Predictive Biomarkers and Personalized Medicine

Reduced Expression of miRNA-27a Modulates Cisplatin Resistance in Bladder Cancer by Targeting the Cystine/Glutamate Exchanger SLC7A11

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Abstract

Purpose: Resistance to cisplatin-based chemotherapy is a major obstacle to bladder cancer treatment. We aimed to identify microRNAs (miRNA) that are dysregulated in cisplatin-resistant disease, ascertain how these contribute to a drug-resistant phenotype, and how this resistance might be overcome.

Experimental Design: miRNA expression in paired cisplatin-resistant and -sensitive cell lines was measured. Dysregulated miRNAs were further studied for their ability to mediate resistance. The nature of the cisplatin-resistant phenotype was established by measurement of cisplatin/DNA adducts and intracellular glutathione (GSH). Candidate miRNAs were examined for their ability to (i) mediate resistance and (ii) alter the expression of a candidate target protein (SLC7A11); direct regulation of SLC7A11 was confirmed using a luciferase assay. SLC7A11 protein and mRNA, and miRNA-27a were quantified in patient tumor material.

Results: A panel of miRNAs were found to be dysregulated in cisplatin-resistant cells. miRNA-27a was found to target the cystine/glutamate exchanger SLC7A11 and to contribute to cisplatin resistance through modulation of GSH biosynthesis. In patients, SLC7A11 expression was inversely related to miRNA-27a expression, and those tumors with high mRNA expression or high membrane staining for SLC7A11 experienced poorer clinical outcomes. Resistant cell lines were resensitized by restoring miRNA-27a expression or reducing SLC7A11 activity with siRNA or with sulfasalazine.

Conclusion: Our findings indicate that miRNA-27a negatively regulates SLC7A11 in cisplatin-resistant bladder cancer, and shows promise as a marker for patients likely to benefit from cisplatin-based chemotherapy. SLC7A11 inhibition with sulfasalazine may be a promising therapeutic approach to the treatment of cisplatin-resistant disease.

Introduction

Around 7.6 million individuals die from cancer annually (1). Despite recent advances, the prognosis for advanced tumors remains poor. One of the front-line treatments for bladder cancer is cisplatin-based combination chemotherapy, but the effectiveness of this treatment is severely limited through the development of cisplatin resistance. Most patients with advanced bladder cancer typically show a good initial response to treatment, but ultimately 90% of these patients will suffer a recurrence of cisplatin-resistant disease (2). In solid tumors, methods to lower cisplatin concentration within cells, such as increased drug efflux, reduced influx, or sequestration seem to be amongst the predominant mechanisms of resistance. The latter may be achieved by a variety of compounds, including glutathione (GSH). This thiol-containing tripeptide is synthesized by nearly all cells; it is a strong electron donor and protects against the harmful effects of various endogenous stresses by quenching reactive hydroxyl-free radicals, other oxygen-centered free radicals, and radical centers on DNA and other biomolecules (3). In this way, GSH is also able to protect cells from the cytotoxic effects of various chemotherapeutics, including cisplatin (4), and radiotherapy. A rate-limiting step in GSH synthesis is the availability of cysteine (5), which provides the cysteine moiety of GSH. Cystine import is carried out by the heterodimeric \( \chi \)-cystine-glutamate transporter (6), which is composed of SLC3A2 (also known

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Translational Relevance

Cisplatin-based chemotherapy is the first-line systemic treatment for advanced bladder and ovarian cancers. Most patients with these cancers eventually suffer recurrence with cisplatin-resistant disease. Mechanisms by which resistance is achieved are varied and better understanding of these is essential for the development of re-sensitization strategies. We found consistent dysregulation of a panel of microRNAs (miRNA) during the development of cisplatin resistance in bladder cancer, and showed that one of these miRNAs (miRNA-27a) targets a cysteine/glutamate exchanger, which mediates glutathione synthesis SLC7A11. Cancer samples from patients showed that dysregulation of these molecules stratified clinical outcomes in patients treated with cisplatin. Cisplatin resistance in our cells could be reversed using siRNA to the target genes and sulfasalazine, a U.S. Food and Drug Administration–approved drug known to inhibit SLC7A11.

as 4F2HC) and SLC7A11 (also known as xCT). Upregulation of SLC7A11 has been reported as a mechanism of cisplatin resistance in ovarian cancer (7).

Resistance to cisplatin treatment in bladder cancer is common, and can be mediated through one or more of a large number of pathways (8). The changes in protein expression that underpin these pathways may arise through genetic or epigenetic means (8). The latter include changes in DNA methylation and microRNA (miRNA) expression (9). These short single-stranded RNAs play key roles in many carcinogenic processes (10) and primarily act as negative regulators of genes through the interaction with the 3′ untranslated region (UTR) of target mRNAs resulting in either mRNA destruction or inhibition of translation (11). Alterations in expression of particular miRNAs following the development of cisplatin resistance with functional consequences for target mRNAs is well documented in various cancer cell lines including those derived from breast (12) and ovarian cancers (13). Here, we present evidence that a change in expression of miRNA-27a contributes to cisplatin resistance in bladder cancer through modulating the expression of the SLC7A11 and as a result, increasing levels of intracellular GSH. We demonstrate a way to reverse resistance that has clinical potential and suggest that the biochemical consequences of this mechanism of resistance may be used to stratify drug choice in patients with advanced cancer.

Materials and Methods

Cell lines, single cell clones, and cisplatin-resistant variants

Cisplatin-resistant cell lines were generated from heterogeneous cells and sensitive single cell clones using EJ/T24 and RT112 bladder cancer lines. Cells were grown in increasing drug concentrations (10%–20% increase per passage at 70% confluence) for 4 months. Primary cells were purchased from American Type Culture Collection and grown in Dulbecco’s Modified Eagle Medium with 10% fetal calf serum. The adapted cell lines are deposited at the Health Protection Agency. A2780-DDP cells were obtained from Sigma.

Clonogenic survival assay

Five hundred to 2,500 cells were plated in triplicate onto 100-mm dishes 4 hours before the addition of increasing doses of cisplatin as indicated. Ten days later, when colonies could be observed, they were fixed and stained with methylene blue in ethanol (4 g/L). Colonies consisting of more than 50 cells were subsequently counted. Each colony was assumed to represent one cell surviving from the original number plated and the surviving fraction for each dose calculated compared with untreated control. When siRNA-depleted cells were used, they were transfected as above for 48 hours, then replated in the presence or absence of increasing doses of cisplatin.

Quantification of miRNA expression

Total RNA was extracted using the mirVana Kit (Ambion). The expression of 357 miRNAs (miR) was determined using real-time PCR (RT-PCR) with microfluidic cards (Human miRNA v1.0, Applied Biosystems) used as per the manufacturer’s instructions and analyzed on an ABI 7900HT RT-PCR system. Relative expression was calculated from the ΔCt value for each miRNA, calculated by the subtraction of the plate mean Ct value from the Ct value for each specific miRNA. ΔΔCt values between resistant and sensitive lines were calculated and used to generate fold changes in expression using the expression 2−ΔΔCt.

Standalone TaqMan quantitative PCR (qPCR) assays (Applied Biosystems), used according to the manufacturer’s instructions were also used to confirm downregulation of miRNA-27a in resistant cells.

Measurement of cisplatin adducts in DNA

Cells were treated with cisplatin for 2 hours, harvested at relevant time points, and frozen at −80°C until analysis. Genomic DNA was extracted using a DNeasy Kit (Qiagen), treated with RNaseA (Sigma) to remove cisplatin-damaged RNA. Exactly 1 μg DNA from each sample was loaded into a dot blotter (Bio-Rad), washed thoroughly with 20× saline-sodium phosphate-EDTA buffer, and baked (80°C, 30 minutes). The DNA was then blocked with 5% milk, probed using R-C18 monoclonal antibody (Oncolysle) raised against 1,2 (GG) intrastrand adducts (14), and then with horseradish peroxidase-conjugated anti-rat immunoglobulin G (#7707– NEB). Dots were developed using a chemiluminescent substrate (GE Healthcare), visualized and quantified using a LAS-3000 imager (Fujifilm).

Expression of cisplatin transport and sequestration molecules

The expression of mRNAs important for cisplatin transport (ATP7A, ATP7B, SLC31A1; CTR1) and GSH biosynthesis
[SLC3A2 (4F2HC), GCLC, GCLM, GSS, GSR, SLC7A11 (xCT)] was measured using RT-PCR of cDNA generated by reverse transcription of cellular RNA using random hexamers. Primers were validated using melting curve analysis, and by inspection of an electrophoretic gel for a single band of the expected size. miRNA quantification was normalized to the average of β-actin and U1 RNA expression and fold changes in resistant cells were calculated using ΔΔCt values. The expression of ATP7A, SLC31A1, and SLC7A11 proteins were measured using Western blotting using primary antibodies (SC-32900 and SC-66847; Santa Cruz Biotechnology) and NB300–318 (Novus).

Cellular glutathione concentration
Levels of intracellular GSH were measured using a GSH-Glo glutathione assay kit (Promega). In triplicate, 5,000 cells were plated, left for 4 hours before measuring the luminescence with a SpectraMax luminometer (Molecular Devices), according to the manufacturer’s instructions. Tris (2-carboxyethyl)phosphine (Thermo) was added to reduce oxidized GSH and ascertain the levels of total GSH present in each sample.

Restoration of underexpressed miRNA expression
To modulate miR expression, cells were transfected with pre-miR miRNA precursors or relevant scrambled negative control RNAs (Applied Biosystems) using Lipofectamine RNAiMAX (Invitrogen). Six-well plates were loaded with 5 pmol pre-miR in 500 μL serum-free medium to which 5 μL RNAiMAX was added 20 minutes before 100,000 cells. Cells were used after 48 to 72 hours and desired alteration of miR expression level was confirmed using a TaqMan miRNA qPCR (Applied Biosystems).

Luciferase reporter assay
The first 1,100 bp of the SLC7A11 3’UTR containing the miRNA-27a–binding site was cloned from EJ-R/EJ mixed cDNA and ligated into pMIR REPORT (Invitrogen). The resulting pMIR REPORT + SLC7A11 3’UTR plasmid was subjected to a round of site-directed mutagenesis to introduce two mutations into the miRNA-27a–binding site. Dual luciferase assays were conducted in a 6-well plate format, pMIR REPORT + SLC7A11 3’UTR and pMIR REPORT + Mutated SLC7A11 3’UTR (100 ng) was transfected into 70% confluent HEK293 cells, along with miScript miRNA-27a inhibitor, or scrambled RNA control (50 pmol/L; Applied Biosystems) and pRL-TK Renilla luciferase plasmid (0.7 ng). Forty-eight hours posttransfection, firefly and Renilla luciferase were quantified sequentially using the Dual Luciferase Assay Kit (Promega), and luminescence was measured using the manufacturer’s recommended luminometer (Promega Glomax). Firefly luciferase expression was quantified and normalized to Renilla luciferase expression.

siRNA knockdown and small-molecule inhibition of SLC7A11
SLC7A11 was knocked down using a commercially available ON-TARGET smartpool RNA from Thermo Fisher. Sulfasalazine was obtained from Sigma. For clonogenic assays, cells were pretreated for 48 hours with siRNA/sulfasalazine before replating and treating with cisplatin.

Patients and tumors
To evaluate cell culture findings in human bladder cancer, we examined the tumors from 354 patients in two separate cohorts (Supplementary Tables S1 and S2). The first cohort included 139 primary tumors representing all disease phenotypes, collected prospectively from the Royal Hallamshire Hospital, (Sheffield, United Kingdom). We microdissected sections of fresh-frozen tissue to enrich for tumor cells (>90%) and extracted total RNA, as above. The second cohort included 215 patients treated with adjuvant chemotherapy for invasive bladder cancer. Of these, the majority (n = 149) were from a phase III, multicentre randomized controlled trial conducted by the German Arbeitsgemeinschaft Urologische Onkologie (AUO) comparing two adjuvant regimens (cisplatin/methotrexate vs. methotrexate/vinblastine/adriamycin/cisplatin; AUO-AB 05/95: ref. 15). For each patient, we obtained archived formalin-fixed paraffin-embedded (FFPE) tissue and extracted 3 × 1.5 mm cores (33–31A P/25, Miltex Inc) from cancerous regions to construct a tissue microarray (TMA) as detailed elsewhere (16, 17). TMA content and construction were controled by a certified uropathologist (A. Hartmann).

Tissue array
Protein expression was determined using automated immunohistochemistry (program: SCC1, Benchmark XT, Ventana Medical Systems, Oro Valley, AZ). For SLC7A11, we used a polyclonal antibody (xCT, Thermo-Scientific; Cat.-No. PA1–16893) at 1:400 dilution for 30 minutes. Sections were visualized with diaminobenzidine chromogen and counterstained in a modified Mayer hematoxylin. Negative controls were performed using normal serum rather than SLC7A11 antibody. Membranous staining was scored for intensity and percentage of positive cells. High SLC7A11 expression was defined as strong intensity in 15% or more of cells.

Statistical analyses
Associations between miR expression and cisplatin sensitivity were tested using fold changes in resistant and sensitive cells. We identified miRs that consistently showed more than a 2-fold difference (either gain or loss) in expression between resistant and sensitive cells, and selected those in which this reached statistical significance (defined as P < 0.05, Student t test). We compared these with miR expression profiles in primary bladder cancer (18) generated using the same array. Hierarchical clustering was performed using Cluster 3.0 and visualized in Tree view (Eisen Lab). MiRNAs that were differentially expressed in resistant compared with sensitive cells were used to hierarchically cluster samples according to the cell line and presence/absence of chemoresistance. Correlation between continuous variables was assessed using Pearson correlation coefficient within SPSS.
For patient outcomes, we compared RNA and protein expression with tumor pathology and subsequent phenotype. Associations between clinicopathological features and tumor behavior were examined using univariable and stepwise multivariable Cox proportional hazards regression models (SPSS Vsn. 14.0; SPSS Inc.). Outcomes with respect to time were plotted using the Kaplan–Meier method and compared using a log-rank test within SPSS (Vsn. 19.0 SPSS Inc.). Tumor progression was defined as the presence of pathologic, radiologic, or clinical evidence of an increase in tumor stage and measured from the time of surgery to the time of proven event. Where cisplatin sensitivity was compared between cell lines or between control cells and cells treated with a drug, precursor miRNA or siRNA statistical significance was calculated using Student two-tailed t test.

Results

miRNA expression is altered in cisplatin-resistant bladder cancer cells

To identify changes in miRNA expression associated with cisplatin resistance, we compared matched cisplatin-sensitive and -resistant bladder cancer cell lines. The latter were derived by culturing the bladder cancer cell line EJ, or single cell clones derived from the original heterogeneous EJ (namely clones D4 and G7) in media augmented with increasing cisplatin concentrations for several months. This resulted in three cisplatin-resistant cell lines (EJ-R, D4-R, and G7-R), all of which were able to tolerate much higher concentrations of cisplatin than any of the parental lines (Fig. 1A). IC\textsubscript{50} concentrations for cisplatin were typically <1 \textmu mol/L for sensitive lines and 10 \textmu mol/L for resistant lines. The expression of 160 miRNAs was measured using RT-PCR. A decrease in miRNA expression was observed in the cisplatin-resistant cells compared to the parental lines in 48 miRNAs. The complete list of miRNAs is detailed in Table 1. A hierarchical clustering analysis using 32 selected miRs (red, low expression; green, high expression) stratifies cells according to cisplatin resistance (orange, resistant; white, sensitive) regardless of cell line or method of derivation.

Figure 1. Characterization of cisplatin-resistant cell lines and miRNA expression. A, clonogenic survival assays of cisplatin-treated bladder cancer cell lines and their cisplatin-resistant derivatives (designated as respective cell line followed by R). Error bars, SD; n = 4. B, fold-change in expression of C. 160 miRNAs in three paired sensitive and resistant lines. C, hierarchical clustering using 32 selected miRs (red, low expression; green, high expression)
lines. MiRNA expression profiling in these paired cell lines revealed that although the expression of the majority of miRNAs remained unchanged (Fig. 1B), a miRNA signature could be identified that stratified cisplatin resistance (Fig. 1C). A second set of paired cisplatin-sensitive and -resistant bladder cancer cell lines, generated in the same way using RT112 cells as the parental line (RT112 and RT112-R), were also included in this analysis, and displayed a similar miRNA profile (Fig. 1C).

Cisplatin induces fewer 1,2 (G,G) intrastrand adducts in resistant cells

Resistance to cisplatin can occur either before DNA adduct formation via changes in cellular metabolism of cisplatin or postadduct formation via changes in DNA damage repair. To initially characterize the mechanism by which cisplatin resistance had arisen in our cells, we measured cisplatin-induced 1,2 (G,G) intrastrand DNA cross-links using monoclonal antibodies directed against these adducts in EJ and EJ-R cells (14). Two hours following treatment-resistant cells displayed significantly fewer 1,2 (G,G) intrastrand cross-links compared with sensitive cells (Fig. 2A; \( P < 0.001 \) for all concentrations of cisplatin above 1 \( \mu \)mol/L). B, densitromic plot of extent of 1,2 (G,G) intrastrand cisplatin-DNA adduct repair in EJ/EJ-R for 48 hours following a 2-hour treatment with 12 \( \mu \)mol/L cisplatin. Error bars, SD; \( n = 3 \). C, example dot blot used to generate data visualized in Fig. 3B.

Cisplatin resistance is mediated through increased expression of SLC7A11 and increased production of glutathione

The expression of copper transporter proteins are known to be responsible for the efflux (ATP7A/ATP7B; ref. 19) and influx (CTR1; ref. 20) of cisplatin, and their dysregulation has been linked with cisplatin resistance in some cancers, most notably ovarian (21). Initial analysis of mRNA levels of these copper transporters revealed that ATP7B mRNA was significantly downregulated by approximately 50% in the three resistant lines, whereas ATP7A mRNA was significantly upregulated by around 40% (Fig. 3A). Increased mRNA expression of the cisplatin efflux protein ATP7A was not considered a likely candidate for the modulation of cisplatin resistance; however, as this increased mRNA expression was not accompanied by an increase in protein levels as evidenced by Western blot analysis (Fig. 3D). CTR1 mRNA was typically 3-fold upregulated in all resistant cell lines when compared with corresponding sensitive cell lines (Fig. 3A). This is the opposite of what would be expected were these influx and efflux proteins to be involved in the regulation of cisplatin resistance and, like ATP7A, this altered mRNA expression was not reflected in levels of the CTR1 protein (Fig. 3D). This suggests that changes in the cellular import and export of cisplatin are unlikely to be responsible for the resistance of our bladder cancer cell lines to cisplatin.
Following import into cells, cisplatin is able to form conjugates with GSH leading to its sequestration and detoxification (22), and increased GSH levels have been observed in other cisplatin-resistant cell lines (23). We found significantly increased concentrations of both reduced (GSH) and oxidized glutathione (GSSG) GSH in cisplatin-resistant cells (Fig. 3B). Resistant cells displayed levels of GSH and GSSG approximately double that of the sensitive parental line, suggesting that increased GSH production might be responsible for cisplatin resistance. We examined mRNA expression of the six major genes whose protein products are concerned with GSH biogenesis in three paired cisplatin-sensitive (EJ, D4, G7) and -resistant (EJ-R, D4-R, G7-R) cell lines (Fig. 3C). Many of these genes displayed a degree of small but significant upregulation in resistant cells. However, one gene, SLC7A11, was particularly significantly overexpressed in resistant cells, displaying a 10-fold increase in expression in resistant lines and a high degree of statistical significance. We also examined the expression of SLC7A11 protein and found it to be overexpressed in resistant cells (Fig. 3D). SLC7A11 is one part of the heterodimeric x_cysteine/glutamate exchanger, and as cystine bioavailability is a critical factor in the rate of GSH production, it may represent the point at which the increased rate of GSH production in resistant cells is regulated. This suggests that changes in the expression of genes involved in GSH production, particularly SLC7A11, might be responsible for resistance in our resistant cell lines.

Overexpression of miRNA-27a reduces levels of SLC7A11 and intracellular glutathione, and resensitizes resistant cells to cisplatin

To determine whether SLC7A11 was a candidate for regulation by miRNA, we examined the 3'UTR for sites of potential miRNA interaction using TargetScan (www.targetscan.org) and compared them with our expression profiles. Of the miRNAs predicted to target the 3'UTR of SLC7A11, we observed that miRNAs-25 and -27a were significantly downregulated in our resistant lines and had
appeared in our panel of dysregulated miRs (Fig. 1C), in keeping with consequential upregulation of SLC7A11. MiRNA-32 was also shown to have the potential to interact with the 3’UTR of SLC7A11, and although it did not appear in our signature of dysregulated miRs, it did show consistent downregulation in all resistant cells compared with their sensitive parental lines. However, transfection of resistant cells with a commercially available pre-miR precursor to miRNA-27a, but not miRNA-25 or -32, lowered the expression of SLC7A11 (Fig. 4A) and increased sensitivity by 100-fold following a 12 μmol/L treatment with cisplatin (Fig. 4B). This suggests that reduced expression of miRNA-27a can induce cisplatin resistance in bladder cancer cells, by reducing the level of cellular SLC7A11. Downregulation of miRNA-27a in EJ-R cells was also further confirmed by standalone qPCR (Supplementary Fig. S1). The restoration of cisplatin sensitivity following increased expression of miRNA-27a was associated with an increase in 1,2 (G,G) intrastrand adducts following cisplatin treatment (Fig. 4C) and a decrease in intracellular GSH (Fig. 4D), supporting the hypothesis that miRNA-27a downregulation protects cells from the cytotoxic effect of cisplatin via alterations in GSH biosynthesis. To determine whether the SLC7A11 3’UTR can be a direct target of miRNA-27a in living cells, we constructed a luciferase reporter assay using the wild-type and a mutant SLC7A11 miR-27 seed sequence. Inhibition of miRNA-27a production in HEK293 cells using a commercially available inhibitory RNA significantly increased.

Figure 4. Effect of miRNA-27a manipulation on SLC7A11 expression, cisplatin sensitivity, and cisplatin genotoxicity in resistant bladder and ovarian cancer cells. A, Western blot analysis of SLC7A11 expression following transfection of cisplatin-resistant cells with miRNA-27a, miRNA-25, or miRNA-32, (miR-27a, -25, -32). B, effect of increased miRNA-27a expression on cisplatin sensitivity of EJ-R cells compared with a scrambled control oligo as measured by clonogenic assay. Error bars, SD; n = 3. C, effect of increased miRNA-27a expression of level of cisplatin-induced DNA damage in EJ-R cells compared with untreated and scrambled RNA controls. Error bars, SD; n = 3. D, effect of increased miRNA-27a expression on levels of reduced (black bars) and total (white bars) GSH. * = P < 0.01. Error bars, SD; n = 3. E, effect on luciferase expression of a miRNA-27a inhibitor in HEK293 cells compared with scrambled control. The luciferase gene was augmented with a portion of the 3’ UTR of SLC7A11 containing the miRNA-27a–binding site, or a similar construct with the miRNA-27a abolished by site-directed mutagenesis (mutant). F, effect of increased miRNA-27a expression (miR-27a) or transfection with a scrambled control (Scrm) on cisplatin sensitivity, the cisplatin resistant ovarian cancer cell line A2780-cDDP as measured by clonogenic assay. Error bars, SD; n = 3.
expression of luciferase reporter augmented with the relevant part of the SLC7A11 3'UTR. This effect was abolished by mutation of the miRNA-27a seed sequence (Fig. 4E).

The cisplatin sensitivity of a resistant ovarian cancer cell line can also be increased by modulation of miRNA27a levels

Cisplatin resistance though SLC7A11 was first reported when the drug-resistant ovarian cancer line A2780-cDDP was found to have higher cystine uptake and intracellular GSH concentrations than its cisplatin-sensitive parent (7). Resistance could be induced with transfection of components of the Xc_− transporter (SLC7A11 and SLC3A2). We transfected A2780-cDDP with pre-miR-27a, and similar to EJ-R cells, transfection resulted in a 10-fold increase in cisplatin sensitivity, suggesting that altered levels of miRNA-27a can effect cisplatin sensitivity in other cancer types.

SLC7A11 is a key modulator of cisplatin resistance in bladder cancer cells

Evidence of the importance of SLC7A11 in the regulation of cisplatin resistance is demonstrated by the increased sensitivity of formerly resistant EJ-R cells to cisplatin when transfected with siRNA against SLC7A11 (Fig. 5A and B). Taken together, these data suggest that direct targeting of the SLC7A11 mRNA 3'UTR by miRNA-27a, alters GSH levels in cells, which can then sequester and detoxify cisplatin, resulting in decreased cisplatin-induced DNA adduct formation and consequently cellular resistance to cisplatin.

Inhibition of SLC7A11 with sulfasalazine, at clinically relevant doses, resensitizes cisplatin-resistant bladder cancer cells

The Xc_− transporter has recently been implicated as a causative mechanism for seizures in patients with glioma (24). Specifically, upregulation of Xc_− led to high peritumoral concentrations of glutamate, a neuro-excitatory amino acid that precipitated seizures (25). Although the role of Xc_− is unknown in glioma, seizures could be reduced in a model using the proven Xc inhibitor sulfasalazine (26) at doses equivalent to those used to treat Crohn inflammatory bowel disease (250 μmol/L) in humans (24). We evaluated the potential for sulfasalazine to induce cisplatin sensitivity in our cells through Xc_− inhibition. We found that this dose of sulfasalazine increased the cisplatin sensitivity of resistant cells more than 10-fold compared with a dimethyl sulfoxide control (Fig. 5C).

miRNA-27a/b expression correlates with increased SLC7A11 expression and poor prognosis in patients with bladder cancer

To examine the clinical significance of our current findings, we studied tumors from two distinct patient cohorts. The first included 139 primary tumors representing the disease spectrum. Although our in vitro data implicate miRNA-27a in cisplatin resistance, its similarity to miRNA-27b and the fact that both were downregulated in resistant cells, combined with our previous observation that both are abnormally expressed in primary bladder cancer (18), lead us to examine the expression of both in this first cohort. We found that the expression of miRNAs-27a/27b was directly correlated (r = 0.75; P < 0.001) and inversely related to SLC7A11 mRNA expression (r = -0.64; P > 0.001; Fig. 6A and Supplementary Table S1). When outcome was analyzed, tumors with high SLC7A11 and/or low miRNA-27a/27b expression progressed more frequently to advanced disease following treatment, compared with others (log-rank P < 0.04; Fig. 6B). The second cohort included the tumors from 215 patients treated with chemotherapy as part of a randomized control trial comparing

Figure 5. Effect of SLC7A11 knockdown and inhibition on cisplatin sensitivity. A, use of siRNA directed against SLC7A11 to reduce SLC7A11 protein levels in EJ-R cells 48 hours posttransfection. B, effect of SLC7A11 knockdown compared with scrambled RNA control on cisplatin sensitivity of EJ-R cells as measured by clonogenic assay. Error bars, SD; n = 3. **, P < 0.005. C, effect of sulfasalazine pretreatment on cisplatin sensitivity of EJ-R cells as measured by clonogenic assay. Error bars, SD; n = 3. *, P < 0.1.
two cisplatin-based regimens (15) with gemcitabine–cisplatin. High membranous expression of SLC7A11 was identified in 22 of the 215 (10.2%) tumors and was associated with poor cisplatin response ($P < 0.04$; Fig. 6C). In particular, patients whose tumors had high SLC7A11 protein expression responded to cisplatin less frequently and for a shorter duration than those with little or no expression. In multivariable analysis, SLC7A11 expression [HR 2.0; 95% confidence interval (CI), 1.1–3.5; $P < 0.017$] and extent of lymph node involvement (HR 4.4; 95% CI, 1.1–18.5; $P = 0.04$) were the only variables independently predicting progression and cancer-specific survival (Fig. 6D and Supplementary Table S2).

Discussion

Many previous studies have examined alterations in miRNA expression during the development of cisplatin resistance in various cancer lines including those derived from hepatocellular carcinoma (27), ovarian cancer (28), non–small cell lung cancer (29), and germline tumors (29). None of these studies identified miRNA-27a as a potential marker for or mediator of cisplatin resistance. The action of specific miRNAs tends to be highly specific to particular tissues, and consistent with this miRNA-27a has been reported as having both oncogenic and tumor-suppressive roles in various tumors. In cancer types such as breast (30) and prostate (31), miRNA-27a has been considered an onco-miR, with high levels of expression being associated with a poor prognosis, whereas in acute leukemia, it has been suggested that miRNA-27a functions as a tumor suppressor (32). Consistent with our data suggesting miRNA-27a as a key mediator of drug resistance and prognosis in the context of bladder cancer, a recent paper found miRNA-27a to be consistently downregulated in FFPE bladder tumor material from patients who experienced disease progression when compared with material from those patients who experienced a complete response (33).

MiRNA-27a forms part of a larger signature of miRNA expression that stratifies cisplatin resistance in bladder cancer. Although many of these miRNAs do not have functional roles in the mediation of cisplatin resistance, we have uncovered a critical role for miRNA-27a, namely its decreased expression in cisplatin-resistant bladder cancer modulating SLC7A11 upregulation and therefore, allowing for increased cystine import and increased GSH synthesis. This linked our miRNA signature with well-established and widely studied mechanisms of cisplatin resistance (34).

When designing our experiments, we were interested in understanding whether our mechanism of drug resistance would arise de novo with carcinogenesis (i.e., in cells not exposed to cisplatin) or be acquired during cisplatin treatment. It is likely that both occur in cancer, as many hallmarks of carcinogenesis produce drug resistance as a secondary phenotype (e.g., apoptosis avoidance). In an attempt to model these different scenarios, we derived drug-resistant cells from two different cell populations, heterogeneous cells and expansions of single cell clones. Although resistance in former may reflect the selection of de novo resistant-cells, the latter models acquired resistance by the conversion of sensitive to resistant cells. It was
interesting to see that our resistance mechanism was common to all derived cells, regardless of initial population. Indeed, miRNA profiling revealed many other similarities between the resistant cell lines.

Our findings have direct clinical implications for patient care. First, analysis of tumor material revealed that miRNA-27a downregulation and associated SLC7A11 upregulation are not unique to a cell culture model of cisplatin resistance and occur in tumors in vivo. Second, we identified that cisplatin resistance could be reversed by either reinstatement of the miRNA, by siRNA-induced knockdown of SLC7A11, or by inhibition of SLC7A11 with a small-molecule inhibitor. Because the latter is possible with clinically achievable doses of sulfasalazine, our findings highlight the need to evaluate this drug in combination with cisplatin within a clinical trial. Indeed, the side-effect profiles of these agents suggest little cross-toxicity, beyond bone marrow suppression. Third, the expression of miRNA-27a/27b and SLC7A11, or increased intracellular GSH within tumors may be used to predict cisplatin response in individual patients. This could guide chemotherapy choice toward alternative agents not detoxified by GSH in tumors likely to be resistant.

Recent radiologic advances have enabled the spatial and temporal resolution of glutamate (26) and GSH (35) in vivo. Our findings suggest that this imaging modality could determine glutamate concentrations within a tumor and could be used as a surrogate for cisplatin resistance (36). This approach could inform the initial chemotherapy regimen and monitor drug resistance before clinical relapse occurs.

In summary, we have identified a novel mechanism of cisplatin resistance in cancer namely increased expression of microRNA-27a mediated regulation of intracellular GSH. The direct clinical implications of our work are that this mechanism may be overcome by clinically achievable doses of sulfasalazine and could be predicted using MRI spectroscopy to measure tumor glutamate concentrations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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