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ABSTRACT

Patients with high-risk localized prostate cancer have a high recurrence rate following primary therapy. Neoadjuvant chemotherapy has been shown to be beneficial in reducing recurrence rates in some tumor types, but has yet to be of proven benefit in prostate cancer. Further, current clinical, pathological and molecular markers poorly predict the response and resistance of chemotherapy, and the molecular mechanisms of chemotherapy resistance are largely unknown. We utilized tissue resources from a unique prospective phase II clinical trial of neoadjuvant chemotherapy with docetaxel and mitoxantrone in patients with high-risk localized prostate cancer to identify molecular alterations after chemotherapy, and correlated these alterations with clinical and pathological indicators of tumor response. We hypothesized that this approach may identify molecular signatures of chemotherapy resistance and uncover mechanisms or pathways suitable for targeting with the objective of improving tumor responses to chemotherapy. Gene expression changes after chemotherapy were measured in 31 patients who completed 4 cycles of docetaxel and mitoxantrone neoadjuvant chemotherapy. After excluding possible ischemia-related genes, the expression of 53 genes were significantly altered after chemotherapy. Several cytokines were significantly up-regulated including IL-8, CCL2, GDF15, CXCL10, and IL1B. Overexpression of GDF15 or treatment with GDF15 protein in DU145 cells conferred resistance to docetaxel and mitoxantrone. Using PSA decline greater than 40% as a cut-point to distinguish good from poor responders, we were able to identified 33 significantly-altered genes. IL8 was not only shown to be activated after chemotherapy but also have higher expression levels in the group of poor responders compared with good responders. Alterations of molecular signatures after administration of docetaxel and mitoxantrone in patients with high-risk localized prostate cancer were recognized. Correlations between expression changes after chemotherapy and clinical outcome using a criterion of PSA decline greater than 40% identified candidate genes and pathways that may contribute to chemotherapy resistance and response. Of these, cytokines may have important roles accounting for the mechanisms of chemotherapy resistance and response.

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INTRODUCTION:

Chemotherapy with docetaxel and/or mitoxantrone has been shown to be beneficial for some patients with advanced hormone refractory prostate cancer [1, 2]. However, there are no useful clinical and pathological markers to predict who will benefit from receiving these agents. In addition, the mechanisms used by tumor cells to circumvent the cytotoxic effects of chemotherapy are poorly understood, and thus cannot be effectively targeted to enhance tumor responses. Our hypothesis is that identifying *in vivo* gene expression changes before and after neoadjuvant chemotherapy will uncover the molecular mechanisms of prostate tumor response and resistance to cytotoxic drugs. Once identified, these tumor resistance mechanisms can be exploited through the design of combination therapies targeted toward inhibiting resistance pathways.

BODY:

Between January 2001 and November 2004, 57 patients with high-risk localized prostate cancer (defined as TNM > cT2b or T3a or PSA $\geq$ 15 ng/ml or Gleason grade $\geq$ 4+3) were recruited for a phase II trial clinical trial of neoadjuvant chemotherapy. The design of the clinical trial has been previously described [3, 4]. Figure 1 shows the schema of the study design. From each patient, ten standard prostate biopsies (bilateral at the apex, bilateral medial and lateral at mid-gland, bilateral medial and lateral at the base of the gland) were obtained under ultrasound guidance and snap-frozen in liquid nitrogen prior to chemotherapy. At the time of radical prostatectomy, cancer-containing tissue samples were snap frozen immediately after prostate removal. Evaluation of tissue samples identified the presence of adequate numbers of cancer cells in both pre-treatment and post-treatment samples for 31 subjects. We used laser capture microdissection techniques to specifically collect cancer epithelia from pre-treated biopsy specimens and post-treated radical prostatectomy specimens. Total RNA and cy3-cy5 labeled cDNA were generated based on the standard protocol in our lab. The strategy of hybridization is depicted in Figure 1.

Chemotherapy-Induced Profiles:

A chemotherapy-induced profile was generated by a direct head-to-head hybridization between post-treated cancer epithelia and pre-treated cancer epithelia. Cancers surviving through docetaxel and mitoxantrone treatment are presumably enriched for resistant clones with molecular pathways contributing to cell survival. Hence, chemotherapy-induced profiles may reflect general mechanisms of chemotherapy resistance and response. After excluding 441 ischemia-related genes recognized in our previous study [5], we identified 53 genes with significant alterations (p-value < 0.001) in post-treated specimens compared with pre-treated specimens by a random variance t-test provided (Table 1). Several altered genes encode cytokines such as IL-8, CCL2, GDF15, CXCL10 and IL1B. Cytokines may have important roles in modulating chemoresistance in cancer cells. A previous study has shown that IL-8, IL6, and CCL2 expression increased in paclitaxel-resistant ovarian cancer cell sublines [6]. GDF15 has been reported to be upregulated after neoadjuvant chemotherapy with epirubicin and cyclophosphamide or taxol in breast cancer [7]. These findings support our result using human tissues exposed to chemotherapy *in vivo*, and identified general pathways and gene expression changes that appear to be common across different cancers and/or chemotherapy agents.

The ultimate objective of this study is to identify chemotherapy resistance mechanisms that could be exploited as therapeutic targets to increase treatment responses. To this end, we further analyzed the functional categories of the 53 chemotherapy-altered genes based on GO biological processes using EASE software [8]. We found a significant enrichment of genes involved in cellular stress responses including categories of cell death and responses to abiotic stimulus, external stimulus, and chemical substances (Table 2). These findings are consistent with a cellular reaction to exogenous toxic agents. We also found a significant enrichment of genes involved in pathways of signal transduction, regulation

of transcription, cell communication, chemokine activity and inflammatory responses. These findings suggest that chemokines may play important roles in mediating chemotherapy resistance and response. Although chemokine activation after chemotherapy could be explained by generalized inflammatory reactions induced by cell death after chemotherapy, growing evidence indicates that chemokines are important survival factors for cancer cells under chemotherapy treatment [6, 9]. Chemokines and their receptors have the therapeutic advantage of modulation by agonists or antagonist such as small molecules or antibodies.

GDF15 Influences Chemotherapy Resistance

Cytokines have been shown to be associated with chemotherapy resistance and exert cytoprotective effects [6, 9]. Of the cytokine-encoding transcripts that we found to be differentially expressed, Growth Differentiation Factor 15 (GDF15), alias Macrophage Inhibitory Cytokine 1; a TGF- β superfamily member, has been reported to be associated with cancer progression and metastasis [10]. The role of GDF15 in chemotherapy resistance has yet to be determined. We first validated the expression results derived from the microarray analyses. GDF15 mRNA abundance of pre-treated biopsy and post-treated prostatectomy samples was measured by real-time PCR in aRNA obtained from the LCM material. Twenty-eight of 31 samples had measurable increases in GDF15 expression (90%) with 23/31 showing a 2-fold or greater change (Figure 1). The consistency of these findings suggests that GDF15 induction could be a generic response to chemotherapy stress, or could represent an important modulator of resistance in that there were no complete tumor responses in any of the subjects treated with 4 cycles of neoadjuvant therapy.

To explore these possibilities, we evaluated whether over-expression of GDF15 could confer cellular chemoresistance using an *in vitro* cell culture system. We over-expressed GDF15 by transfecting the DU145 cell with plasmids designed to express GDF15 and green fluorescent protein (GFP) (a kind gift from Dr. Breit [11]). Two clones of DU145 cells overexpressing different levels of GDF15 and a DU145 line expressing only the GFP vector were treated with increasing concentrations of docetaxel or mitoxantrone for 3 days. The percentage of viable cells was determined using the MTS assay (Figure 2). We found that GDF15 expressing cells exhibited significantly greater resistance to both docetaxol and mitoxantrone-induced cytotoxicity, and the differences in cell survival were enhanced with increasing drug concentrations. To determine if GDF15 could exert a cytoprotective function via a paracrine mechanism, we treated parental DU145 cells with various concentrations of recombinant GDF15 protein and the chemotherapy drugs at around LD50 concentrations. We found that concentrations of GDF15 ≥ 1 ng/ml resulted in measurable increases in cell viability. Following 72 hours of exposure to docetaxel, 1 ng/ml GDF15 increased cell viability by 16% over no GDF15 treatment group, and 50 ng/ml increased viability by 52% (Figure 3). A similar protective effect was also observed in cells treated with mitoxantrone (Figure 3).

Expression Profiles Reflect Differential Prostate Cancer Responses to Chemotherapy

One objective of studies designed to analyze tumor gene expression is the identification of inherent molecular differences that could define the variability often observed in treatment responses. Such biomarkers could assist in predicting outcomes and stratify patients as appropriate for a given therapy. For correlations with tumor gene expression, we considered three parameters to reflect the clinical responses of prostate cancers to chemotherapy. The first, and potentially most direct endpoint involves the pathological assessment of tumor response. However, detailed histopathological reviews of all radical prostatectomy samples in this study did not reveal any patient with a complete response, and partial pathological responses are difficult to accurately quantify, as the pre-treatment assessment of tumor volume is based on sampling by needle biopsies rather than having a complete organ for comparison. A second endpoint involves the determination of disease-free or overall survival. For

patients treated by radical prostatectomy, PSA serum levels are a good indicator of persistent or recurrent tumor when a threshold of 0.4 ng/ml and rising is used as an indicator of ultimate progression to metastasis [4]. However, for the cohort of patients enrolled in this study, the average follow-up time is too short (mean: 3.3 years, range: 1.2- 5.1) to fit survival data into a model. A third clinical parameter we evaluated involves measuring a PSA response based on the percentage of PSA decline after chemotherapy. PSA response is an immediate endpoint calculated from the serum PSA level measured before and after chemotherapy. We have previously reported that the serum androgen levels were not affected by the chemotherapy protocol employed here [4]. Thus PSA concentrations likely reflect changes in cancer cell numbers, though could potentially represent chemotherapy-mediated changes in cellular secretory mechanisms or tumor vasculature. We chose an arbitrary cut-point of PSA decline greater than 40% to distinguish good versus poor responders, which divided the study population into approximately equal groups.

Evaluating only those genes we previously determined to exhibit significant chemotherapy-associated changes, we assessed if any were differentially expressed between the good (16 patients) and poor responders (15 patients) based on PSA declines of >40%. We identified 33 genes that met these criteria (p -value <0.005) (Figure 5). Several response-associated genes have known biological functions that are plausibly associated with a differential response to cytotoxic treatment. For example, the expression of interleukin 8 (IL-8) was not only activated after chemotherapy across the majority of patients, but also had higher expression levels in the cohort of poor responders compared with good responders. This finding suggests that IL-8 may exhibit a dose-response effect in prostate cancer chemoresistance. Two genes with functions related to the detoxification or elimination of drugs or toxins were also significantly associated with treatment response. The membrane organic cation transporter, solute carrier member 22A3 (SLC22A3) is a member of a family of proteins that function to eliminate small organic cations and an arrays of toxic compounds. Glycine-N-acyltransferase (GLYAT) modulates the conjugation of glycine to carboxylic xenobiotics prior to excretion. Higher expression of these two detoxification genes in the poor responder group suggests that a pathway of enhanced cellular detoxification pathway might contribute to drug resistance.

KEY RESEARCH ACCOMPLISHMENTS:

- We have identified alterations of gene expression in 31 prostate cancers resulting from *in vivo* exposure to the cytotoxic chemotherapy drugs docetaxel and mitoxantrone
- We have validated one of the chemotherapy-induced genes, GDF15 in an in vitro cell culture system. GDF15 may act as a paracrine or autocrine factor to enhance chemotherapy resistance in prostate cancer.
- We have correlated a chemotherapy-induced expression profile with PSA decline and identified genes that distinguished good responders from poor responders.
- We have generated an intrinsic expression profile by comparing transcript levels between benign epithelia and cancer epithelia from pre-treated needle biopsy specimens. However, to date, we have not found significant gene alterations correlating with clinical data.

REPORTABLE OUTCOMES:

Manuscript in preparation: Identification of Molecular Alterations Contributing to Chemotherapy Resistance in Prostate Carcinoma.

CONCLUSION:

We have identified genes with altered expression in post-treated prostate tumor tissue compared with pre-treated tissue and in poor responders compared with good responders. We also showed one of the significantly altered genes in post-chemotherapy samples, GDF15 may confer chemoresistance. Thus,

expression profiling in the neoadjuvant setting is a feasible way to delineate possible chemoresistant genes and pathways. Further mechanism-based studies and correlations with clinical outcomes such as disease free or biochemical relapse free survival are needed and are ongoing.

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APPENDICES:

Table 1. Differentially expressed genes in post-treated samples compared with pre-treated samples.

Unigene	HUGO	Description	Ratio	p-value
Hs.624	IL8	Interleukin 8	3.27	< 1e-07
Hs.144513	TMEFF2	Transmembrane protein with EGF-like and two follistatin-like domains 2	2.39	0.00054
Hs.303649	CCL2	Chemokine (C-C motif) ligand 2	2.18	0.00010
Hs.466871	PLAUR	Plasminogen activator urokinase receptor	2.05	0.00000
Hs.413924	CXCL10	Chemokine (C-X-C motif) ligand 10	1.81	0.00070
Hs.504609	ID1	Inhibitor of DNA binding 1 dominant negative helix-loop-helix protein	1.80	0.00021
Hs.293736	ADNP	Activity-dependent neuroprotector	1.74	0.00000
Hs.515258	GDF15	Growth differentiation factor 15	1.71	0.00006
Hs.502829	SF1	Splicing factor 1	1.61	0.00001
Hs.76884	ID3	Inhibitor of DNA binding 3 dominant negative helix-loop-helix protein	1.56	0.00062
Hs.76753	ENG	Endoglin (Osler-Rendu-Weber syndrome 1)	1.50	0.00053
Hs.472651	BLCAP	Bladder cancer associated protein	1.49	0.00007
Hs.2178	HIST2H2BE	Histone 2 H2be	1.47	0.00052
Hs.244139	TNFRSF6	Tumor necrosis factor receptor superfamily member 6	1.46	0.00041
Hs.2030	THBD	Thrombomodulin	1.45	0.00001
Hs.270055	SH3GL3	SH3-domain GRB2-like 3	1.44	0.00048
Hs.126256	IL1B	Interleukin 1 beta	1.43	0.00035
Hs.549393	FOSL2	FOS-like antigen 2	1.41	0.00054
Hs.520140	SRF	Serum response factor	1.38	0.00055
Hs.470943	STAT1	Signal transducer and activator of transcription 1 91kDa	1.37	0.00056
Hs.159161	ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha	1.35	0.00014
Hs.435490	TMPPRSS7	Transmembrane protease, serine 7	1.34	0.00013
Hs.59332	SPRED2	Sprouty-related EVH1 domain containing 2	1.34	0.00010
Hs.405662	CRABP2	Cellular retinoic acid binding protein 2	1.33	0.00004
Hs.516490	TANK	TRAF family member-associated NFKB activator	1.33	0.00001
Hs.429581	RTN4	Reticulon 4	1.32	0.00027
Hs.380906	MYADM	Myeloid-associated differentiation marker	1.30	0.00090
Hs.501309	CIRBP	Cold inducible RNA binding protein	1.29	0.00007
Hs.512908	ARPP-19	Cyclic AMP phosphoprotein 19 kD	1.27	0.00022
Hs.495960	ATP6AP2	ATPase H+ transporting lysosomal accessory protein 2	1.26	0.00084
Hs.487325	PRKACB	Protein kinase cAMP-dependent catalytic beta	1.26	0.00085
Hs.493096	PBX1	Pre-B-cell leukemia transcription factor 1	1.26	0.00071
Hs.370725	OSBPL1A	Oxysterol binding protein-like 1A	1.23	0.00087
Hs.73799	GNAI3	Guanine nucleotide binding protein alpha inhibiting activity polypeptide 3	1.22	0.00078
Hs.534312	TOR1A	Torsin family 1 member A (torsin A)	1.19	0.00066
Hs.444600	LAT1-3TM	LAT1-3TM protein	1.18	0.00055
Hs.517948	DHX30	DEAH (Asp-Glu-Ala-His) box polypeptide 30	1.18	0.00033
Hs.480073	HNRPD	Heterogeneous nuclear ribonucleoprotein D	1.16	0.00098
Hs.459779	DNAJA3	DnaJ (Hsp40) homolog subfamily A member 3	0.86	0.00061
Hs.248785	AGPAT3	1-acylglycerol-3-phosphate O-acyltransferase 3	0.84	0.00076
Hs.272062	PTPRF	Protein tyrosine phosphatase receptor type F	0.84	0.00075
Hs.433702	EIF5	Eukaryotic translation initiation factor 5	0.83	0.00041
Hs.302977	C12orf4	Chromosome 12 open reading frame 4	0.82	0.00088
Hs.25669	NCOA5	Nuclear receptor coactivator 5	0.79	0.00013
Hs.356247	ACY1L2	Aminoacylase 1-like 2	0.79	0.00018
Hs.301277	KIAA0543	KIAA0543 protein	0.79	0.00033
Hs.524183	FKBP4	FK506 binding protein 4 59kDa	0.76	0.00027
Hs.32417	SARG	Specifically androgen-regulated protein	0.75	0.00003
Hs.438545	SLC2A9	Solute carrier family 2 (facilitated glucose transporter), member 9	0.73	0.00004
Hs.411490	FAM36A	Family with sequence similarity 36 member A	0.72	0.00003
Hs.284491	PDXK	Pyridoxal (pyridoxine vitamin B6) kinase	0.68	0.00001
Hs.302738	SLC26A2	Solute carrier family 26 (sulfate transporter) member 2	0.63	0.00018
Hs.533977	TXNIP	Thioredoxin interacting protein	0.57	0.00004

Table 2. EASE functional categories of differentially expressed gene between pre- vs post-treated samples

GO biological process	EASE score	Gene Symbol
Signal transduction	0.004	ARHGDIA; CCL2; CRABP2; CXCL10; GNAI3; IL1B; IL8; OSBPL1A; PLAUR; PRKACB; PTPRF; SH3GL3; SRF; STAT1; TANK; TNFRSF6; TXNIP
Regulation of transcription	0.007	ADNP; CIRBP; CRABP2; FOSL2; HIST2H2BE; HNRPD; ID1; KIAA0543; NCOA5; PBX1; SF1; SRF; STAT1
Cell communication	0.010	ARHGDIA; CCL2; CRABP2; CXCL10; ENG; GNAI3; IL1B; IL8; OSBPL1A; PLAUR; PRKACB; PTPRF; SH3GL3; SRF; STAT1; TANK; TNFRSF6; TXNIP
Chemokine activity	0.019	CCL2; CXCL10; IL8
Response to abiotic stimulus	0.021	CCL2; CIRBP; CXCL10; IL8; OSBPL1A; PLAUR
Response to external stimulus	0.029	CCL2; CIRBP; CXCL10; IL1B; IL8; OSBPL1A; PLAUR; STAT1; TNFRSF6; TXNIP
Response to chemical substance	0.040	CCL2; CXCL10; IL8; PLAUR
Cell death	0.043	CCL2; FOSL2; IL1B; RTN4; STAT1; TNFRSF6
Inflammatory response	0.046	CCL2; CXCL10; IL1B; IL8

Figure 1. Study design

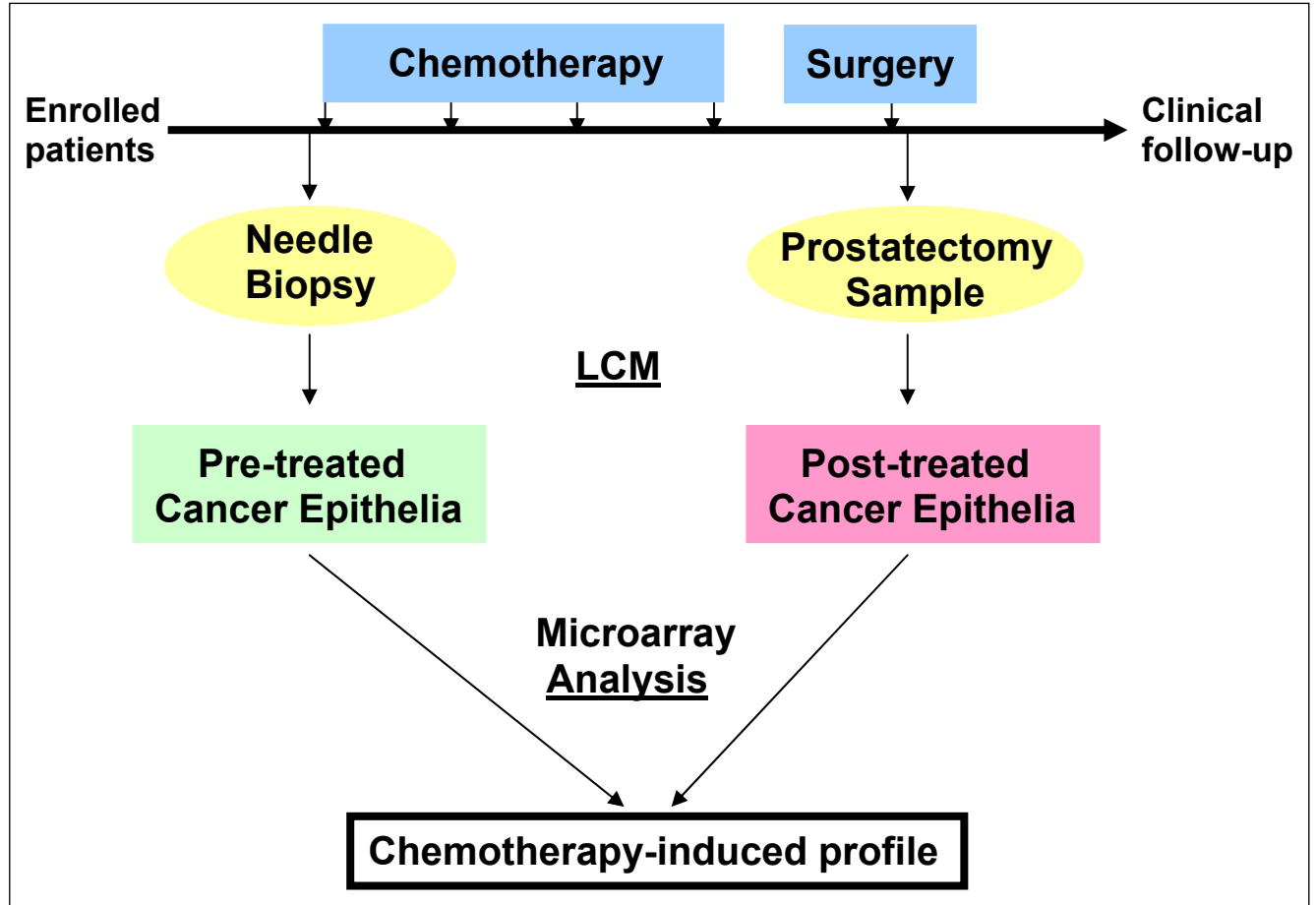


Figure 2. Real-time PCR results of GDF15 expression in post-treated specimens normalized to pre-treated specimens.

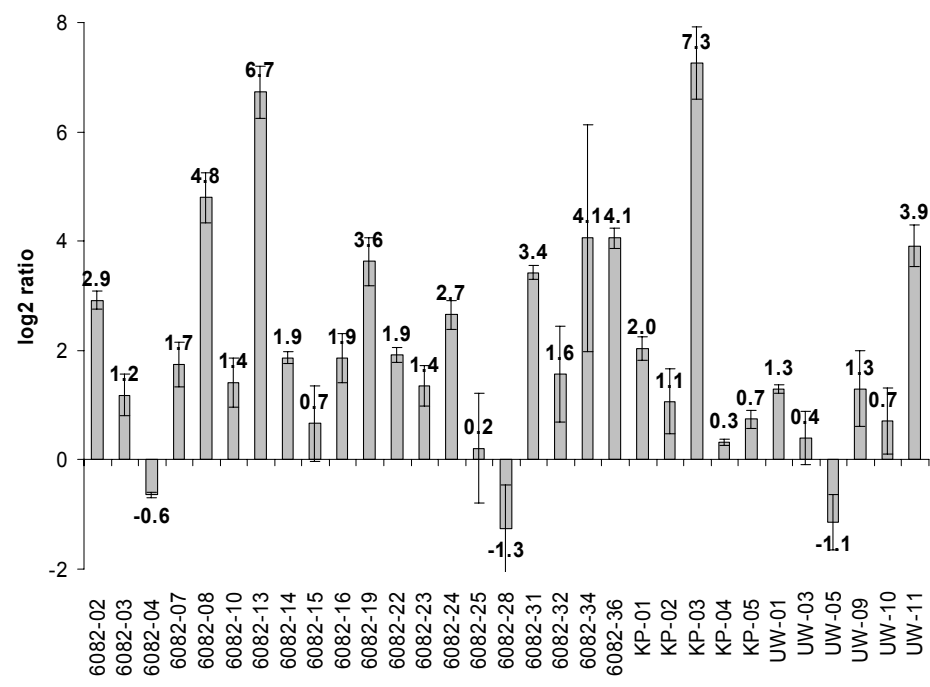
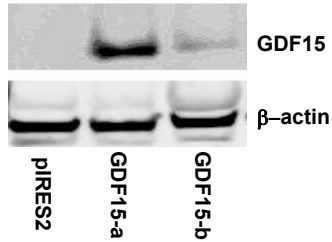


Figure 3. A. Western blot of GDF15 over-expressed cells and empty vector cells. B. MTS assay of GDF15 over-expressed cells vs. empty vector cells. * p-values < 0.05 by student *t*-test.

A. Western blot



B. MTS assay

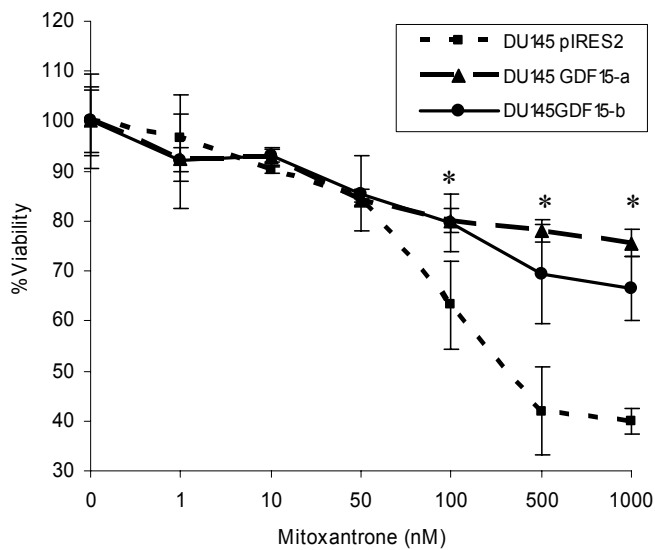
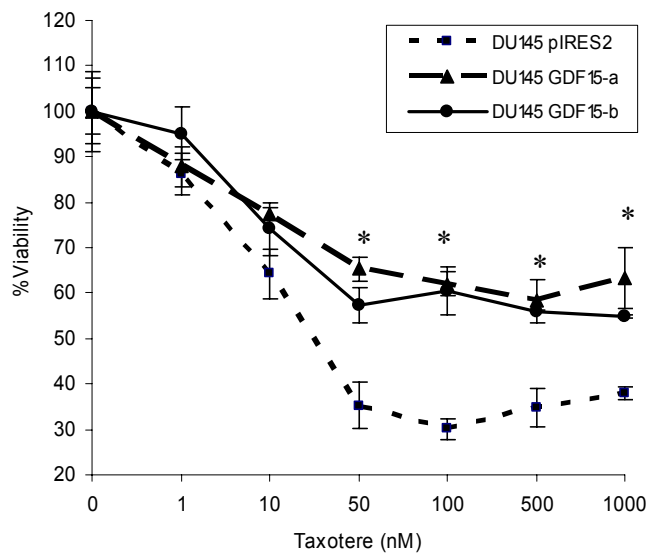


Figure 4. MTS assay of cell lines treated with series concentration of recombinant GDF15 protein. Percentage of viability was calculated by deviding OD490 value of treated cells by the non-treated cell. * p-values < 0.05 by student *t*-test.

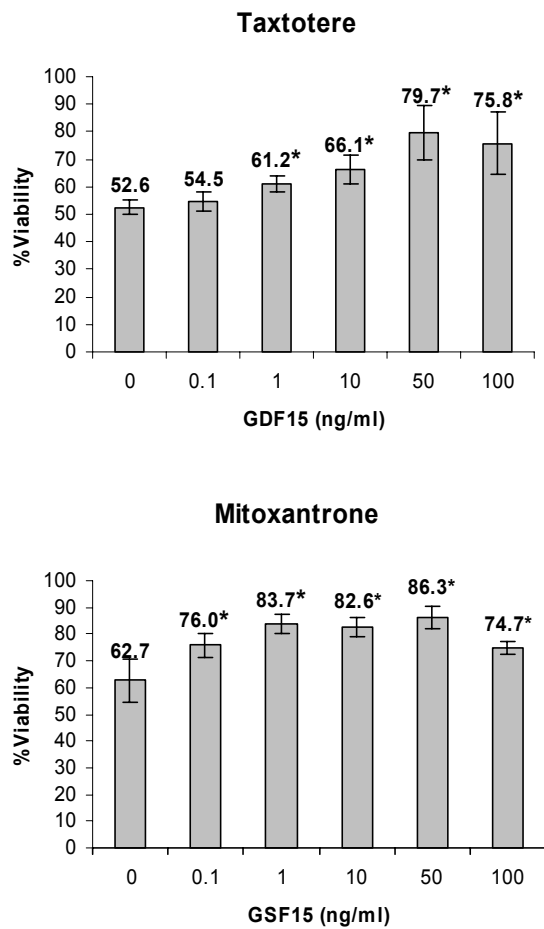


Figure 5. Differentially expressed genes between good and poor responders based on a criterion of PSA decline more than 40% as good response. Heat map on the right-hand side has been median centered. * p-values < 0.001, other p-values < 0.005.

