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THE ROLE OF IGF-1 SIGNALING PATHWAY IN CISPLATIN-RESISTANT LUNG CANCER CELLS

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Abstract

Purpose—The development of drug-resistant phenotypes has been a major obstacle to Cisplatin (CDDP) use in non-small cell lung cancer (NSCLC). We aimed to identify some of the molecular mechanisms that underlie CDDP resistance by using microarray expression analysis.

Methods and Materials—H460 cells were treated with cisplatin. Differences between cisplatin-resistant lung cancer cells (CDDP-R) and parental H460 cells were studied using Western blot, MTS and clonogenic assays, *in vivo* tumor implantation, and microarray analysis. CDDP-R cells were treated with human recombinant insulin-like growth factor binding protein-3 (IGFBP-3) and siRNA targeting insulin-like growth factor-1 receptor (IGF-1R).

Results—CDDP-R cells illustrated greater expression of the markers CD133 and ALDH, more rapid *in vivo* tumor growth, more resistance to cisplatin- and etoposide-induced apoptosis, and greater survival after treatment with cisplatin or radiation than the parental H460 cells. Also, CDDP-R demonstrated decreased expression of IGFBP-3 and increased activation of IGF-1R signaling as compared to parental H460 cells in the presence of IGF-1. Human recombinant IGFBP-3 reversed cisplatin resistance in CDDP-R cells, and targeting of IGF-1R using siRNA resulted in sensitization of CDDP-R-cells to cisplatin and radiation.

Conclusions—The IGF-1 signaling pathway contributes to CDDP-R resistance to cisplatin and radiation. Thus, this pathway represents a potential target for improved lung cancer response to treatment.

Keywords

cisplatin resistance; IGFBP-3; lung cancer; radiotherapy

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Conflicts of Interest: None

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (1). Cisplatin (CDDP) based combination treatments have been the conventional management for advanced non-small cell lung cancer (NSCLC) for over two decades, however, a major obstacle in using this drug has been the development of CDDP resistance (2). Therefore, the development of more effective treatment targeting molecules associated with resistance is necessary to improve outcomes.

CDDP is frequently used in combination with radiation therapy in the treatment of NSCLC. Patients who receive CDDP based treatments followed by radiotherapy have been noted to have a correlation between their response to CDDP and the subsequent response to radiotherapy (3). In addition, *in vitro* studies have revealed that the acquirement of CDDP resistance in cell lines may result in the acquisition of cross resistance to radiotherapy (4). Thus, identifying the molecular mechanisms associated with CDDP resistance may provide a target to overcome resistance to combined modality treatment.

High throughput techniques comparing the gene signature of CDDP resistant cells with normal cancer cells reveal genes that are differentially expressed between these two cell populations. In this study, cells isolated following cisplatin exposure (CDDP-R cells) expressed markers associated with lung cancer stem cells. Microarray gene expression analysis comparing CDDP-R cells with parental H460 cells found that Insulin-like growth factor-binding protein-3 (IGFBP-3) was a highly ranked hub gene that was down-regulated in CDDP-R cells.

IGFBP-3 regulates IGF-1 bioactivity by sequestering IGF-1 in the extracellular milieu, thereby inhibiting its mitogenic and antiapoptotic actions (5). Overexpression of IGFBP-3 inhibits the growth of NSCLC cells by inducing apoptosis (6). Reduced IGFBP-3 expression in NSCLC has been associated with decreased tumor cell sensitivity to cisplatin (7). Therefore, we investigated the role of IGFBP-3 and the IGF-1R pathway in chemotherapy- and radiation-resistant cells and its potential as a treatment target in NSCLC. We found that IGF-1R is highly active in CDDP-R cells and that siRNA treatment of CDDP-R cells results in the recovery of their sensitivity to cisplatin and radiation therapy. Thus, the IGF-1/IGF-1R pathway holds promise as a therapeutic target to overcome resistance to chemotherapy and radiation therapy in NSCLC.

Material and Methods

Cell lines and reagents

NCI-H460 cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in RPMI1640 culture medium supplemented with 10% FBS (Invitrogen). CDDP-R cells were selected as described (8). Briefly, after H460 cells were treated by 3 μ M cisplatin for seven days, the survival cells were trypsinized and cultured in 0.8% methyl cellulose that was supplemented with 20ng/mL EGF (BD Biosciences), bFGF, and 4 μ g/mL Insulin (Sigma). EGF, bFGF (20ng/mL), and insulin (4 μ g/mL) were added every second day for 14 days to allow the cells to form spheres. Spheres were diluted with PBS to make a single-cell suspension and then plated in 100mm dishes with RPMI 1640 supplemented with 10% FBS. Cisplatin and etoposide were obtained from Sigma-Aldrich. Human recombinant IGF-1 and human recombinant IGFBP-3 (hrIGFBP-3) were purchased from R&D Systems (Minneapolis, MN). 5'AZA-2'DC was obtained from Sigma (St. Louis, MO) and cells were treated with 10 μ M for 72h.

RNA extraction and microarray

Cells were plated in 6-well plates and allowed to reach 80% confluency. 1ml of Trizol (Invitrogen; Carlsbad, CA) was added into each well, and then RNA was extracted following the manufacturer's guidelines. RNA was further purified by the RNeasy kit (Qiagen). Sample integrity was confirmed on the Agilent Bioanalyzer, and then samples were quantitated at 260nm on the Nanodrop spectrophotometer (Thermo Fisher Scientific). 200ng of the total input RNA was used in the Affymetrix Gene 1.0 ST arrays for the target labeling reactions. The reactions, hybridization and data process were performed in the Vanderbilt Functional Genomics Shared Resources (FGSR) according to manufacturer protocol using the Affymetrix reagent kits (# 900652). Three biological replicates were profiled for each cell line. The microarray data were normalized by the Robust Multi-chip Average method (RMA) (9) and then differential genes were identified based on both the Significance Analysis of Microarrays (SAM) (FDR < 0.1) and the fold change > 2. The microarray data was submitted to Gene Expression Omnibus (GEO ID GSE21656). Additional details are provided in the supplementary methods section.

siRNA and transfections

Parental and CDDP-R H460 cells were transfected 24h after seeding in a 6-well plate. IGF-1R siRNA and control siRNA (Santa Cruz Biotechnology) (25pmol) in 100µl of serum-free, antibiotic-free, opt-MEM (Invitrogen) were mixed with 5µl Lipotectamine RNAimax transfection reagent (Invitrogen) and dissolved in 100µl of the same medium and allowed to stand at room temperature (RT) for 20m. The 200µl transfection solutions were added to each well containing 2ml medium and incubated for 6h before being replaced by 2ml fresh medium supplemented with 10% FBS and antibiotics.

Cell viability assay

MTS assay was performed using tetrazolium compound based CellTiter 96® AQueous One Solution Cell Proliferation assay (Promega). Parental and CDDP-R cells were seeded in 96 well plate at 2,000cells/well. Cells were treated with various concentrations of cisplatin the following day. For siRNA studies, cells were transfected 48h before treatment with cisplatin. For hrIGFBP-3 treatment, CDDP-R cells were seeded at 5,000cells/well and allowed to attach overnight before exposure to 30µg/ml hrIGFBP-3 or 3µM cisplatin in serum free RPMI1640, either alone or in combination. MTS assay was performed 72h after treatment.

Western blot analysis

Cells were washed twice with ice-cold PBS and then lysed in M-Per mammalian lysis buffer (Thermo Scientific). The protein concentration of the lysates was determined with the Bradford reagent (Bio Rad), and equal amounts of protein were subjected to SDS-PAGE of a 10% or 15% gel. Separated proteins were transferred to a nitrocellulose membrane, which was then exposed to 5% nonfat dried milk in TBS containing 0.1% Tween 20 (0.1% TBST) for 1h at room temperature and incubated overnight at 4°C with antibodies against ALDH (R&D Systems), CD133 (Abcam), caspase-3, phospho-IGF-IR (Tyr1135/1136), total IGF-IR (Cell Signaling Technology), IGFBP-3(R&D Systems) or actin (Sigma). The membranes were then washed with 0.1% TBST before being incubated with horseradish peroxidase-conjugated goat antibodies to rabbit or mouse (Santa Cruz Biotechnology). Immune complexes were detected with chemiluminescence reagents (Perkin-Elmer Life Science).

Reverse transcription-PCR

2µg of total RNA was reverse transcribed using hexamer primer and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a final volume of 20µl. 2µl of the cDNA

was used for the PCR reactions to amplify the IGFBP-3 gene. The sequences of primers used were described previously (7). Actin was used as a control.

DNA extraction, bisulfite treatment, and MSP assay

Genomic DNA was isolated using DNeasy kit (Qiagen) from parental and CDDP-R H460 cells. For bisulfite treatment, the extracted DNA (1µg) was modified using the EZ DNA Methylation Kit™ (Zymo Research, Orange, CA). The methylation specific primers were designed based on the CpG island sequence from gene browser (<http://genome.ucsc.edu/>). MSP primers are designed using methprimer (<http://www.urogene.org/methprimer/index1.html>). The primers used to amplify the methylated (M) IGFBP-3 promoter were M(S) 5'-AATCGTAGAAGATATTAAATTCGA-3' and M (AS) 5'-AACCAAAAAAATAAACAAACACGT-3'. Primers for the unmethylated (U) IGFBP-3 were U(S) 5'-AATTGTAGAAGATATTAAATTTGA-3' and U(AS) 5'-ACCAAAAAAATAAACAAACACATT-3'. Bisulfite-modified DNAs were mixed with 10× PCR Buffer, 150µM of deoxynucleotide triphosphates, 0.3µM of primers and 1 unit of HotStarTaq (Qiagen). The PCR condition for both methylated and unmethylated promoter consisted of 15m at 95°C, 40 cycles of 15s of 94°C, 30s of 55°C and 30s of 72°C followed 10m elongation. The PCR products were resolved by electrophoresis in 3% agarose gels containing ethidium bromide.

Clonogenic survival assay

Cells were irradiated with 0–6Gy (dose rate of 1.8Gy/min) using ¹³⁷Cs irradiator (J.L. Shepherd and Associates). After irradiation, cells were incubated at 37°C for 8–10 days. Cells were fixed for 15m with 3:1 methanol/acetic acid and stained for 15m with 0.5% crystal violet (Sigma) in methanol. After staining, colonies were counted by the naked eye (cut-off of 50 viable cells). The surviving fraction was calculated as (mean colony counts)/(cells inoculated)×(plating efficiency), with plating efficiency defined as (mean colony counts)/(cells inoculated for irradiated controls). The dose enhancement ratio (DER) was calculated as the dose (Gy) of radiation that yielded a surviving fraction of 0.2 for control siRNA treated cells divided by that for IGF-1R siRNA treated cells.

Animals and tumor xenograft assay

All animal studies were approved and handled following Institutional Animal Care and Use Committee (IACUC) guidelines (IACUC-approved protocol M/08/095). Female athymic nude mice (5–6wks old) were purchased from Harlan Laboratories (Indianapolis, IN). Exponentially growing parental H460 and CDDP-R H460 cells were trypsinized and washed with PBS and then diluted into 1×10^6 cells per 100µL PBS. The cell suspension was injected s.c. into the left or right flank of each mouse ($n = 5$). Following tumor visibility, tumor size was measured with a digital caliper every two days. The tumor volume was calculated by (length×width×height)/2.

Statistical analysis

All data are presented as mean ± S.D. Two tailed Student's t-test was performed for statistical difference between two groups. Wilcoxon two sample test was performed to compare the times required for tumor size to quadruple in a xenograft model.

Results

CDDP-R H460 cells exhibit higher levels of markers associated with lung cancer stem cells and demonstrate more aggressive tumor growth

Lung cancer stem cells are known to express specific cell surface markers including CD133 (10) and ALDH (11). Cell lysates of parental and CDDP-R H460 cells were probed for CD133 and ALDH and subjected to Western blot analysis. Higher levels of CD133 and ALDH were detected in the CDDP-R cells as compared to parental cells (Figure 1A). An equal number of parental or CDDP-R H460 cells (1×10^6) were inoculated into nude mice and quantitation of tumor growth demonstrated faster growth in the CDDP-R xenografts (Figure 1B). The tumor volume quadrupling time was significantly shorter in CDDP-R xenografts, 5.4 days (95% CI, 4.9–5.9 days), as compared to parental H460 xenografts, 7.8 days (95% CI, 7.4–8.2 days), ($p < 0.03$).

CDDP-R H460 cells are resistant to treatment with radiation and cisplatin

To determine differences in the response of parental and CDDP-R cells to chemotherapy, cells were treated with either cisplatin (20 μ M) or etoposide (100 μ M) for 16h. Cleavage of caspase-3 was detected by Western blot as a marker for chemotherapy-induced apoptosis. Significant attenuation of apoptosis was observed in CDDP-R cells compared to parental controls (Figure 2A). To determine whether CDDP-R cells were also resistant to radiation-induced apoptosis, both cell types were irradiated with 20Gy and collected 48h later. Immunoblotting demonstrated less caspase-3 cleavage in CDDP-R H460 cells than in parental cells (Figure 2B). These data suggest that CDDP-R H460 cells are more resistant to apoptosis induced by either chemotherapy or radiotherapy. Consistent with less apoptosis seen by Western blot, MTS cell viability assays showed ~40% of CDDP-R cells survived following 20 μ M cisplatin treatment compared to 15% of parental cells (Figure 2C). Clonogenic assay confirmed these findings (data not shown), as we observed a plating efficiency of 49% in CDDP-R cells (DMSO 117%) following 1 μ M cisplatin, as compared to a plating efficiency of 17% in the parental cells (DMSO 103%). Similarly, clonogenic assay showed that CDDP-R cells were more resistant to radiation (DER = 1.21, $p < 0.01$) than parental cells (Figure 2D).

Reduced IGFBP-3 level in CDDP-R H460 cells

To determine the potential molecular differences that contributed to the chemo- and radioresistance of CDDP-R cells, microarray analyses were performed to compare the expression profiles of the parental and CDDP-R cells. Of the 20,364 genes examined in the microarray, 180 were found as differentially expressed genes (DEGs). 73 genes were up-regulated in CDDP-R cells and 107 were down-regulated. A heatmap is provided in the supplementary methods section. Next, a protein interaction network (PIN) was integrated with the microarray data. The PIN consists of 10,374 nodes and 50,909 edges. Nodes represent gene products and edges represent physical interactions between the interconnected nodes. DEGs were used as seeds for a Perturbed Subnetwork (PSN) search. This analysis resulted in a PSN with 212 genes and 404 edges. The giant component size, a measure of the group of nodes with the greatest number of members, was 195 (92%), indicating that the majority of the PSN is connected. We then used a data fusion model to prioritize genes considering both their degrees in the PSN and the change in expression. A Joint Rank Score (JRS) was calculated for each gene and the top 10 genes were identified (Table 1). Interestingly, IGFBP-3 ranked highest based on the fold change (–4.3) and the degree (12) in the PIN and, thus, it was selected as a prioritized gene for further characterization (Table 1, Figure 3). Additional details regarding the bioinformatics analyses are provided in the supplementary methods.

DNA hypermethylation plays a role in the inactivation of genes and subsequent treatment resistant phenotypes in cancer. To determine whether hypermethylation contributes to the reduced levels of IGFBP-3 seen in CDDP-R cells, cells were treated with the de-methylator 5'AZA-2'DC. Western blot and RT-PCR were performed on cells to find how de-methylation affected IGFBP-3 protein and DNA levels. Treatment with 5'AZA-2'DC increased IGFBP-3 protein and DNA levels in both parental and CDDP-R cells, suggesting that methylation is at least partially responsible for down-regulating IGFBP-3 levels (Figure 4A). MSP assay confirmed increased IGFBP-3 promoter methylation in CDDP-R cells compared to parental H460 cells (Figure 4B).

IGF-1 signaling is enhanced in CDDP-R H460 cells and addition of hrIGFBP-3 reverses cisplatin resistance in CDDP-R cells

To determine whether the reduced levels of IGFBP-3 transcript resulted in lower levels of IGFBP-3 protein, Western blot analysis was performed. Decreased levels of IGFBP-3 were expressed in CDDP-R cells than in parental H460 cells (Figure 5A). Since IGFBP-3 sequesters IGF-1 in the extracellular space, decreased levels of IGFBP-3 likely result in increased downstream activation of the IGF-1 receptor (IGF-1R). To test this hypothesis, immunoblots probing phosphorylated IGF-1R were performed following induction by recombinant IGF-1. A greater induction of phospho-IGF-1R was observed in CDDP-R H460 cells than in the parental cell line (Figure 5A and 5B) suggesting that CDDP-R cells can up-regulate the IGF1-R signaling pathway to a greater extent than parental H460 cells. To confirm that decreased IGFBP-3 expression contributes to the ability of CDDP-R cells to up-regulate IGF-1R signaling in response to IGF-1, hrIGFBP-3 was added to cells in increasing concentrations which reversed this effect (Figure 5B).

CDDP-R cells were treated with hrIGFBP-3 that sequesters IGF-1 and prevents binding to the IGF-1R. As shown by MTS assay, CDDP-R cells treated with 30 μ g/ml hrIGFBP-3 prior to 3 μ M cisplatin were significantly more sensitive to treatment than CDDP-R cells treated with cisplatin alone (Figure 5C).

IGF-1R inhibition results in sensitization to radiation and cisplatin

To determine whether inhibition of the IGF-1R signaling pathway could sensitize CDDP-R cells to cisplatin and radiation, siRNA against IGF-1R was transfected into the parental and CDDP-R cell lines. Western blot showed that the IGF-1R level was dramatically reduced at 48h after transfection (Figure 6A). MTS assay showed that inhibition of IGF-1R signaling by siRNA enhanced cisplatin-induced cell death in CDDP-R cells, as well as in parental cells (Figure 6B). Similarly, Clonogenic survival assays showed that siRNA against IGF-1R sensitized CDDP-R cells to radiation (DER = 1.17, $p < 0.05$) but this time had no effects on the parental cells (Figure 6C). Taken together, these findings suggest that the IGF-1 pathway is a potential target to overcome cisplatin- and radiation- resistance in lung cancer.

Discussion

In this study, we described CDDP-R cells that expressed higher levels of the cell surface markers CD133 and ALDH (Figure 1A) compared to parental H460 cells. These markers have been found in lung cancer stem cells and have been associated with cells which have stem cell like properties (10–12). While CD133 expression is correlated with treatment resistance, studies have not shown it to be a prognostic marker for the survival of NSCLC patients (8). In addition, CD133+ and CD133– A549 and H446 lung cancer cell subpopulations contain similar numbers of cancer stem cells, suggesting that the precise role of CD133 in cancer stem cells is still undiscovered (13). However, high ALDH1 protein expression has been identified as a poor prognostic marker, possibly because ALDH+ lung

tumor cells have more lung cancer stem cells than ALDH⁻ cells (14). Of note, ALDH⁺ cells isolated from NCI-H358 and NCI-H125 lung cancer cell lines show enrichment of tumorigenic CD133⁺ cancer cells (15). As the precise role of these cell surface markers has not been completely deciphered, the expression of CD133 and ALDH is not sufficient to suggest that CDDP-R cells are cancer stem cells. However, these findings hint that further testing of stem cell properties in CDDP-R cells is warranted.

CDDP-R cells in a xenograft lung cancer model quadrupled tumor size more rapidly (Figure 1B) than did the parental H460 cells. This suggests that resistant cells may be more aggressive than their parental cells. In addition, CDDP-R cells were also more resistant to both chemotherapy and radiation (Figure 2) compared to parental H460 cells. This data is consistent with previous findings which suggest that cells which acquire cisplatin resistance develop cross resistance to radiotherapy through similar mechanisms (4, 16).

To elucidate genes that play a role in the differential phenotype of CDDP-R cells, microarray analyses were performed (Figure 3). The gene expression data was ranked as described in the Results (Table 1). The most highly ranked gene was IGFBP-3, which modulates the IGF-1R signaling pathway. Thus, it is possible that this pathway contributes to the acquisition of treatment resistance by CDDP-R cells. Remarkably, the second-most highly ranked gene encodes transglutaminase, which was recently shown to protect epithelial ovarian cancer cells from cisplatin-induced apoptosis, providing an internal control for our microarray data collecting and processing (17).

Our finding that IGFBP-3 is suppressed in CDDP-R cells is consistent with previous studies. Hypermethylation of the IGFBP-3 promoter may contribute to this suppression (7). Such hypermethylation is seen in 61.5% of stage I NSCLC and is associated with a poor prognosis (18) in addition to contributing to cisplatin resistance in NSCLC cells (7). We found that treatment of the CDDP-R cells with the demethylator 5'AZA-2'DC increased both DNA and protein levels of IGFBP-3 (Figure 4), further suggesting hypermethylation as a mechanism of suppression. Further, treatment of CDDP-R cells with hrIGFBP-3 recovered sensitivity to cisplatin (Figure 5C). This suggests that the suppressed IGFBP-3 levels observed in CDDP-R cells partially contribute to the cisplatin-resistant phenotype of these cells. Similarly, studies in lung and colon cancer cells have shown tumor inhibition with hrIGFBP3 (19). Based on preliminary data, research assessing the feasibility or effectiveness of IGFBP3 analogues for cancer treatment in humans is warranted.

In concordance with decreased IGFBP-3, CDDP-R cells illustrated increased IGF-1R signaling in the presence of IGF-1 compared to parental H460 cells (Figure 5A). We hypothesized that these differences may contribute to treatment resistance of CDDP-R cells (Figure 7). Increased IGF-1R activation in breast cancer cell lines leads to increased Akt activation and resistance to tamoxifen (20). Inhibition of IGF-1R signaling has previously been found to increase the sensitivity of NSCLC cells to radiation (21) and to enhance the sensitivity of small cell lung cancer (SCLC) to etoposide and carboplatin (22). Using MTS and clonogenic assays, we demonstrated that the knockdown of IGF-1R rescued the cisplatin and radiation sensitivity of CDDP-R cells (Figure 6). These findings suggest that this pathway represents a promising target for overcoming treatment resistance. Fortunately, over 30 agents that target IGF-1R are already in preclinical or clinical studies and seem to have a favorable toxicity profile (23). In a recent phase II study, patients with NSCLC had higher responses rates when treated with the IGF-1R inhibitor figitumumab plus paclitaxel and carboplatin than when treated with paclitaxel and carboplatin alone (54% versus 42%, respectively) (24).

Profiling the gene expression of CDDP-R cells is important for identifying potential targets for overcoming therapeutic resistance. Molecular profiling of tumor tissue from patients with NSCLC allows tailoring of individualized therapy. The gene expression profiling for cisplatin resistance in lung cancer could be an important first step in heralding the age of personalized medicine.

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References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics: 2009. *CA Cancer J Clin.* 2009; 59:225–249. [PubMed: 19474385]
2. Wang G, Reed E, Li QQ. Molecular basis of cellular response to cisplatin chemotherapy in non-small cell lung cancer (Review). *Oncol Rep.* 2004; 12:955–965. [PubMed: 15492778]
3. Coughlin CT, Richmond RC. Biologic and clinical developments of cisplatin combined with radiation: concepts, utility, projections for new trials, and the emergence of carboplatin. *Semin Oncol.* 1989; 16:31–43. [PubMed: 2669136]
4. De Pooter CM, Scalliet PG, Elst HJ, et al. Resistance patterns between cis-diamminedichloroplatinum(II) and ionizing radiation. *Cancer Res.* 1991; 51:4523–4527. [PubMed: 1873796]
5. Butt AJ, Firth SM, King MA, et al. Insulin-like growth factor-binding protein-3 modulates expression of Bax and Bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. *J Biol Chem.* 2000; 275:39174–39181. [PubMed: 10998426]
6. Lee HY, Chun KH, Liu B, et al. Insulin-like growth factor binding protein-3 inhibits the growth of non-small cell lung cancer. *Cancer Res.* 2002; 62:3530–3537. [PubMed: 12068000]
7. Ibanez de Caceres I, Cortes-Sempere M, Moratilla C, et al. IGFBP-3 hypermethylation-derived deficiency mediates cisplatin resistance in non-small-cell lung cancer. *Oncogene.* 2010; 29:1681–1690. [PubMed: 20023704]
8. Salnikov AV, Gladkikh J, Moldenhauer G, et al. CD133 is indicative for a resistance phenotype but does not represent a prognostic marker for survival of non-small cell lung cancer patients. *Int J Cancer.* 2010; 126:950–958. [PubMed: 19676044]
9. Bolstad BM, Irizarry RA, Astrand M, et al. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics.* 2003; 19:185–193. [PubMed: 12538238]
10. Tirino V, Camerlingo R, Franco R, et al. The role of CD133 in the identification and characterisation of tumour-initiating cells in non-small-cell lung cancer. *Eur J Cardiothorac Surg.* 2009; 36:446–453. [PubMed: 19464919]
11. Moreb JS, Baker HV, Chang LJ, et al. ALDH isozymes downregulation affects cell growth, cell motility and gene expression in lung cancer cells. *Mol Cancer.* 2008; 7:87. [PubMed: 19025616]
12. Eramo A, Lotti F, Sette G, et al. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ.* 2008; 15:504–514. [PubMed: 18049477]
13. Meng X, Li M, Wang X, et al. Both CD133+ and CD133– subpopulations of A549 and H446 cells contain cancer-initiating cells. *Cancer Sci.* 2009; 100:1040–1046. [PubMed: 19385971]
14. Ishii H, Iwatsuki M, Ieta K, et al. Cancer stem cells and chemoradiation resistance. *Cancer Sci.* 2008; 99:1871–1877. [PubMed: 19016744]
15. Levina V, Marrangoni AM, DeMarco R, et al. Drug-selected human lung cancer stem cells: cytokine network, tumorigenic and metastatic properties. *PLoS One.* 2008; 3:e3077. [PubMed: 18728788]

16. Frit P, Canitrot Y, Muller C, et al. Cross-resistance to ionizing radiation in a murine leukemic cell line resistant to cis-dichlorodiammineplatinum(II): role of Ku autoantigen. *Mol Pharmacol*. 1999; 56:141–146. [PubMed: 10385694]
17. Cao L, Petrusca DN, Satpathy M, et al. Tissue transglutaminase protects epithelial ovarian cancer cells from cisplatin-induced apoptosis by promoting cell survival signaling. *Carcinogenesis*. 2008; 29:1893–1900. [PubMed: 18667446]
18. Chang YS, Wang L, Liu D, et al. Correlation between insulin-like growth factor-binding protein-3 promoter methylation and prognosis of patients with stage I non-small cell lung cancer. *Clin Cancer Res*. 2002; 8:3669–3675. [PubMed: 12473575]
19. Alami N, Page V, Yu Q, et al. Recombinant human insulin-like growth factor-binding protein 3 inhibits tumor growth and targets the Akt pathway in lung and colon cancer models. *Growth Horm IGF Res*. 2008; 18:487–496. [PubMed: 18502161]
20. Miller TW, Perez-Torres M, Narasanna A, et al. Loss of Phosphatase and Tensin homologue deleted on chromosome 10 engages ErbB3 and insulin-like growth factor-I receptor signaling to promote antiestrogen resistance in breast cancer. *Cancer Res*. 2009; 69:4192–4201. [PubMed: 19435893]
21. Iwasa T, Okamoto I, Suzuki M, et al. Inhibition of insulin-like growth factor 1 receptor by CP-751,871 radiosensitizes non-small cell lung cancer cells. *Clin Cancer Res*. 2009; 15:5117–5125. [PubMed: 19671857]
22. Warshamana-Greene GS, Litz J, Buchdunger E, et al. The insulin-like growth factor-I receptor kinase inhibitor, NVP-ADW742, sensitizes small cell lung cancer cell lines to the effects of chemotherapy. *Clin Cancer Res*. 2005; 11:1563–1571. [PubMed: 15746061]
23. Zha J, Lackner MR. Targeting the insulin-like growth factor receptor-1R pathway for cancer therapy. *Clin Cancer Res*. 2010; 16:2512–2517. [PubMed: 20388853]
24. Gualberto A, Karp DD. Development of the monoclonal antibody figitumumab, targeting the insulin-like growth factor-1 receptor, for the treatment of patients with non-small-cell lung cancer. *Clin Lung Cancer*. 2009; 10:273–280. [PubMed: 19632947]

Figure 1. CDDP-R H460 cells exhibit higher levels of markers associated with lung cancer stem cells and demonstrate more aggressive tumor growth

A. ALDH and CD133 were detected by immunoblotting of the total cell lysates of parental and CDDP-R cells. B. Growth curves showing parental and CDDP-R mean tumor volumes and standard errors (n=6); *, p<0.05; **, p<0.001.

Figure 2. CDDP-R H460 cells are resistant to chemo- and radio-therapies

A, B. Parental and CDDP-R H460 cells were exposed to cisplatin (20μM for 16h), etoposide (100μM for 16h), or 20Gy radiation, and cleaved caspase-3 was detected by Western blot. C. Parental and CDDP-R H460 cells were treated with various doses of cisplatin. Cell viability was measured by MTS assay. Shown are mean surviving fractions and standard deviations of each treatment group relative to control. **, $p < 0.001$. D. Clonogenic survival assay was performed with parental H460 and CDDP-R H460 cells and surviving colonies normalized for plating efficiency is shown. DER=1.21 ($p < 0.01$). Experiments were performed in triplicate.

Figure 3. The holistic view of protein subnetwork (PSN) shows IGFBP-3 and its interacting proteins

Shown is the subnetwork with IGFBP-3 and the full PSN (main view and inset, respectively). The IGFBP-3 node is boxed in both views. Each node represents a gene and the connecting lines represent physical protein interactions between the end nodes. Color changes reflect the expression fold-changes of cisplatin-resistant cells as compared to parental cells for each gene (red shows increased expression, green shows decreased expression).

Figure 4. Treatment with the de-methylator 5'AZA-2'DC increases protein and DNA levels of IGFBP-3

A. Parental and CDDP-R H460 cells were treated with 10 μ M 5Aza-dC for 72h. Cell lysates were harvested for Western blot and probed for IGFBP-3. RNA was collected for reverse transcription, which was performed following the manufacture's protocol and the IGFBP-3 gene was amplified. Actin was used as control. B. Genomic DNA was isolated from parental and CDDP-R H460 cells and primers were used to amplify the methylated (M) and unmethylated (U) IGFBP-3 promoter regions. Shown are qualitative levels of methylated and unmethylated IGFBP-3 promoters in parental and CDDP-R cells.

Figure 5. Reduced IGFBP-3 levels lead to enhanced IGF signaling in CDDP-R cells and hrIGFBP-3 reverses CDDP-R resistance to cisplatin

A, B. Parental and CDDP-R H460 cells were grown in 10% FBS medium, serum free medium, or medium supplemented with IGF-1 or insulin. Cells supplemented with IGF-1 were also exposed to 0.5 or 1 μg/ml of hrIGFBP-3. Lysates were collected and probed for phospho-IGF-1R, total IGF-1R, and IGFBP-3 by Western blot. Actin was used as control. C. CDDP-R cells were seeded at 5,000 cells/well in 96-well plates and allowed to attach overnight. Cells were then exposed to 30 μg/ml hrIGFBP-3 or 3 μM cisplatin, either alone or in combinations. After 72h, MTS assay was performed. Shown is a bar graph of mean survival fractions of each treatment group relative to control and standard errors. *: p<0.05, **: p<0.01. Experiments were performed in triplicate.

Figure 6. IGF-1R inhibition sensitizes CDDP-R cells to cisplatin and radiation

A. Parental and CDDP-R cells were transfected with control siRNA or IGF-1R siRNA. After 48h, cells were lysed and probed for IGF-1R by Western blot. Actin was used as control. B. Transfected cells were treated with 0, 3, or 20μM cisplatin. After 72h, MTS assay was performed according to the manufacture's instruction and absorbance at 490nm was recorded. Shown are bar graphs representing mean survival fraction relative to control and standard deviations. *: $p < 0.01$. C. Transfected cells were treated with 0, 2, 4, or 6Gy and shown are surviving colonies normalized for plating efficiency. Experiments were performed in triplicate.

Figure 7. CDDP-R cells have greater IGF pathway activation than the parental H460 cell line
 Binding of IGFBP-3 to IGF-1 prevents receptor binding and downstream pathway activation. With decreased expression of IGFBP-3 in CDDP-R cells, IGF-1 signaling through IGF-1R is maximized, leading to increased metabolism, proliferation, and survival through the Ras-raf-MAPK and PI3K-PDK/AKT-TOR-S6K pathways.

Table 1

Top 10 genes based on the Joint Rank Score (JRS)

Gene ID	Gene Symbol	Gene Name	JRS	Fold Change	Degree
3486	IGFBP3	Insulin-like growth factor binding protein 3	197.25	-4.30	12
7052	TGM2	Transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	182.5	3.09	9
1272	CNTN1	contactin 1	179.75	5.41	5
4739	NEDD9	Neural precursor cell expressed, developmentally down-regulated 9	174.25	-3.27	6
5069	PAPPA	Pregnancy-associated plasma protein A, pappalysin 1	167.5	-4.43	4
4922	NTS	Neurotensin	164.25	-15.35	3
6616	SNAP25	Synaptosomal-associated protein, 25kDa	162.25	2.89	5
4162	MCAM	Melanoma cell adhesion molecule	161.25	-7.04	3
10382	TUBB4	Tubulin, beta 4	160.75	-2.85	5
9047	SH2D2A	SH2 domain protein 2A	159.25	-5.40	3