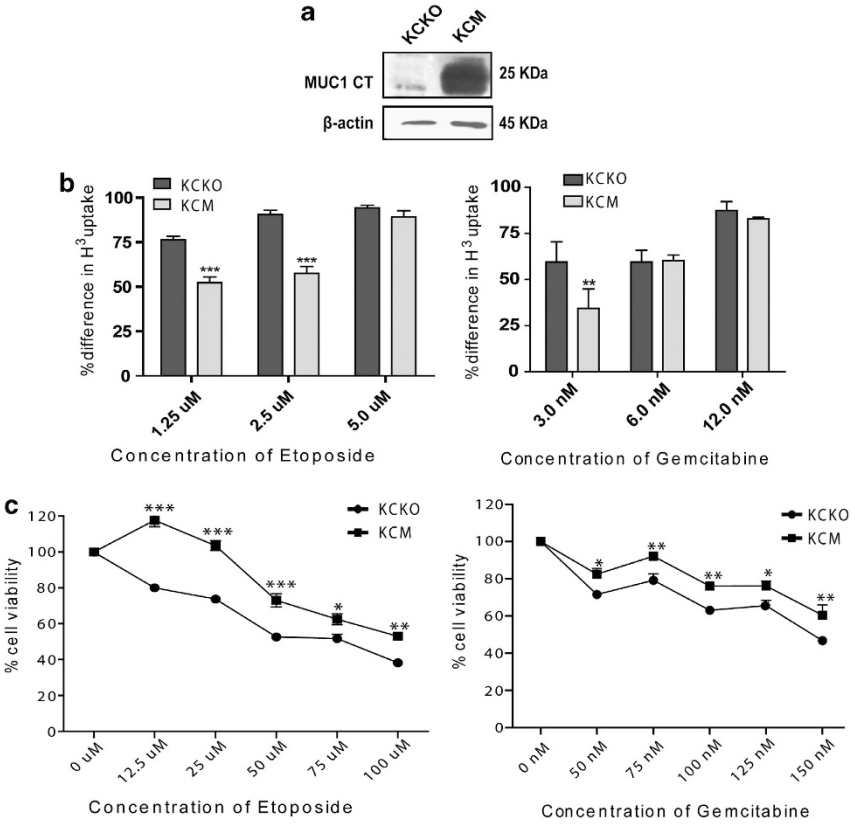

Figure 2. Endogenous expression of MUC1 in PC cells confers resistance to cytotoxic drugs. (**a**) Western blot analysis of endogenous Muc1/MUC1 expression in mouse cells lines, KCKO and KCM using CT2 antibody. Note: CT2 is the only antibody that recognizes both mouse and human Muc1/MUC1. (**b**) Percent difference in H^3-thymidine uptake in KCKO and KCM cells following 24 h treatment with etoposide and gemcitabine. Significant differences between KCKO and KCM cells at varying concentrations of the drugs are shown as *P*-values ($n = 4$) (**$P < 0.01$, ***$P < 0.001$). (**c**) Cell viability in KCKO and KCM cells following 24 h treatment with etoposide and gemcitabine. Significant differences between KCKO and KCM cells at varying concentrations of the drugs are shown as *P*-values ($n = 6$) (*$P < 0.1$, **$P < 0.01$, ***$P < 0.001$).

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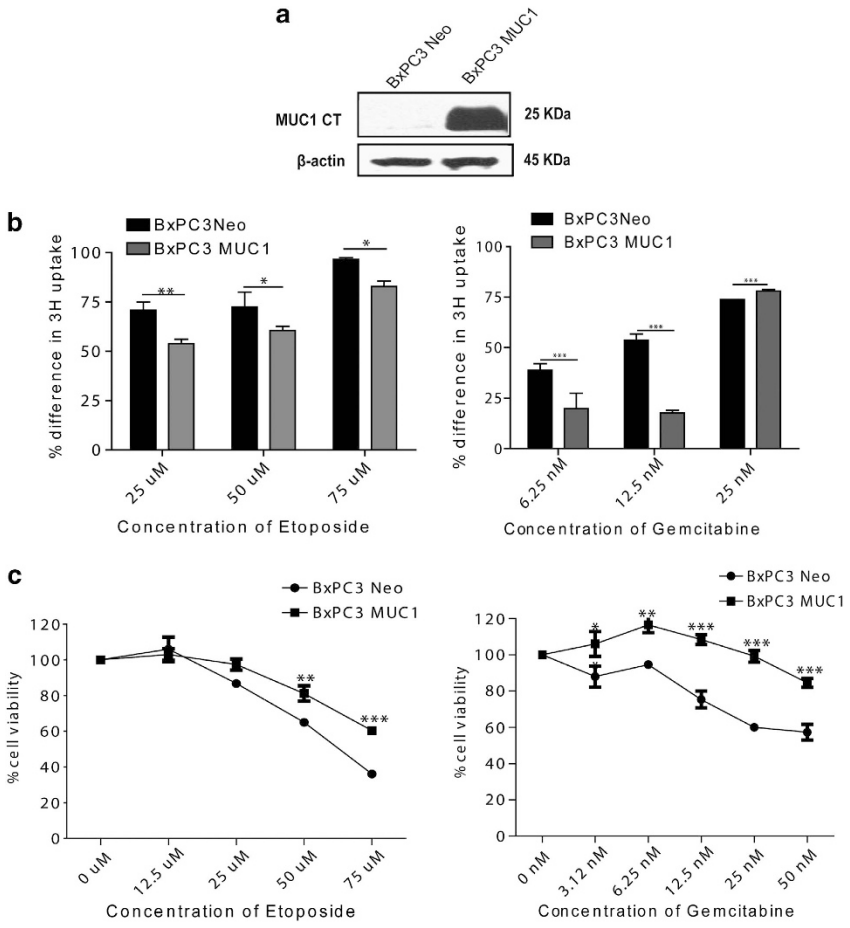

Figure 3. Exogenous expression of MUC1 in BxPC3 cells confers resistance to cytotoxic drugs. (**a**) Western blotting analysis of MUC1 expression in BxPC3 cells using CT2. (**b**) Percent difference in H^3-thymidine uptake of BxPC3 Neo and MUC1 cells following 24 h treatment with etoposide and gemcitabine ($n=4$). Significant differences between BxPC3.Neo and MUC1 are shown (**$P<0.01$). (**c**) Cell viability in BxPC3 Neo and BxPC3 MUC1 cells following 24-h treatment with etoposide and gemcitabine. Significant differences between BxPC3 Neo and BxPC3 MUC1 cells at varying concentrations of the drugs are shown as *P*-values ($n=6$) (*$P<0.1$, **$P<0.01$, ***$P<0.001$).

compared with only 15.5% cell death in BxPC3 MUC1 cells (Figure 3c, right panel). These results suggested that MUC1 confers resistance to gemcitabine and etoposide in PC cells.

MUC1 regulates expression of multidrug resistance genes in PC cells *in vitro*

Previously we have published proteomics data that showed KCM cells express eightfold higher P-glycoprotein, fourfold higher MRP-1 and twofold higher MRP-5 protein compared with KCKO cells.[19] Therefore, we first determined the mRNA level of some of these MDR genes. Consistent with those results, we found significantly higher m-RNA levels of the *Abcc1, Abcc3, Abcc5, Abcb1a* and *Abcb1b* genes in KCM vs KCKO cells using RT–PCR (Figure 4a, left panel) Similarly, in BxPC3 MUC1, the m-RNA levels of *ABCC1, ABCC3, ABCC5* and *ABCB1* genes were significantly higher compared with BxPC3 Neo cells (Figure 4a, right panel). To validate this finding, we determined the protein expression of MRP-1 by western blotting, and, as expected, we observed significantly higher expression of MRP-1 in KCM cells compared with KCKO cells, and in BxPC3 MUC1 compared with BxPC3 Neo cells (Figure 4b and Supplementary Table 4).

Tumors lacking MUC1 or expressing low levels of MUC1 have lower expression of MRP-1

All of the data so far have been shown in cells grown *in vitro*. To answer if this is true *in vivo*, we determined the MRP-1 protein expression in spontaneously occurring PDA.MUC1 (KCM) and PDA.Muc1KO (KCKO) tumors, as well as in BxPC3 Neo and MUC1 tumors grown in nude mice. Immunohistochemical analysis was performed on tumor sections from ~16-week-old KCM and ~24-week-old KCKO mice, and a representative section from each tumor type is shown in Figure 4c (left panel). Significantly higher expression of MRP-1 protein was observed in KCM as compared with KCKO tumor sections (Figure 4c). MRP1 levels in the tumor lysates isolated from BxPC3 Neo and BxPC3 MUC1-xenografted tumors were determined by western blotting. BxPC3 MUC1 tumors showed higher MRP-1 expression compared with BxPC3 Neo tumors (Figure 4d). Interestingly, the tumor sample (sample 3) that had higher MUC1 expression compared with the other MUC1-positive tumor sample (sample 4) also showed higher MRP-1 expression (Supplementary Table 5). The data suggest that a positive correlation exists between MUC1 overexpression and upregulation of *mdr* genes in PC cells.

Knockdown of Akt decreases MRP1 expression in MUC1 high PC cells and sensitizes them to chemotherapeutic drugs

Often in tumor cells, reduced sensitivity to chemotherapeutic drugs is due to enhanced activation of the anti-apoptotic or prosurvival pathways, which includes the PI3K/Akt pathway. We first determined the activation status of PI3K/Akt pathway in KCKO, KCM, BxPC3.Neo and BxPC3.MUC1 cells. Protein lysates from these cell lines were subjected to immunoblotting using

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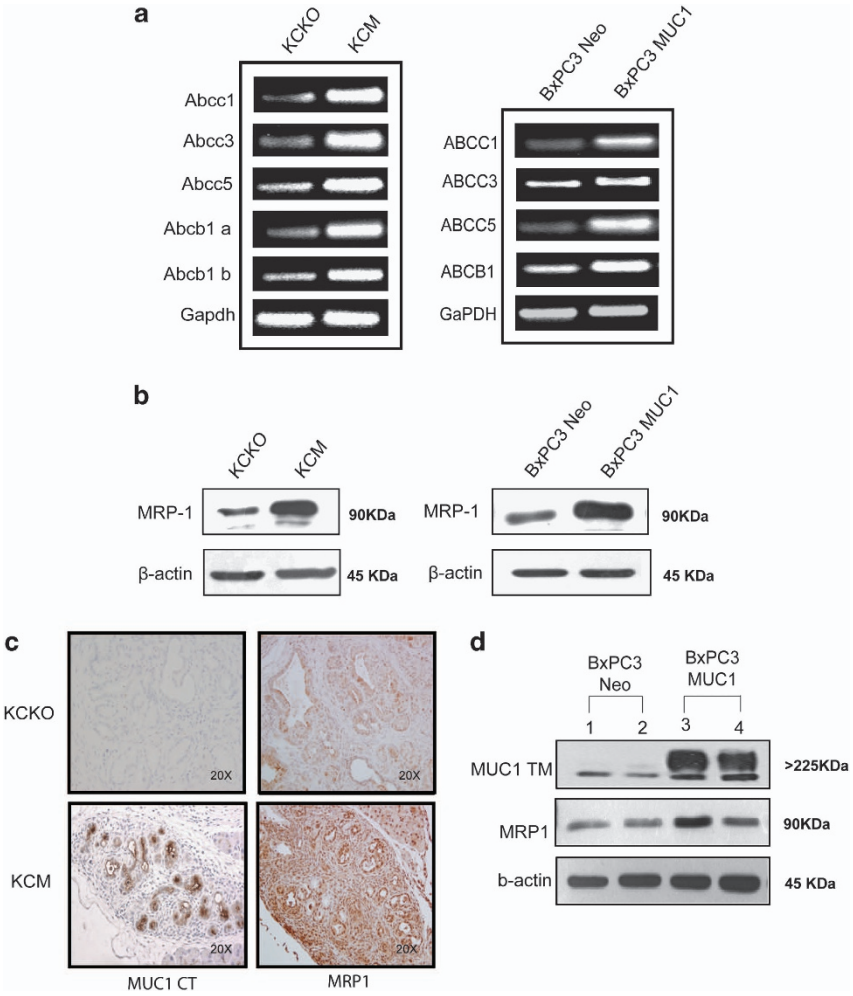

Figure 4. MUC1-positive PC cells express elevated levels of MDR genes *in vitro* and *in vivo*. (**a**) RT-PCR data showing fold changes in the m-RNA level of MDR genes that are associated with multidrug resistance. (**b**) Levels of MRP1 protein in BxPC3 Neo, MUC1, KCKO and KCM cell lysates analyzed by western blot. (**c**) Immunohistochemical analysis of MRP1 expression in the tumor sections from KCKO (24-week-old) and KCM (16-week-old) mice. Note: Two different time points were deliberately selected, as the tumor burden in the KCKO mice at 24 weeks is equivalent to the tumor burden in 16-week-old KCM mice. (**d**) Levels of MRP1 protein in BxPC3 Neo and MUC1 tumor lysates were determined by western blot.

anti-phospho-Akt (p-Akt) and Akt antibodies. Significantly higher levels of pAkt were found in MUC1-positive PC cells (KCM and BxPC3 MUC1) compared with MUC1-low or -null PC cells (KCKO and BxPC3 Neo) (Figure 5a and Supplementary Table 5). The levels of total Akt remained same in all cell lines, indicating enhanced activation of the PI3K/Akt pathway in KCM and BxPC3 MUC1cells. This finding positively correlates with the results presented previously in MUC1-overexpressing fibroblasts.[11]

To test the contribution of Akt on MRP1 expression and drug resistance, we transiently knocked down Akt and evaluated the levels of MRP1 expression by western blot and drug sensitivity by MTT assay. The levels of Akt, MRP1 and MUC1 are shown in Figures 5b–d. Upon Akt knockdown, we observed a 5.4-fold decrease in MRP1 expression in Capan-1 cells and 4.6-fold decrease in MRP1 expression in BxPC3 MUC1 cells (Figures 5b and c, and Supplementary Tables 7 and 8). Furthermore, ~40% and 25% increase in cytotoxicity was observed in Akt siRNA-treated BxPC3 MUC1 cells upon treatment with 50 μM of etoposide and 25 nM of gemcitabine, respectively (Figure 5e). These data indicated that Akt pathway had an important role in MUC1-induced MRP1 expression, and drug resistance in Capan1 and BxPC3 cells.

Interestingly, we also observed a subsequent decrease in MUC1 expression upon downregulation of Akt in Capan-1 and BxPC3 MUC1 cells (Figures 5b and c). When Akt was transiently knocked down in Capan-1 and BxPC3 MUC1 cells, a respective 3.2-fold and 2.5-fold decrease in MUC1 expression was observed (Supplementary Tables 7 and 8). These data indicate that *MUC1* gene is also under regulation of PI3K/Akt pathway. Hence, abrogation of the Akt pathway causes a significant decrease in MUC1 expression, which in turn negatively affects MRP1 expression.

However, we did not see a significant decrease in MUC1 and MRP1 expression in KCM cells upon Akt knockdown (1.2-fold decrease) (Figure 5d and Supplementary Table 9). Consequently, we did not detect a significant increase in cytotoxicity in Akt siRNA-treated KCM cells upon treatment with etoposide and gemcitabine (data not shown). These data indicate that in KCM cells, *MUC1* gene is not strongly regulated by PI3K/Akt pathway. This observation further led to the possibility that in KCM cells, an Akt-independent mechanism must be involved in MUC1-induced MRP1 expression and drug resistance. It is of interest that BxPC3 and Capan-1 are human cells while KCM is a mouse cell line.

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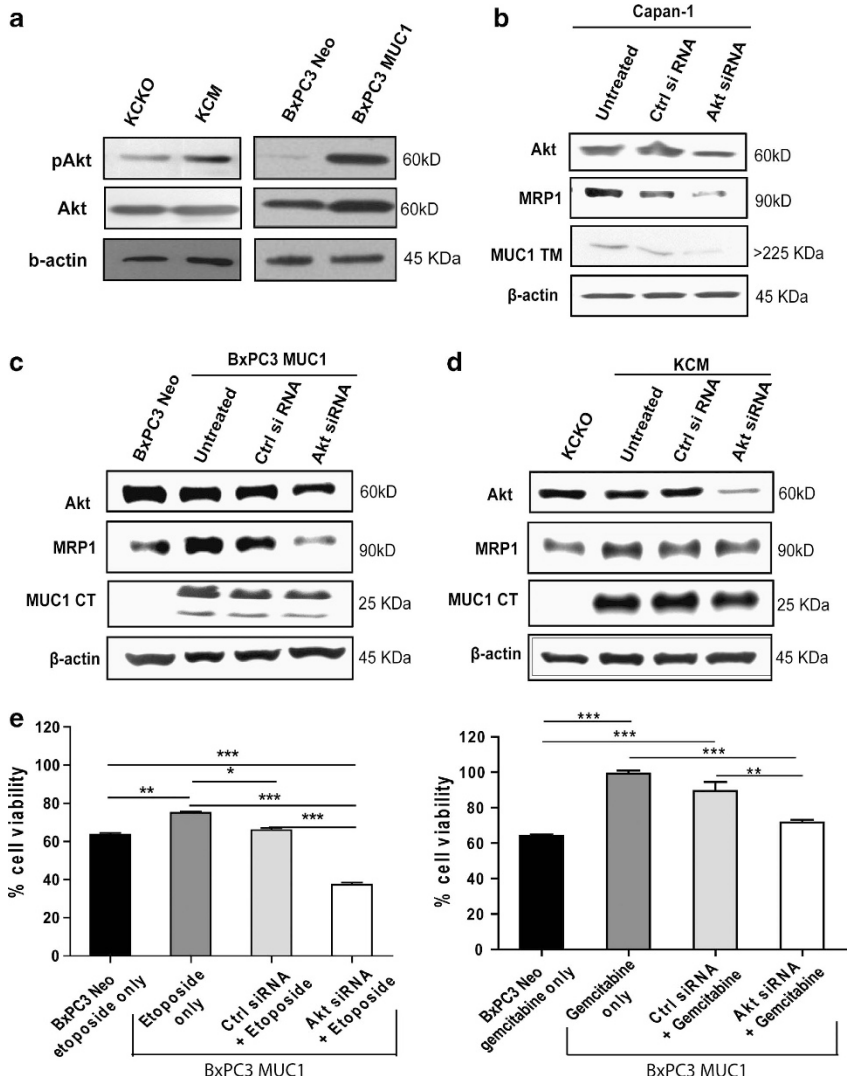

Figure 5. Enhanced activation of the prosurvival pathways in MUC1-positive PC cells. MUC1 induces MRP1 expression via Akt- dependent and -independent pathways. (**a**) BxPC3 Neo, MUC1, KCKO and KCM cell lysates were subjected to western blot analysis to determine phosphorylation of Akt. Level of unphosphorylated Akt served as control for phosphorylation. β-Actin served as loading control. (**b**–**d**) Cells were treated with with 100 nM of Akt siRNA for 48 h, and the lysates were immunoblotted to evaluate the levels of Akt, MRP1 and MUC1. β-Actin served as a loading control. (**e**) Cells growing in a 6-well plate were left untreated (WT) or treated with either control siRNA or Akt siRNA (100 nM). Thirty-six hours post treatment, cells were trypsinized, and equal number of cells were re-plated in a 96-well plate. The cells were allowed to adhere and, at 48 h, were left untreated or treated with 50 μM of etoposide and 25 nM of gemcitabine. MTT assay was performed to measure cytotoxicity 24 h post drug treatment.

MUC1.CT interacts within the promoter region of the *ABCC1* gene

Several studies have shown that MUC1 CT associates with mediators of signal transduction and transcriptional regulation, and thereby modifies the expression of specific target genes.[21,22] In this study, we wanted to investigate the occupancy of MUC1 CT in the promoter region of *ABCC1/Abcc1* gene, which can be indicative of MUC1's role as a modulator of *ABCC1/Abcc1* gene expression. First, we demonstrate that MUC1 CT localizes to the nucleus of MUC1-positive PC cells. Nuclear and cytosolic fractions were extracted from KCKO, KCM, BxPC3 Neo and BxPC3 MUC1 cells, and the lysates were immunoblotted to determine the cellular localization of MUC1 CT in these PC cells. As expected, we found MUC1 CT localizing to the nucleus of KCM cells (Figure 6a, left top panel) and BxPC3 MUC1 cells (right top panel Figure 6a). Lamin A/C and MEK1 served as controls for the extraction process. Lamin A/C is a nuclear protein, and hence is found only in the nuclear fractions (middle panels, Figure 6a). MEK1 is a cytosolic protein and is found only in the cytosolic fractions (bottom panels Figure 6a).

Next, we evaluated the occupancy of MUC1 CT in the genomic regions of the *ABCC1/Abcc1* gene upstream the transcription start site (Figure 6b). Sheared DNA was immunoprecipitated using MUC1 CT specific antibody CT2. IgG antibody was used as a control. The immunoprecipitated DNA was amplified by PCR using primers spanning around 1000 bp upstream (ChIP region I) and 2000 bp upstream (ChIP region II) of the *ABCC/Abcc1* gene transcription start site (Figure 6b). In Capan-1 cells, we observed a strong interaction between MUC1 CT and ChIP region I of *ABCC1* gene (6.5-fold enrichment with CT2 antibody relative to IgG) (Figure 6c and Supplementary Table 10). Similarly, in KCM cells, a strong interaction was observed between MUC1 CT and ChIP region I of *Abcc1* gene (3.2-fold enrichment with CT2 antibody relative to IgG) (Figure 6c and Supplementary Table 11). However, no interaction was observed between MUC1 CT and ChIP region II

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in Capan-1 and KCM cells (Figure 6c). KCKO cells, which are null for MUC1, did not show any interaction between MUC1 CT, and ChIP region I and II of *Abcc1* gene (Figure 6c). These data indicated that the interaction of MUC1 CT with the promoter region of *ABCC1/Abcc1* gene around ChIP region I is specific. However, in BxPC3 MUC1 cells, a very weak interaction between MUC1 CT and ChIP region I of *ABCC1* gene was observed (1.1-fold enrichment compared with IgG, Supplementary Table 12 and Figure 6c). BxPC3 Neo cell also showed weak binding of MUC1 CT around the same gene locus. This is most likely because BxPC3 cells express low levels of endogenous MUC1 and are not null for the same (Figure 6c). The interaction between MUC1 CT and ChIP region II was not observed in BxPC3 cells (Figure 6b).

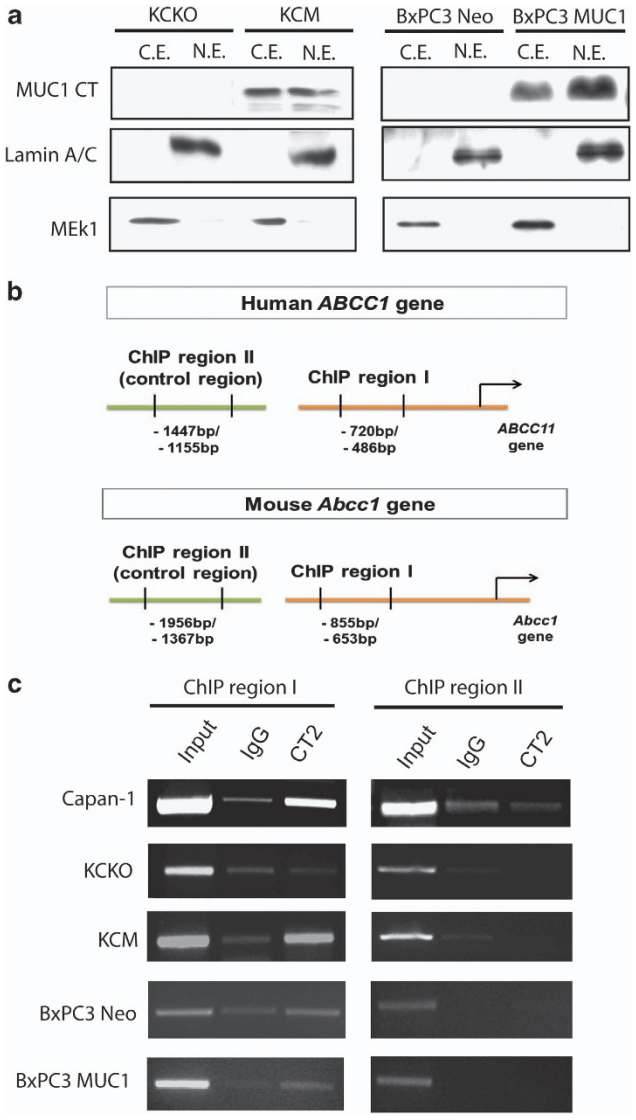

Figure 6. ChIP–PCR assay reveals an interaction between the MUC1 CT and the ABCC1 promoter region. (**a**) Nuclear lysates of KCKO, KCM, BxPC3 Neo and BxPC3 MUC1 cells were subjected to immunoblotting to determine the nuclear localization of MUC1 CT. Lamin and MEK1 were used as controls for nuclear and cytosolic fractions, respectively. (**b**) Schematic representation of the primers that were designed to PCR amplify the promoter region of human *ABCC1* gene (top panel) and mouse *Abcc1* gene (bottom panel) in ChIP assay. (**c**) ChIP–PCR; lanes include: Input DNA, DNA precipitated using control IgG and CT2, and amplified by PCR using Taq polymerase and separated by 2% agarose gel.

DISCUSSION

The ability of tumor cells to escape the cytotoxic effect of chemotherapeutic agents may result from genetic alterations that affect cell cycle, apoptosis or accumulation of drugs inside the cell. Several studies in breast, colon and thyroid cancers have shown that MUC1 attenuates stress-induced or chemotoxic agents-induced apoptosis by blocking the release of cytochrome *c* from mitochondria.[11,12,23] In this study, we demonstrate additional mechanisms by which MUC1 enables PC cells to escape chemotherapeutic drug-mediated cell death.

We found that cells expressing full-length MUC1 are less sensitive to genotoxic drugs than cells lacking or expressing low levels of MUC1, indicating a direct correlation between MUC1 expression and chemoresistance in PC (Figures 1, 2 and 3).

Here, for the first time, we provide evidence that in PC cells, *mdr* gene expression is directly correlated with MUC1 expression (Figure 4). Previous work has shown that hyperactivation of PI3K/Akt pathway is able to regulate expression of *mdr* genes, including *ABCC1, ABCC3, ABCC5 and ABCB1* genes.[10] Studies have demonstrated that MUC1 oncoprotein induces transformation in rat fibroblasts or desensitizes thyroid cancer cells to chemotherapy induced apoptosis through activation of Jak/Stat and PI3K/Akt pathways.[11,12] So, we evaluated if MUC1 induced expression of the *mdr* gene *ABCC1/Abcc1* via activating the PI3K/Akt pathway. We found that in a subset of human PC cells (BxPC3 MUC1 and Capan-1), MUC1-induced MRP1 expression was via the Akt pathway with a pattern that suggests increased refractoriness of these cells to genotoxic drugs. Accordingly, abrogation of the PI3K/Akt pathway resulted in increased responsiveness of these cells to etoposide and gemcitabine (Figure 5). We also found the evidence for existence of a positive feedback loop between MUC1 expression and PI3K/Akt signaling cascade. PC cells with high MUC1 expression exhibited hyperactivation of the PI3K/Akt pathway, which in turn upregulated MUC1 expression in those PC cells. However, it is beyond the scope of the current study to determine how Akt pathway regulates MUC1 expression. In the future, we would like to investigate the mechanism in further

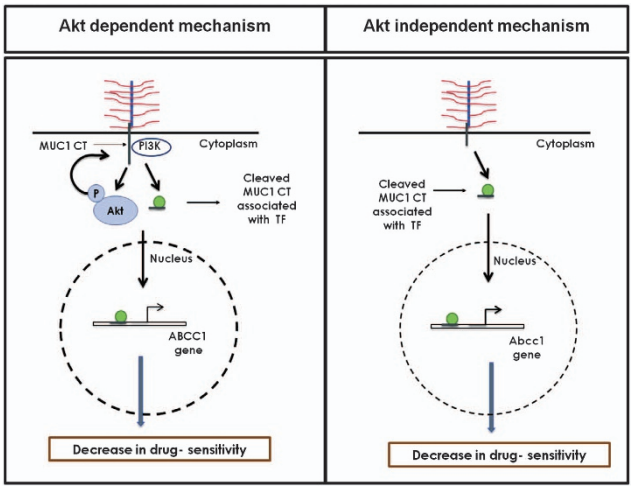

Figure 7. Schematic illustration of the two possible pathways by which MUC1 regulates MRP1 gene expression in PC cells. In human PC cell lines, Capan-1 and BxPC3 MUC1, MUC1-induced *ABCC1* gene expression is dependent on PI3K/Akt pathway. The CT of MUC1 stimulates the PI3K/Akt pathway, which in turn increases MUC1 expression (left panel). In murine PC cell line, KCM, MUC1-induced MRP1 expression is independent of the PI3K/Akt pathway (right panel). The CT of MUC1 translocates to the nucleus and binds to the promoter of *ABCC1/Abcc1* gene, possibly acting as a part of the transcriptional complex that drives the expression of this gene (left and right panels).

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detail. Interestingly, in the mouse PC cells, KCM, MUC1-induced MRP1 expression was independent of PI3K/Akt pathway even though the pAkt was significantly higher in the KCM vs KCKO cells. These data underscored the possibility of involvement of an alternative mechanism involved in MUC1-induced MRP1 expression (Figure 6). Thus, we report for the first time that two alternate mechanisms may be involved in MUC1-induced MRP1 expression in PC cells (Figure 7).

Interestingly, we found a strong association between MUC1 CT and the promoter region of the *ABCC1/Abcc1* gene (Figure 6). These preliminary data raise a possibility that MUC1 might be part of the transcriptional complex that regulates expression of the *ABCC1/Abcc1* gene. The 5′-untranslated promoter region of the human *ABCC1* gene contains several putative binding sites, such as GC elements (− 91 to + 103) that bind Sp1, AP1 sites (− 511 to − 492) that bind a complex of cJun/cFos and E box elements (− 1020 to − 2008) that bind N-myc.[23–25] We found MUC1 CT associating with the promoter region of the *ABCC1/Abcc1* gene within ChIP region I. Both mouse and human ChIP region I contain putative AP1, CREB1, GATA1, c-Ets1 and MZF1 binding motifs, as predicted by the transcription factor binding site prediction tools that uses TRANSFAC and JASPAR core databases (data not shown). MUC1 as such does not have a DNA-responsive domain, and studies so far have shown that it binds to DNA via transcription factors such as NF-κB, cJun, β-catenin and HIF-1α.[21,26,27] Thus, in future we intend to investigate in detail what MUC1 CT is doing at the promoter region of *ABCC1/Abcc1* gene and also the transcription factor that is involved in MUC1-mediated *MRP1* gene expression.

Taken together, our study shows that, in PC cells, MUC1 overexpression leads to chemoresistance, and that MUC1 CT associates directly with the promoter region of the *ABCC1/Abcc1* gene. Thus, the data provide new insights into the mechanisms by which MUC1 can interfere with the effectiveness of chemotherapy in PC. As MUC1 acts as a vital component that minimizes the efficacy of chemotherapy, it could be considered as a key molecular target for sensitizing cancer cells to conventional or novel treatments. The CT of MUC1 can be targeted to inhibit its ability to initiate signaling cascades, and also to block its nuclear translocation and subsequent binding to the promoter regions of its target genes. MDR modulators did not gain much popularity in the clinic owing to their ability to regulate more than one transporter and subsequently causing severe side effects in patients.[6] As an alternative strategy, MUC1 CT can be targeted to downregulate the expression of *mdr* genes or the activity of these efflux pumps.

MATERIALS AND METHODS

Cell culture and establishment of stable cell lines expressing MUC1

BxPC3 cells (American Type Culture Collection, Manassas, VA, USA) are a human PC cell line that express very little endogenous MUC1. For retroviral infection, GP2-293 packaging cells (stably expressing the gag and pol proteins) were co-transfected with the full-length MUC1 construct or an empty vector expressing the VSV-G envelope protein as previously described.[20,28] Cells were treated with 0.5 mg/ml of G418, beginning 48 h post infection. Three independent infections of the constructs were carried out with similar results. Expression of the constructs was stable throughout the span of experiments. Cells infected with vector alone were used as control and designated Neo. For MUC1-infected cells, MUC1-positive cells were sorted using the FACS Aria (BD Biosciences, San Jose, CA, USA). For Neo-infected cells, MUC1-negative cells were sorted. Capan-1 is a human PC cell line that expresses high levels of endogenous MUC1.

Mouse model and mouse cell lines

In our laboratory, mice that develop spontaneous pancreatic ductal adenocarcinoma (PDA) were generated by mating the P48-Cre with the LSL-KRAS^{G12D} mice.[29] PDA mice were further mated with the MUC1.Tg mice (that express human MUC1) to generate PDA.MUC1 mice or with the Muc1 knockout mice to generate PDA.MUC1KO mice.[19,30] All these mice were on the C57/B6 background. Cell lines were generated from the primary tumors of PDA.MUC1 and PDA.Muc1 KO mice, and were designated as KCM and KCKO, respectively.

Transient knockdown using siRNA

The method is previously described in Sahraei *et al.*[28] In brief, cells were seeded in a six-well plate and were allowed to reach 40% confluency. The cells were then transfected with 100 nm of MUC1 siRNA (Smart genome pool) or 100 nm of Akt siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA and Cell Signaling, Boston, MA, USA). Cells of the control group were treated with 100 nm of scrambled siRNA (Dharmacon, Thermo Fischer Scientific, CO, USA; Santa Cruz and Cell Signaling). Lipofectamine (Invitrogen, San Diego, CA, USA) was used for the delivery of siRNA into the cells over a period of 5–6 h in serum-free Opti-MEM. Forty-eight hours post transfection, MUC1 and Akt expression were evaluated by western blot. For MTT assay, 36 h post siRNA treatment, cells were trypsinized, re-plated in a 96-well plate and treated with or without the drugs. MTT assay was performed 24 h post drug treatment. The calculations were done as follows.

The viability of each treatment group without drug treatment (that is, WT alone, control siRNA alone and Akt siRNA alone) was considered as 100%. The viability following drug treatment on each of these treatment groups was calculated using the following expression:

% viability of drug-treated WT cells = (OD of drug-treated WT cells/OD of WT cells) × 100% viability of drug + control (or Akt) siRNA-treated cells = (OD of drug + control (or Akt) siRNA treated cells/OD of control (or Akt) siRNA treated cells) × 100

Preparation of nuclear extract

Cells were grown in a 10-cm plate. When the cells reached around 80% confluency, they were scraped off the plate, and a nuclear extraction kit (EMD Millipore, Billerica, MA, USA) was used to isolate the nuclear and cytosolic fractions.

Western blots

Equal quantities of cell lysates were loaded on SDS–PAGE gels. MUC1 antibodies were a gift from Dr Sandra Gendler. pAkt and Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), MRP-1, Lamin A/C and β-actin were purchased from Santa Cruz (CA, USA), and MEK1 was purchased from Abcam (Boston, MA, USA). The antibodies were used according to the manufacturer's recommendations.

MTT assay and H^3-thymidine incorporation assays

10×10^3 cells were plated in quadruplicate in normal growth medium in 96-well plates and were permitted to grow for 18 h. Cells were left untreated or treated with etoposide (Sigma-Aldrich, St Louis, MO, USA) and gemcitabine (Sigma-Aldrich) for 24 h. Next, MTT (Biotium) solution was added (10 μl per well) to the cells, incubated for additional 3–4 h. In the final step, media was removed, formazan was dissolved in DMSO (200 μl per well) and the absorbance was read on an ELISA plate reader.

For H^3-thymidine assay, 5×10^3 cells were plated and treated as described above. Twenty-four hours post drug treatment, H^3-thymidine (Perkin Elmer, Waltham, MA, USA) was added in fresh medium (1 μCi per well), and cells were permitted to grow for another 24 h. At this time, cells were washed to remove excess radioactivity, trypsinized and harvested onto a filter plate, which was then read on a TopCount plate reader. The data have been represented as % difference in H^3-thymidine uptake, which represents the % decrease in proliferation or % in growth arrest. The following formula was used for calculations:

% difference in H^3-thymidine uptake = ((c.p.m. untreated − c.p.m. treated)/c.p.m. untreated × 100)

Semiquantitative RT-PCR

Total RNA was extracted from cells by TRIzol (Invitrogen) according to the manufacturer's protocol. One to two micrograms of the extracted RNA was used as template for RT-PCR reaction (Access quick RT-PCR kit, Promega, Madison, WI, USA). Sequence of the primers is available upon request.

Immunohistochemistry

BxPC3 Neo and MUC1 cells (1×10^6 cells per mouse) were implanted subcutaneously in nude mice, and 30 days later tumors were collected for immunohistochemical analysis and protein lysate as described

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previously.[20] In brief, paraffin-embedded blocks of formalin-fixed tumor sections were made by the Histology Core at Mayo Clinic. Four-micron-thick sections were prepared for immunohistochemical staining. MRP1 expression in the tumor was determined using anti-MRP1 antibody (1:50 dilution, Santa Cruz) followed by appropriate secondary antibody (1:100 dilution, Dako).

Slides were examined under a light microscope and pictures were taken at $\times 20$.

Chromatin immunoprecipitation

Cells grown to near 80% confluence were cross-linked with formaldehyde (Sigma) at room temperature for 10 min. Cross-linked chromatin prepared with a commercial ChIP assay kit (EZ-Magna ChIP; Millipore) was immunoprecipitated with 20 μg of normal Armenian hamster IgG (Santa Cruz Biotechnology, CA, USA) and 20 μg of anti-MUC1 CT antibody (CT2). MUC1 CT binding site on the *ABCC/Abcc1* promoter was amplified by PCR using the input DNA (1%) or DNA isolated from precipitated chromatin as templates and using primers flanking the promoter region 1000 bp upstream (ChIP region I) and 2000 bp upstream (ChIP region II) of *ABCC1/Abcc1* gene (Figure 6b). ChIP region II was used as a negative control for binding of MUC1 CT to the promoter region. Sequence of the primers is available upon request.

Statistical analysis

Statistical analysis was performed with GraphPad software, La Jolla, CA, USA.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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