

Identification of Candidate Biomarkers of Therapeutic Response to Docetaxel by Proteomic Profiling

Liangli Zhao,¹ Brian Y. Lee,¹ David A. Brown,³ Mark P. Molloy,⁴ Gavin M. Marx,⁵ Nick Pavlakis,⁵ Michael J. Boyer,⁶ Martin R. Stockler,⁶ Warren Kaplan,² Samuel N. Breit,³ Robert L. Sutherland,¹ Susan M. Henshall,¹ and Lisa G. Horvath^{1,6}

¹Cancer Research Program and ²Peter Wills Bioinformatics Centre, Garvan Institute of Medical Research; ³Centre for Immunology, St. Vincent's Hospital; ⁴Department of Chemistry and Biomolecular Science, Australian Proteome Analysis Facility, Macquarie University; ⁵Royal North Shore Hospital; ⁶Sydney Cancer Centre, Royal Prince Alfred Hospital, Sydney, Australia

Abstract

Docetaxel chemotherapy improves symptoms and survival in men with metastatic hormone-refractory prostate cancer (HRPC). However, ~50% of patients do not respond to Docetaxel and are exposed to significant toxicity without direct benefit. This study aimed to identify novel therapeutic targets and predictive biomarkers of Docetaxel resistance in HRPC. We used iTRAQ-mass spectrometry analysis to identify proteins associated with the development of Docetaxel resistance using Docetaxel-sensitive PC3 cells and Docetaxel-resistant PC3-Rx cells developed by Docetaxel dose escalation. Functional validation experiments were performed using recombinant protein treatment and siRNA knockdown experiments. Serum/plasma levels of the targets in patient samples were measured by ELISA. The IC₅₀ for Docetaxel in the PC3-Rx cells was 13-fold greater than the parent PC-3 cell line ($P = 0.004$). Protein profiling identified MIC-1 and AGR2 as respectively up-regulated and down-regulated in Docetaxel-resistant cells. PC-3 cells treated with recombinant MIC-1 also became resistant to Docetaxel ($P = 0.03$). Conversely, treating PC3-Rx cells with MIC-1 siRNA restored sensitivity to Docetaxel ($P = 0.02$). Knockdown of AGR2 expression in PC3 cells resulted in Docetaxel resistance ($P = 0.007$). Furthermore, increased serum/plasma levels of MIC-1 after cycle one of chemotherapy were associated with progression of the cancer ($P = 0.006$) and shorter survival after treatment ($P = 0.002$). These results suggest that both AGR2 and MIC-1 play a role in Docetaxel resistance in HRPC. In addition, an increase in serum/plasma MIC-1 level after cycle one of Docetaxel may be an indication to abandon further treatment. Further investigation of MIC-1 as a biomarker and therapeutic target for Docetaxel resistance in HRPC is warranted. [Cancer Res 2009;69(19):7696–703]

Introduction

Prostate cancer remains the third most common cause of cancer death in men in the developed world (1). As prostate cancer progresses it becomes refractory to hormone manipulation. Docetaxel chemotherapy offers both symptomatic and survival benefits in men with metastatic hormone-refractory prostate

cancer (HRPC; refs. 2, 3); however, only 48% to 50% of men treated with Docetaxel have a prostate-specific antigen (PSA) response with a >50% decrease in their serum PSA. A high proportion of patients experience significant toxicity with 42% experiencing nausea, vomiting, or both, 30% any grade of sensory neuropathy and 26% ≥ 1 serious adverse event (2). Clearly, it would be ideal to know before or early in the treatment schedule which patients will respond to Docetaxel to avoid use of a toxic drug in an elderly population. Furthermore, identifying new pathways of resistance to Docetaxel may result in new therapeutic options.

Several pathways have been implicated in Docetaxel resistance including the Clusterin (4), Bcl-2 (5), Stat1 (6), sphingosine kinase-1 (7), PIM1 kinase (8), IL-6 (9), and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways (10). Anti-sense oligonucleotide inhibition of Bcl-2 (11), inhibition of PI3K signaling with CCI-779, mammalian target of rapamycin inhibitor (10), and sphingosine kinase-1 inhibition with B-5354c (7) have all resulted in improved tumor kill in HRPC xenograft models. However, a phase II trial of Oblimersen Sodium, an antisense inhibitor of Bcl-2, and Docetaxel in HRPC resulted in response rates comparable with, but not better than, those seen in the landmark Docetaxel randomized control trials (2, 3, 5). As yet no drug has made a clinical impact on Docetaxel resistance.

Identifying predictive biomarkers in men with HRPC is complex. The evolution to metastatic HRPC is the result of many changes in the biology of the cancer (12) and may develop 10 years or more after the original diagnosis. Therefore, tissue from the original cancer many years earlier does not reflect what the cancer becomes in its more advanced stage. Men with HRPC do not have tumor tissue that is easily accessible for rebiopsy as 80% have bone metastases only. Plasma/serum biomarkers are the practical alternative for this patient group and the strategy most likely to result in direct translation to the clinical setting. Serum IL-6 levels have been associated with Docetaxel response (9) but there are no plasma/serum biomarkers used in clinical practice. The aim of this study was to identify potential plasma/serum biomarkers that might predict response to Docetaxel and assess the functional role of these molecules in Docetaxel resistance *in vitro*.

Materials and Methods

Cell culture and selection of Docetaxel-resistant clones. The human androgen-independent prostate carcinoma cell line, PC3, was obtained from American Type Culture Collection and was maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) with 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were initially cultured in 1 ng/mL Docetaxel and maintained until the Docetaxel-sensitive clones died. The surviving PC3 cells repopulated the flask and continued to divide through four passages. This process was repeated using a Docetaxel concentration of 5 ng/mL and

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Lisa Horvath, Sydney Cancer Centre, Royal Prince Alfred Hospital, Missenden Road, Camperdown, NSW 2050, Australia. Phone: 612-9515-7680; Fax: 612-9519-1546; E-mail: lisa.horvath@sswahs.nsw.gov.au.

©2009 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-08-4901

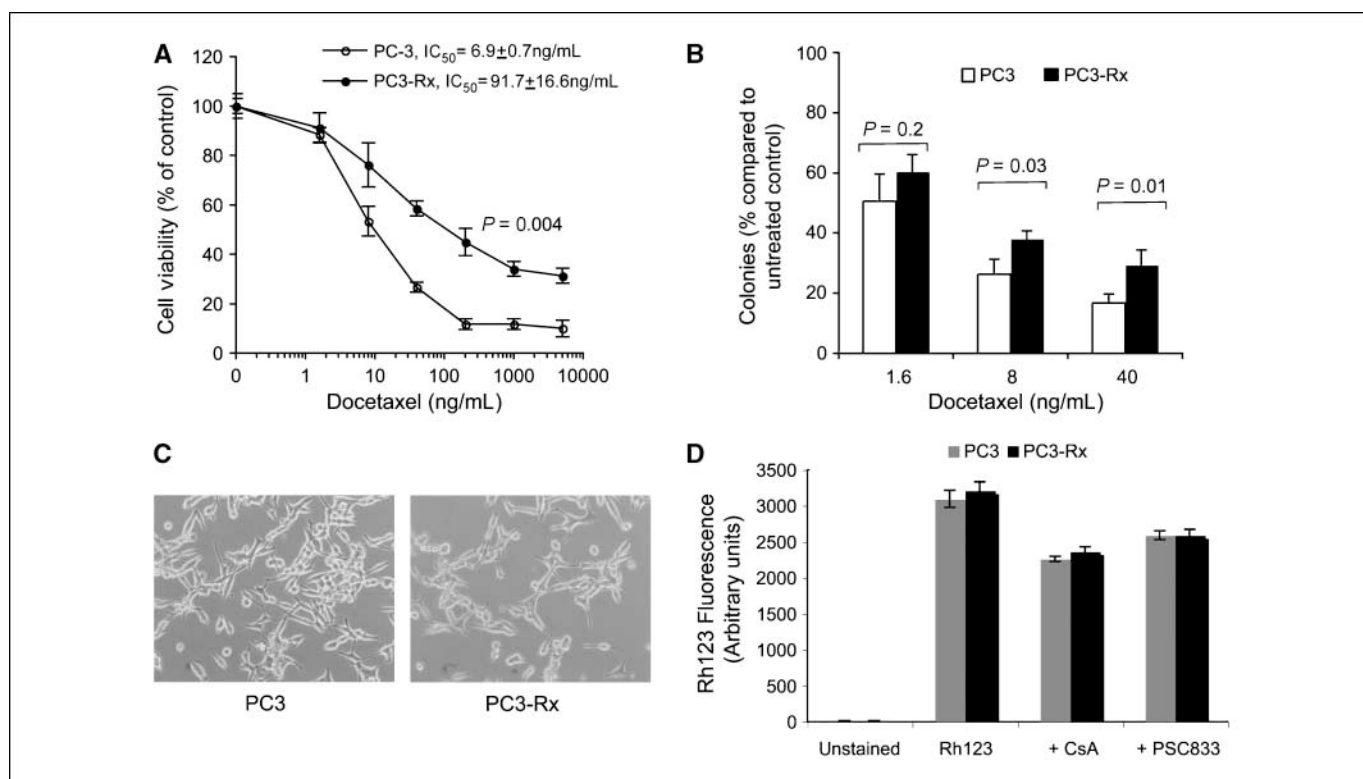


Figure 1. Characterization of PC3-Rx, a Docetaxel-resistant HRPC cell line. **A**, dose response curve assessing cell viability (trypan blue assay) in PC3 and PC3-Rx cells after Docetaxel treatment. **B**, clonogenic assay assessing the differences between PC3 and PC3-Rx cells in the presence of Docetaxel. **C**, morphology of PC3 and PC3-Rx cells detected by phase contrast microscopy. **D**, the accumulation of Rh123 in PC3 and PC3-Rx cells \pm Cyclosporin A (1 mmol/L) or PSC833 (1 mmol/L). Columns, mean of three independent experiments; bars, SEM.

subsequently 10 ng/mL. Once PC3 cells were freely dividing in 10 ng/mL Docetaxel medium, they were considered resistant and labeled PC3-Rx.

Trypan blue cell viability assay. To establish cytotoxicity profiles of Docetaxel, the number of cells surviving the drug treatment was determined by the trypan blue exclusion assay (13). Cells were plated into T25 flasks at 1×10^5 cells/flask. Twenty-four hours later, spent medium was replaced with fresh medium containing Docetaxel at different concentrations (0, 1.6, 8, 40, 200, 1,000, and 5,000 ng/mL). Treated cells were harvested with trypsin/EDTA at 48 h, stained with trypan blue, and counted using a hemocytometer. Cell counts were performed in triplicate for each time point and drug concentration. The cell survival curve was presented as the percentage of surviving cells versus the concentration of Docetaxel. IC_{50} values were defined as the concentration of drug required for 50% cell survival. This was calculated by analysis of the data in Excel using a logistic equation with data fitted using the Levenberg Marquardt algorithm. This experiment was repeated on three separate occasions and the results are expressed as the means \pm SEM.

Clonogenic assays. The cells were plated into six-well plates for 48 h, before the addition of Docetaxel (1.6, 8, 40 ng/mL). After 1 h of incubation, the drug-containing medium was removed and replaced with normal medium. Medium was changed every 3 d for 7 to 10 d until visible colonies formed. Colonies were fixed and stained with 0.5% crystal violet in methanol. Individual stained colonies in each well were visualized by the Bio-Rad Chemidoc system and counted by QuantityOne (Bio-Rad).

Rhodamine accumulation assay. PC3 and PC3-Rx cells were plated at 5×10^5 cells per well into 24-well plates for 24 h. The medium was then replaced with medium containing either vehicle, Cyclosporin A (1 mmol/L), or PSC833 (1 mmol/L; Novartis) and allowed to incubate for 10 min at 37°C before the addition of Rh123 (1 mmol/L; Invitrogen Australia). A final concentration of 0.15% (v/v) ethanol was used for all experiments and controls. Cells were incubated in the dark for 60 min at 37°C, then transferred to ice and maintained at 0°C while they were harvested using

trypsin-EDTA, and analyzed on a FACScan flow cytometer with a 488-nm argon laser using CellQuest™ software (BD). Rh123 fluorescence was measured by a 530-nm band-pass filter and propidium iodide fluorescence measured with a 585-nm band-pass filter. Gates were set to exclude propidium iodide-positive cells and clumps and debris on the basis of forward and side scatter.

iTRAQ-mass spectrometry analysis. Sample preparation, mass spectrometry, and peptide identification were performed as described in (14). Cells were lysed in ice cold lysis buffer [150 mmol/L NaCl, 20 mmol/L HEPES (pH 7.5), 0.15% SDS, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 protease inhibitor cocktail 1 tablet; Roche] to a final concentration of 2 mg/mL. One hundred micrograms of protein for each sample was reduced, alkylated, and enzymatically cleaved using trypsin, then derivatized using iTRAQ 4-plex reagent kit (Applied Biosystems) according to the manufacturer's protocol (14). The iTRAQ-labeled samples were mixed and separated by strong cation exchange chromatography, followed by nanoLC/MS/MS using a QSTAR XL mass spectrometer (Applied Biosystems). Peptides were identified and quantitated from MS/MS data using ProteinPilot V1.0 (Applied Biosystems). All reported data were based on 95% confidence for protein identification as determined by ProteinPilot (ProtScore > 1.3). A further requirement was for a protein P value, which ensured that protein identification and quantitation was based on more than a single peptide hit.

Data analysis. To identify secreted proteins, all data in the July 2006 version of the Secreted Protein Databank (SPD) were downloaded and the associated SwissProt IDs for peptides identified by mass spectrometry were matched to the same University of California at Santa Cruz Table Browser file (Human Assembly March 2006). For all matches, the SPD ranks (0–3) were recorded. These data were then combined in an Excel spreadsheet for analysis of candidate proteins based on SPD rank and proteomic fold-change.

Western blotting analysis. Primary antibodies used were anti-MIC-1 (R&D Systems) and anti-AGR2 (Abnova Corporation). Secondary antibodies were protein G-HRP (Zymed) or antimouse IgG (GE Healthcare UK). The cells were lysed with a radioimmunoprecipitation assay lysis buffer with protease inhibitors (1.0 mmol/L Sodium orthovanadate, 1.0 mmol/L NaF, 1.0 mmol/L PMSE, 0.1 μ mol/L Aprotinin, and 10 μ mol/L Leupeptin). Cell lysates were centrifuged at 14,000 rpm 4°C for 6 min and the protein concentration of the soluble extracts determined using the Bio-Rad (Bradford) protein assay (Bio-Rad). For collection of conditioned media, PC3 and PC3-Rx cells were seeded at a density of 1×10^6 cells in T-75 flasks in RPMI with 10% FBS for 24 h. The media were replaced, the cells incubated for 72 h, and media then collected, centrifuged, and stored at -80°C. Separation of 30 μ g of total protein was performed on 12% acrylamide gels, and transferred to a nitrocellulose membrane before immunoblotting with the relevant primary antibodies. The equal loading of protein sample was verified with β -actin or glyceraldehyde-3-phosphate dehydrogenase-specific antibodies (Sigma).

Recombinant MIC-1 experiments. PC3 cells were treated with human recombinant MIC-1 at 0.01 ng/mL (rMIC-1; R&D Systems) for 72 h, before the exposure to Docetaxel. A dose response curve was established using increasing concentrations of Docetaxel (0, 1.6, 8, 40, 200, 1,000, and 5,000 ng/mL) as described earlier. A clonogenic assay was also performed by treating the cells with 5 ng/mL Docetaxel for 1 h then replacing the medium with fresh nondrug containing medium and allowing colonies to grow over 7 to 10 d as described earlier.

Small interfering RNA transfection. siGenome SMARTpool small interfering RNA (siRNA) against MIC-1 or AGR2, and siCONTROL NonTargeting siRNA were obtained from Dharmacon Research, Inc. The nonsilencing control siRNA, which has no sequence homology to any known human gene sequence, was used as a control for nonsequence-specific effects in all experiments. Cells were transfected with siRNA by electroporation using Nucleofector Technology (Amaxa Biosystems) following the manufacturer's instructions. The optimal amount of siRNA used for transfection was determined empirically for each cell line and the lowest siRNA concentration that gave effective silencing of the targeted protein and caused minimal stress to the cells was used in all subsequent experiments. Control experiments were done in parallel by transfecting the cells with the control siRNA at equivalent concentrations as the

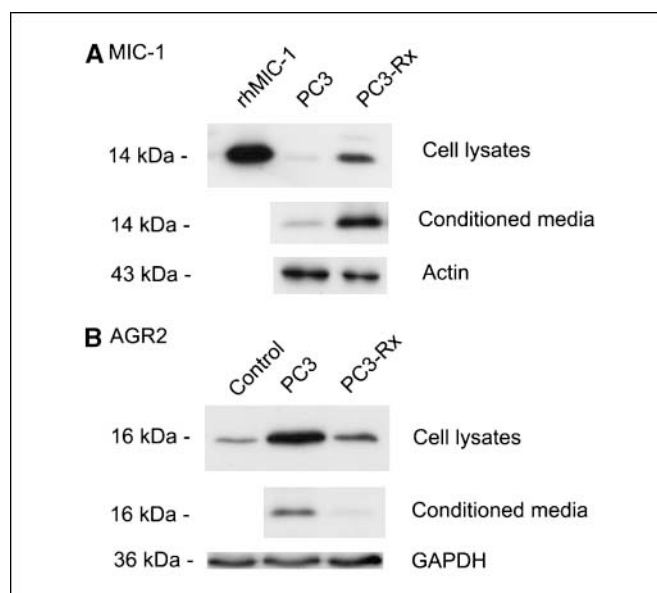


Figure 2. MIC-1 (A) and AGR2 (B) protein expression in the cell lysates and conditioned media of PC3-Rx cells and PC-3 cells by Western blotting.

target siRNA. The efficacy of the siRNA knockdown was assessed by immunoblotting. The effect of MIC-1 and AGR2 knockdown on the Docetaxel sensitivity of PC3-Rx and PC3 cells, respectively, was assessed using increasing concentrations of Docetaxel (0, 1.6, 8, 40, 200, 1,000, and 5,000 ng/mL) as described earlier to produce a dose response curve. Given a small inhibitory effect from the control siRNA and the transfection technique, all analyses were performed comparing the control siRNA to the targeted siRNA conditions.

To assess the synergy between the different pathways that enhance Docetaxel resistance, PC3 cells were treated with (a) siRNA control, (b) rhMIC-1 at 0.01 ng/mL, (c) siRNA targeted to AGR2, or (d) rhMIC-1 0.01 ng/mL + AGR2 siRNA. The cells were then treated with Docetaxel at 40 ng/mL for 48 h at which time cell viability was assessed by the Trypan Blue assay.

Patient samples. Blood samples for biomarker analysis were obtained from 43 men with metastatic HRPc precycle and postcycle one of chemotherapy. Serum samples were obtained from 28 patients treated with Docetaxel and PI-88, an heparanase inhibitor, on a clinical trial (15). Plasma samples were obtained from 15 patients receiving single agent chemotherapy (13 Docetaxel, 2 Mitoxantrone). The serum/plasma concentrations of MIC-1 (pg/mL) were analyzed using a previously described sensitive immunoassay (16). Serum PSA levels were recorded before each cycle of treatment. Patients had a baseline PSA level of ≥ 10 ng/mL. PSA response (partial response, PR) was defined as a 50% or greater decrease in serum PSA compared with the last value assessed before initiation of chemotherapy (2, 3). PSA progression (progressive disease, PD) was defined as an increase in PSA value by 25% over the baseline, and stable disease (SD) was defined as any change in PSA of $\geq 50\%$ decrease or $\leq 25\%$ increase over baseline (2). Confirmatory PSA levels were not required to define a response. Overall survival was defined as the time from commencement of chemotherapy to the time of death or last follow-up. Prospective Eastern Cooperative Oncology Group performance status assessment was available on 28 patients all of whom were performance status of 0 or 1 (17).

Statistical analysis. An unpaired Student's *t* test was used for statistical analysis of comparative *in vitro* data that were expressed as means of at least three independent experiments \pm SEM. The dose response curves were compared using a multiple regression of the percentage of viable cells according to the cell line/cell treatment adjusted for the Docetaxel concentration. As serum MIC-1 levels were not normally distributed, nonparametric methods (Mann-Whitney *U* and Kruskal-Wallis) were used to determine the relationship between the serum/plasma levels of MIC-1 and PSA response to chemotherapy. A Kaplan-Meier analysis and log-rank

Table 1. The characteristics of the secreted proteins with differential expression in PC3-Rx cells compared with PC3 cells

Gene symbol	Protein name	Fold change	Peptides	SPD* rank
MIC-1	Macrophage inhibitory cytokine 1	2.4	8	0
A1BG	α -1B-glycoprotein1 precursor	1.9	1	0
VTNC	Vitronectin precursor	1.7	2	0
RNPEP	Arginyl aminopeptidase	1.6	2	0
FETUA	Fetuin A	1.6	118	0
CYR61	Angiogenic inducer, 61	-1.5	1	0
MASPIN	Serpin peptidase inhibitor	-1.5	1	0
AGR2	Anterior gradient 2 homologue	-2.4	8	0

*A Web-based secreted protein database. Rank0: known secreted proteins.

statistic were used to assess the association between increased MIC-1 levels and overall survival. A Cox Proportional Hazards model was used for bivariate analyses assessing the relationship between change in MIC-1 levels, age, and performance status. A *P* value of <0.05 was required for significance. All reported *P* values are two sided. All statistical analyses were performed using Statview 4.5 software (Abacus Systems).

Results

Identification of novel proteins associated with Docetaxel resistance. The Docetaxel-resistant cell line, PC3-Rx, was developed from the Docetaxel-sensitive, androgen-independent prostate cancer cell line, PC3. The IC₅₀ for Docetaxel in the PC3-Rx cells was 13-fold higher than that in parental PC-3 cells (*P* = 0.004; Fig. 1A). Clonogenic assays also showed significant Docetaxel resistance in the PC3-Rx compared with PC3 cells (Fig. 1B). Despite changes in Docetaxel sensitivity, the morphology (Fig. 1C) and growth rate of the PC3 and PC3-Rx cells were similar (Supplementary Fig. S1). There was no difference in Rh123 accumulation between PC3 and PC3-Rx cells, with/without P-glycoprotein inhibitor, Cyclosporin A, or PSC833 (Fig. 1D). This showed that Docetaxel resistance was not due to drug efflux.

Proteomic profiling was used in the discovery phase to identify potential targets involved in Docetaxel resistance. A single lysate sample of each of the PC3-Rx and PC3 cells were profiled using iTRAQ mass spectrometry. This process identified and quantitated ~1,100 proteins (Supplementary Table S1). Data mining identified secreted proteins with a fold change >1.5, as these proteins would be the most likely to be found in plasma. Based on the fold change criteria, 50 proteins were up-regulated and 35 proteins down-

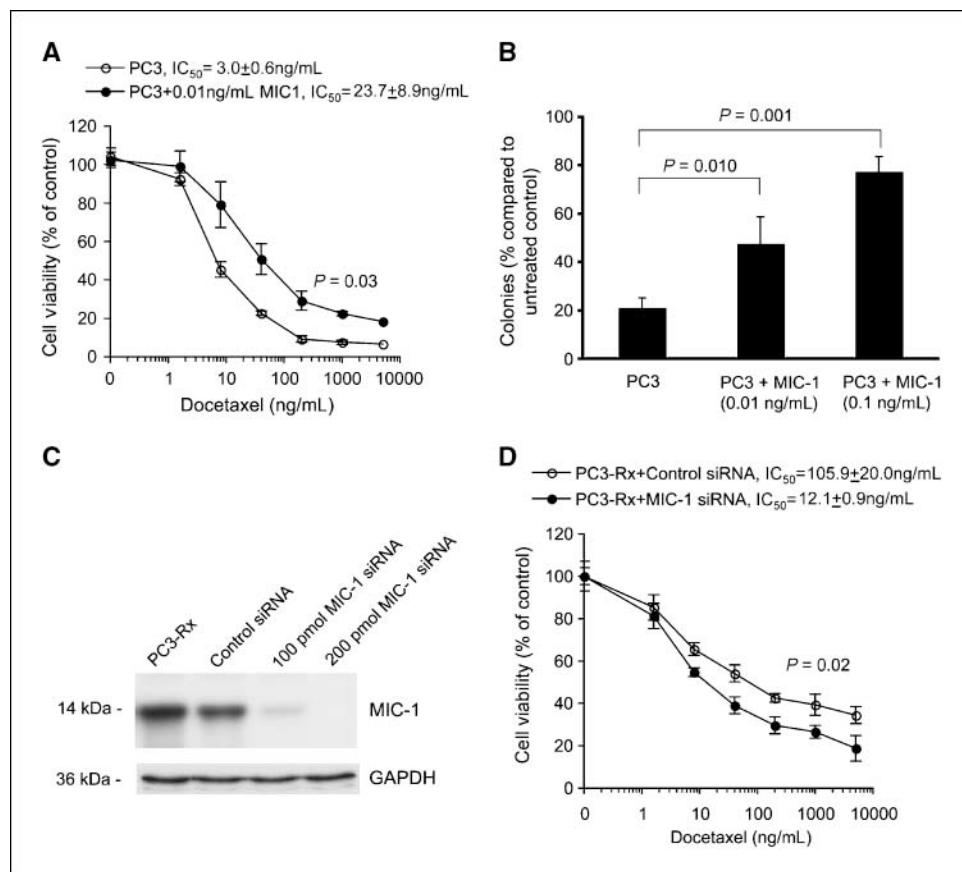
regulated in PC3-Rx cells compared with PC3 cells (Supplementary Fig. S2), but only 7 of these proteins were secreted (Table 1). MIC-1 and AGR2 were the top ranked secreted protein targets (Table 1).

Validation of protein expression. To verify that the candidate proteins were selectively up-regulated or down-regulated in drug-resistant cells, we analyzed protein levels in cell lysates and conditioned media. This confirmed that MIC-1 was expressed at low levels in PC-3 cells and markedly overexpressed in resistant PC3-Rx cell lysates and conditioned media (Fig. 2A). Furthermore, AGR2 was expressed at lower levels in PC3-Rx cells compared with PC-3 cells (Fig. 2B).

MIC-1 and Docetaxel resistance. To delineate a potential functional role of MIC-1 in chemoresistance, Docetaxel-sensitive PC-3 cells were treated with recombinant human MIC-1 (rhMIC-1) at 0.01 ng/mL for 72 hours. This resulted in the PC-3 cells becoming relatively resistant to Docetaxel with an 8-fold increase in the IC₅₀ (*P* = 0.03; Fig. 3A). Clonogenic assays also showed a significant increase in Docetaxel resistance when PC3 cells were treated with MIC-1 (*P* = 0.001; Fig. 3B). Moreover, in the clonogenic assays, there was increased Docetaxel resistance with increasing concentrations of the MIC-1. These results suggest that exogenous MIC-1 promotes Docetaxel-resistance in PC3 cells. MIC-1-targeted siRNA were used to knockdown MIC-1 expression in PC3-Rx cells by 15-fold (Fig. 3C). Cell viability assays showed that MIC-1 siRNA-treated, PC3-Rx cells were significantly more sensitive to Docetaxel treatment than control siRNA-treated PC3-Rx cells with an 8-fold decrease in the IC₅₀ (*P* = 0.02; Fig. 3D).

AGR2 and Docetaxel resistance. The role of AGR2 in Docetaxel resistance was assessed using siRNA targeted to AGR2. AGR2-targeted

Figure 3. Functional validation of MIC-1. **A**, dose response curve assessing the effect of rhMIC-1 treatment on PC3 cells after Docetaxel treatment (trypan blue cell viability assay). **B**, clonogenic assay demonstrating the effect of rhMIC-1 treatment on PC3 cells treated with Docetaxel. **C**, Western blot confirmation of MIC-1 knockdown in PC3-Rx cells by siRNA targeting MIC-1. **D**, dose response curve assessing the effect of MIC-1 knockdown on PC3-Rx cells in the presence of increasing doses of Docetaxel (trypan blue assay). Points, mean of three independent experiments; bars, SEM.



siRNA knocked down AGR2 expression in PC3 cells by 10-fold (Fig. 4A). Cell viability assays showed that AGR2 siRNA-treated, Docetaxel-sensitive PC3 cells developed resistance to Docetaxel compared with PC3 cells treated with control-siRNA with a 5-fold increase in the IC_{50} ($P = 0.007$; Fig. 4B).

To assess for synergy between AGR2 and MIC-1, PC3 cells were treated with either AGR2-siRNA or rhMIC-1 or both and Docetaxel sensitivity assessed. All three conditions resulted in relative Docetaxel resistance compared with parental PC3 cells (Fig. 5C). There was a nonsignificant trend toward increased Docetaxel

resistance in the AGR2-siRNA, rhMIC-1-treated PC3 compared with either of the treatments alone ($P > 0.05$; Fig. 4C).

Serum/plasma MIC-1 levels predict for Docetaxel resistance.

Plasma/serum samples were collected from a cohort of 43 men with metastatic HRPc treated with chemotherapy (13 Docetaxel alone, 28 Docetaxel/PI-88, 2 Mitoxantrone). Docetaxel/PI-88 treatment was part of a phase II clinical trial in which this combination was found to be comparable with Docetaxel alone (15). Given a previous study implicating MIC-1 in Mitoxantrone resistance (18), patients treated with this drug were also included. Of the 43 patients, 26 (60%) had PR, 10 (23%) had SD, and 7 (17%) had PD as best response to treatment. The median follow-up was 52 weeks (range, 7–123 weeks) with 32 of 43 (74%) dead of prostate cancer.

The median pretreatment MIC-1 level was 5,525 pg/mL (range, 730–65,305 pg/mL). Men with a performance status of 0 had a significantly lower pretreatment MIC-1 level compared with men with performance status 1 (mean pretreatment MIC-1 level, 2,855 versus 9,214; $P = 0.01$). This is consistent with data demonstrating that weight loss is associated with elevated MIC-1 levels (19), a factor often associated with poorer performance status. There was no relationship between the pretreatment MIC-1 level and response to treatment ($P = 0.6$).

Of the original cohort, 38 patients (28 Docetaxel/PI-88, 8 Docetaxel alone, 2 Mitoxantrone) had paired samples pre-chemotherapy and postchemotherapy. The mean change in serum/plasma MIC-1 after the first dose of chemotherapy was associated with response to treatment ($P = 0.02$; Fig. 5A) with a correlation between elevated MIC-1 levels after initial treatment and resistance to therapy ($P = 0.006$; Fischer's Exact test). All men who had PD despite treatment had elevated MIC-1 levels after cycle one of treatment (Fig. 5B). Exclusion of the two men treated with Mitoxantrone did not alter the results (data not shown). An increase of $\geq 140\%$ in MIC-1 levels after cycle 1 of chemotherapy was associated with a shorter overall survival (Fig. 5C). Bivariate analysis showed that serum/plasma MIC-1 levels ($P = 0.005$) could predict poorer survival independent of performance status ($P = 0.2$) and age ($P = 0.4$).

Discussion

In this study, protein profiling has successfully identified two secreted molecules that potentially mediate Docetaxel resistance and one serum/plasma protein biomarker that may predict for Docetaxel resistance in the clinical setting. This is the first article to show that down-regulation of AGR2 induces Docetaxel resistance in HRPc cells. Furthermore, it shows that elevated plasma/serum MIC-1 levels after initial Docetaxel treatment can predict for Docetaxel resistance in men with HRPc, indicating that further chemotherapy is not warranted and may be harmful.

AGR2, the human homologue of the *Xenopus laevis* cement gland protein, has been implicated in the progression and metastasis of numerous cancers. AGR2 was originally identified in a screening of genes differentially associated with estrogen receptor status in breast cancer cell lines (20). Subsequently, AGR2 was identified as an androgen-inducible secretory protein over-expressed in prostate cancer epithelium (21). Increased AGR2 is also associated with decreased survival in men with localized prostate cancer (22). Functionally, AGR2 expression promotes tumor growth, cell migration, and transformation *in vitro* (23, 24) and an increased rate of metastasis *in vivo* across a range of cancers (25), although no mechanism has yet been identified. Silencing of AGR2 in a pancreatic cell line improved sensitivity to the cytotoxic drug,

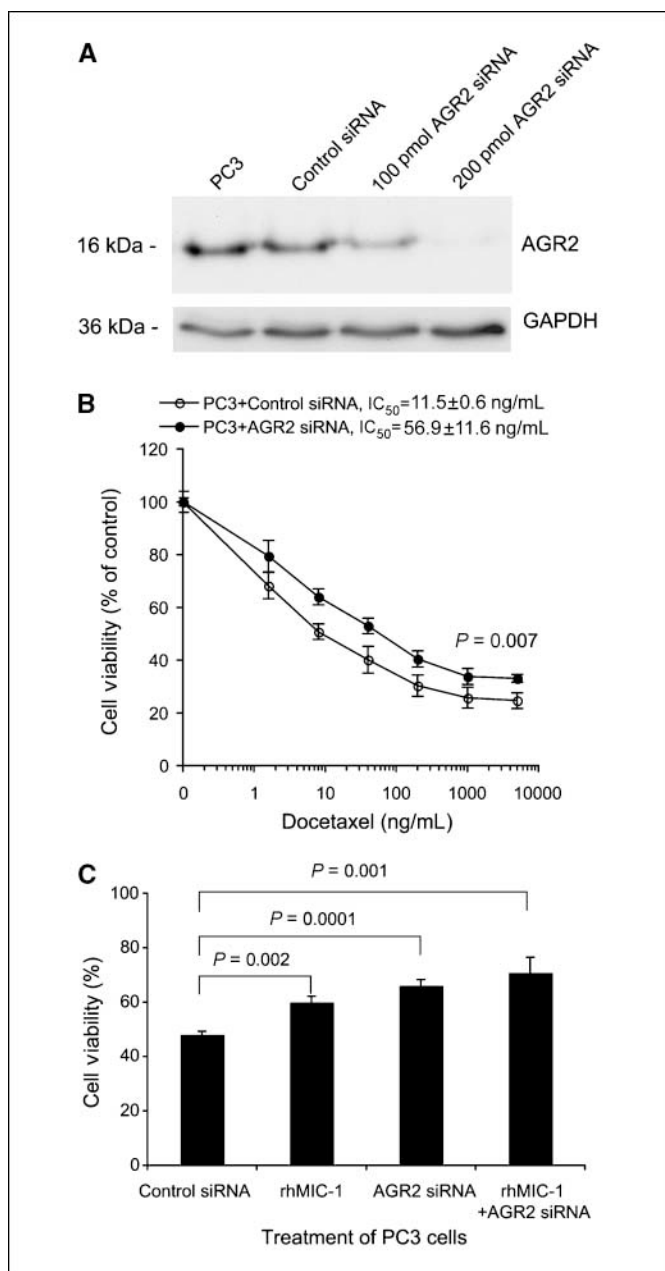


Figure 4. Functional validation of AGR2. **A**, Western blot confirmation of AGR2 knockdown in PC3 cells by siRNA directed against AGR2. **B**, dose response curve assessing the effect of AGR2 knockdown on PC3 cells treated with Docetaxel (trypan blue assay). **C**, interaction between AGR2 and MIC-1. PC3 cells were treated with control siRNA, rhMIC-1 at 0.01 ng/mL, siRNA targeted to AGR2 or rhMIC-1 0.01 ng/mL + AGR2-siRNA. Docetaxel sensitivity was determined by the trypan blue cell viability assay. Points, mean of three independent experiments; bars, SEM.

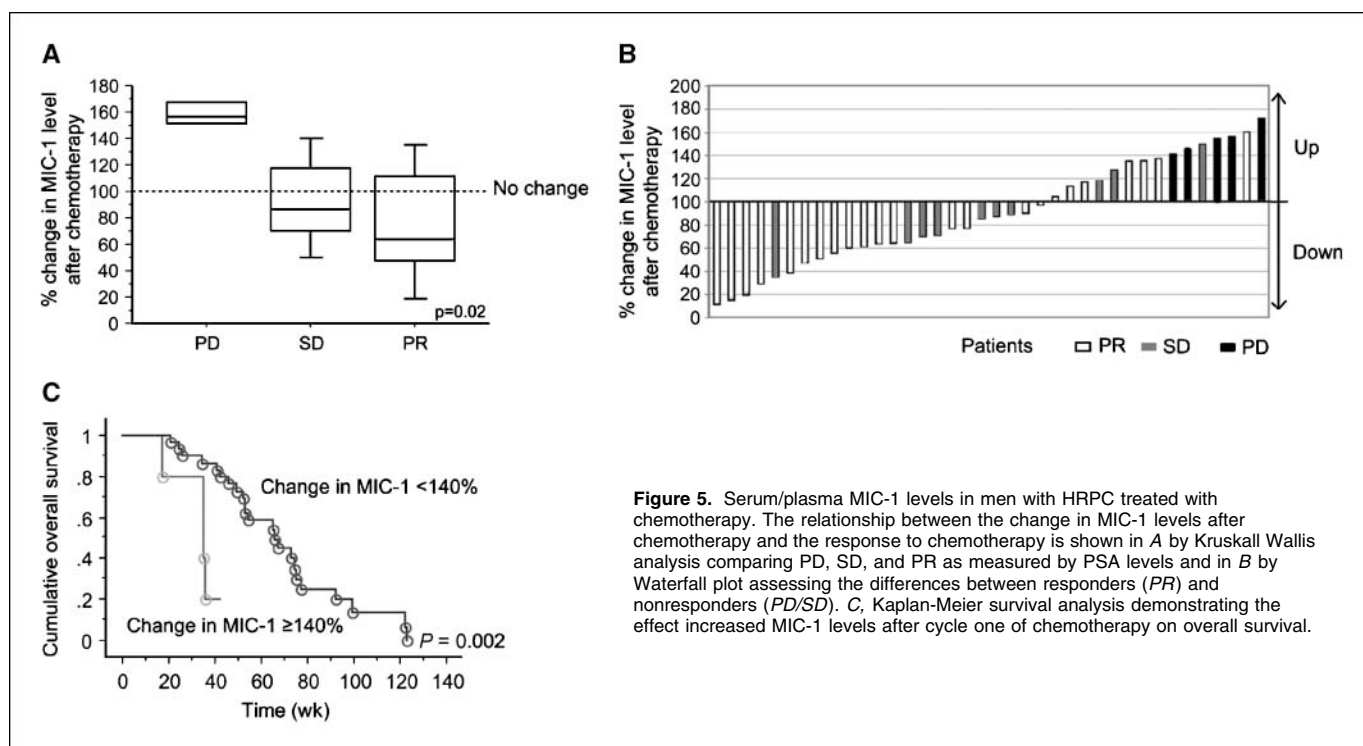


Figure 5. Serum/plasma MIC-1 levels in men with HRPc treated with chemotherapy. The relationship between the change in MIC-1 levels after chemotherapy and the response to chemotherapy is shown in A by Kruskal Wallis analysis comparing PD, SD, and PR as measured by PSA levels and in B by Waterfall plot assessing the differences between responders (PR) and nonresponders (PD/SD). C, Kaplan-Meier survival analysis demonstrating the effect increased MIC-1 levels after cycle one of chemotherapy on overall survival.

Gemcitabine (24). In contrast, our study found that decreased levels of AGR2 were associated with Docetaxel resistance.

The cytokine MIC-1, a member of the transforming growth factor (TGF) β family, has been implicated widely in prostate carcinogenesis and progression. MIC-1 expression is increased in precancerous prostatic intraepithelial neoplasia, with low MIC-1 expression in localized prostatic cancer predicting for increasing stage of disease (26). Furthermore, low levels of stromal expression of the unprocessed propeptide form of MIC-1 predict for biochemical relapse in localized cancer especially in tumors with Gleason scores of ≤ 6 (27). In the context of therapy, MIC-1 is overexpressed in colon cancer cells that become senescent after chemotherapy (28) and MIC-1 expression is elevated in localized breast and prostate cancer tissue after neoadjuvant Docetaxel (18, 29). Although these data imply that MIC-1 is being produced by the tumor cells in response to cytotoxic treatment, there is also data for a functional role for MIC-1 in drug resistance. Increased MIC-1 is associated with 5-fluorouracil resistance in colon cancer cell lines (30) and overexpression of MIC-1 conferred resistance to Docetaxel and Mitoxantrone in advanced prostate cancer (18). Our results are consistent with this but we also found that knock down of MIC-1 confers Docetaxel sensitivity. Furthermore, this is the first time that serum/plasma levels of MIC-1 have been associated with Docetaxel resistance in HRPc. If the changes in serum/plasma MIC-1 reflected tumor cell senescence, then one would expect that patients with a high level would have a better outcome; however, elevated levels of MIC-1 predict for both PD and shorter survival in this study.

The precise mechanism by which MIC-1 exerts its biological effects is still unclear. The activation of the p53 pathway induces MIC-1 expression that corresponds to activating p53 mutations in many cancers (31). High MIC-1 levels *in vitro* and *in vivo* are associated with apoptosis, cell cycle arrest, and antiangiogenic

activity consistent with antitumor activity (31). Conversely, MIC-1 treatment of prostate cancer cells also results in loss of cellular adhesion (32), and increased MIC-1 has been associated with the progression to androgen independence (33) and the formation of lytic bone metastases *in vivo* (34). Furthermore, higher MIC-1 expression in gastric cancer cell lines is associated with a more invasive phenotype suggesting MIC-1 may also have a protumorigenic role (35). These apparently contradictory effects are typical of the TGF β superfamily reflecting factors in the cancer and its environment and the concept that these molecules may have divergent effects at different stages of the disease. Our data are consistent with the more protumorigenic effect described in the advanced stages of metastatic prostate cancer. MIC-1-treated breast and gastric cancer cell lines activate Akt and extracellular signal-regulated kinase 1/2 via transactivation of ErbB2 (36). PI3K/Akt signaling has also been implicated in Docetaxel resistance (37) and is one potential explanation for how MIC-1 modulates Docetaxel resistance.

Although our data suggest that the functional and biomarker aspects of MIC-1 are linked, one could hypothesize that plasma MIC-1 levels increase in PD due to increased tumor burden. Serum MIC-1 levels have been linked to tumor burden in other studies (31, 38); however, in prostate cancer, serum levels of MIC-1 are an independent marker of higher-grade (Gleason sum 7) tumors (16, 39), not stage and lymph node spread, suggesting that these findings are not solely due to tumor burden (39). Furthermore, elevated serum MIC-1 levels predict for bone metastases (16) and cachexia due to prostate cancer (19) with *in vitro* and *in vivo* studies demonstrating the functional role of MIC-1 in these processes (19, 34). Given that the median survival of men with HRPc is 16 months without Docetaxel treatment (2), it is also inconsistent with the tumor growth characteristics that the tumor burden would increase $\geq 40\%$ in the 3 weeks between the

pretreatment and postcycle 1 blood sampling. There is significant evidence that serum MIC-1 reflects biological aspects of prostate cancer rather than simply tumor burden, which is consistent with our data suggesting it predicts, and also plays a biological role, in Docetaxel resistance.

Serum/plasma biomarkers that can predict for chemosensitivity early in treatment will significantly improve patient care for men with HRPc. This group of men is generally over 60 years of age, often with multiple medical comorbidities, and Docetaxel treatment has significant side effects while benefiting only ~50% of the patient group. There was considerable variance in the pretreatment MIC-1 levels most likely due to a number of factors such as tumor volume, bone metastases, and cachexia, which also affect serum MIC-1 level in HRPc. The finding in this study that the change in MIC-1 levels after the first cycle of treatment correlates with PSA response offers new hope for a predictive biomarker. The median time to PSA response in the TAX327 study was 44 days (range, 26–68 days), so most patients had two or more cycles of treatment before response status could be ascertained (40). Identifying response after one cycle of treatment (21 days) would prevent cumulative toxicity in those patients unlikely to benefit from treatment. Although a larger, more homogenous study population will be needed to validate these findings, these data provide new evidence for MIC-1 as a predictive biomarker.

In conclusion, AGR2 and MIC-1 both mediate Docetaxel resistance in HRPc cells *in vitro* and the correlative human data suggest MIC-1 may be a predictive serum/plasma biomarker for Docetaxel response in these patients. This offers the opportunity for therapeutic intervention against Docetaxel resistance by an anti-MIC-1 neutralizing antibody and/or pegylated peptide treatment with AGR2. The MIC-1 data provide the biological principles for the development of a plasma biomarker that could predict for early Docetaxel resistance and direct the appropriate therapeutic intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 12/23/08; revised 7/7/09; accepted 7/28/09; published OnlineFirst 9/22/09.

Grant support: Cancer Institute New South Wales, National Health and Medical Research Council, Hillcrest Foundation, and RT Hall Trust.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Richard Wykes, Jin Sagong, Anne-Maree Haynes, and Ruth Pe Benito for facilitating patient sample and data collection, Jim McBride for IT support, John Allen for assistance with the rhodamine assays, and Nasreen Yaghoutyfam and Rozelle Harvie for technical assistance.

References

- Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics. *CA Cancer J Clin* 2005;55:74–108.
- Tannock IF, de Wit R, Berry WR, et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 2004;351:1502–12.
- Petrylak DP, Tangen CM, Hussain MH, et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med* 2004;351:1513–20.
- Chi KN, Siu LL, Hirte H, et al. A phase I of OGX-011, a 2-methoxyethyl phosphorothioate antisense to clusterin, in combination with docetaxel in patients with advanced cancer. *Clin Cancer Res* 2008;14:833–9.
- Tolcher AW, Chi K, Kuhn J, et al. A Phase II, pharmacokinetic and biological correlative study of Oblimersen sodium and Docetaxel in patients with hormone-refractory prostate cancer. *Clin Cancer Res* 2005;11:3854–61.
- Patterson SG, Wei S, Chen X, et al. Novel role of Stat1 in the development of docetaxel resistance in prostate tumor cells. *Oncogene* 2006;25:6113–22.
- Pchejetski D, Doumerc N, Golzio M, et al. Chemosensitizing effects of sphingosine kinase-1 inhibition in prostate cancer cell and animal models. *Mol Cancer Ther* 2008;7:1836–45.
- Zemskova M, Sahakian E, Bashkirova S, Lilly M. The PIM1 kinase is a critical component of a survival pathway activated by Docetaxel and promotes survival of Docetaxel-treated prostate cancer cells. *J Biol Chem* 2008;283:20635–44.
- Domingo-Domenech J, Oliva C, Rovira A, et al. Interleukin 6, a nuclear factor- κ B target, predicts resistance to docetaxel in hormone-independent prostate cancer and nuclear factor- κ B inhibition by PS-1145 enhances docetaxel antitumor activity. *Clin Cancer Res* 2006;12:5578–86.
- Wu L, Birl DC, Tannock IF. Effects of the mammalian target of rapamycin inhibitor CCI-779 used alone or with chemotherapy on human prostate cancer cells and xenografts. *Cancer Res* 2005;65:2825–31.
- Vilenchik M, Raffo AJ, Benimetskaya L, Shames D, Stein CA. Antisense RNA down-regulation of bcl-xL expression in prostate cancer cells leads to diminished rates of cellular proliferation and resistance to cytotoxic chemotherapeutic agents. *Cancer Res* 2002;62:2175–83.
- Shah RB, Mehra R, Chinnaiyan AM, et al. Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res* 2004;64:9209–16.
- Makarovskiy AN, Stryaporn E, Hixson DC, Akerley W. Survival of docetaxel-resistant prostate cancer cells *in vitro* depends on phenotype alterations and continuity of drug exposure. *Cell Mol Life Sci* 2002;59:1198–211.
- Song X, Bandow J, Sherman J, et al. iTRAQ experimental design for plasma biomarker discovery. *J Proteome Res* 2008;7:2952–8.
- Marx G, Pavlakakis N, McCowatt S, et al. Phase 1/2 study of PI-88, a novel heparanase, in combination with Docetaxel in patients with hormone refractory prostate cancer (HRPC). *Proc Am Soc Clin Oncol GU* 2008; abstract 182.
- Selander KS, Brown DA, Sequeiros GB, et al. Serum macrophage inhibitory cytokine-1 concentrations correlate with the presence of prostate cancer bone metastases. *Cancer Epidemiol Biomarkers Prev* 2007;16:532–7.
- Cancer Therapy Evaluation Program. Common Toxicity Criteria, Version 2.0. Bethesda (MD): National Cancer Institute; 1998. p. 30.
- Huang C-Y, Beer TM, Higano CS, et al. Molecular alterations in prostate carcinomas that associate with *in vivo* exposure to chemotherapy: identification of a cytoprotective mechanism involving growth differentiation factor 15. *Clin Cancer Res* 2007;13:5825–33.
- Johnen H, Lin S, Kuffner T, et al. Tumor-induced anorexia and weight loss are mediated by the TGF- β superfamily cytokine MIC-1. *Nat Med* 2007;13:1333–40.
- Thompson DA, Weigel RJ. hAG2, the human homologue of the *Xenopus laevis* cement gland gene XAG-2, is co-expressed with estrogen receptor in breast cancer cell lines. *Biochem Biophys Res Commun* 1998;251:111–6.
- Zhang J-S, Gong A, Cheville JC, Smith DI, Young CYF. AGR2, an androgen-inducible secretory protein over-expressed in prostate cancer. *Genes Chromosomes Cancer* 2005;43:249–59.
- Zhang Y, Forootan SS, Liu D, Barraclough R, Foster CS, Ke Y. Increased expression of anterior gradient-2 is significantly associated with poor survival of prostate cancer patients. *Prostate Cancer Prostatic Dis* 2007;10:293–300.
- Wang Z, Hao Y, Lowe AW. The adenocarcinoma-associated antigen, AGR2, promotes tumor growth, cell migration, and cellular transformation. *Cancer Res* 2008;68:492–7.
- Ramachandran V, Arumugam T, Wang H, Logsdon CD. Anterior gradient 2 is expressed and secreted during the development of pancreatic cancer and promotes cancer cell survival. *Cancer Res* 2008;68:7811–8.
- Liu D, Rudland PS, Sibson DR, Platt-Higgins A, Barraclough R. Human homologue of cement gland protein, a novel metastasis inducer associated with breast carcinomas. *Cancer Res* 2005;65:3796–805.
- Rasiah KK, Kench JG, Gardiner-Garden M, et al. Aberrant neurotrophin Y and macrophage inhibitory cytokine-1 expression are early events in prostate cancer development and are associated with poor prognosis. *Cancer Epidemiol Biomarkers Prev* 2006;15:711–6.
- Bauskin AR, Brown DA, Junankar S, et al. The propeptide mediates formation of stromal stores of PROMIC-1: role in determining prostate cancer outcome. *Cancer Res* 2005;65:2330–6.
- Chang B-D, Swift ME, Shen M, Fang J, Broude EV, Roninson IB. Molecular determinants of terminal growth arrest induced in tumor cells by a chemotherapeutic agent. *Proc Natl Acad Sci U S A* 2002;99:389–94.
- Modlich O, Prisack HB, Munnes M, Audretsch W, Bojar H. Immediate expression changes after the first course of neoadjuvant chemotherapy in patients with primary breast cancer disease. *Clin Cancer Res* 2004;10:6418–31.
- Shimizu D, Ishikawa T, Ichikawa Y, et al. Prediction of chemosensitivity of colorectal cancer to 5-fluorouracil

- p>by gene expression profiling with cDNA microarrays.
- Int J Cancer*
- 2005;27:371–6.
31. Bauskin AR, Brown DA, Kuffner T, et al. Role of macrophage inhibitory cytokine-1 in tumorigenesis and diagnosis of cancer. *Cancer Res* 2006;66:4983–6.
 32. Liu T, Bauskin AR, Zaunders J, et al. Macrophage inhibitory cytokine 1 reduces cell adhesion and induces apoptosis in prostate cancer cells. *Cancer Res* 2003;63: 5034–40.
 33. Welsh JB, Sapinoso LM, Su AI, et al. Analysis of gene expression identifies candidate markers and pharmacological targets in prostate cancer. *Cancer Res* 2001;61: 5974–8.
 34. Wakchoure S, Swain TM, Hentunen TA, et al. Expression of macrophage inhibitory cytokine-1 in prostate cancer bone metastases induces osteoclast activation and weight loss. *Prostate* 2009;69:652–61.
 35. Lee DH, Yang Y, Lee SJ, et al. Macrophage inhibitory cytokine-1 induces the invasiveness of gastric cancer cells by up-regulating the urokinase-type plasminogen activator system. *Cancer Res* 2003;63:4648–55.
 36. Kim K-K, Lee JJ, Yang Y, You K-H, Lee J-H. Macrophage inhibitory cytokine-1 activates AKT and ERK-1/2 via the transactivation of ErbB2 in human breast and gastric cancer cells. *Carcinogenesis* 2008;29: 704–12.
 37. Riedel RF, Porrello A, Pontzer E, et al. A genomic approach to identify molecular pathways associated with chemotherapy resistance. *Mol Cancer Ther* 2008;7: 3141–9.
 38. Brown DA, Ward RL, Buckhaults P, et al. MIC-1 serum level and genotype: associations with progress and prognosis of colorectal carcinoma. *Clin Cancer Res* 2003;9:2642–50.
 39. Brown DA, Stephan C, Ward RL, et al. Measurements of serum levels of macrophage inhibitory cytokine 1 combined with prostate-specific antigen improves prostate cancer diagnosis. *Clin Cancer Res* 2006;12:89–96.
 40. Berthold DR, Pond GR, Roessner M, de Wit R, Eisenberger M, Tannock IF. Treatment of hormone-refractory prostate cancer with Docetaxel or Mitoxantrone: relationships between prostate-specific antigen, pain, and quality of life response and survival in the TAX_327 study. *Clin Cancer Res* 2008;14:2763–7.