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Reduced expression of microRNA-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 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—MicroRNA expression in paired cisplatin resistant and sensitive cell lines was measured. Dysregulated microRNAs 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. Candidate microRNAs 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 microRNA-27a were quantified in patient tumour material.

Results—A panel of microRNAs were found to be dysregulated in cisplatin resistant cells. MicroRNA-27a was found to target the cystine/glutamate exchanger SLC7A11 and to contribute to cisplatin resistance through modulation of glutathione biosynthesis. In patients, SLC7A11 expression was inversely related to microRNA-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 microRNA-27a expression, or reducing SLC7A11 activity with an siRNA or with sulfasalazine.

Conclusion—Our findings indicate that microRNA-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

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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 appear 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 cystine (5), which provides the cysteine moiety of glutathione. Cystine import is carried out by the hetrodimeric x_c- cystine-glutamate transporter (6), which is comprised of SLC3A2 (also known 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 of 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 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′UTR of target mRNAs resulting in either mRNA destruction or inhibition of translation (11). Alterations in expression of particular microRNAs 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 microRNA-27a contributes to cisplatin resistance in bladder cancer through modulating the expression of the SLC7A11 and as a result, increasing levels of intracellular glutathione. 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 ATCC and grown in DMEM with 10% fetal calf serum. The adapted cell lines are deposited at the Health Protection Agency, UK. A2780-DDP cells were obtained from Sigma, UK.

Clonogenic survival assay

500 – 2500 cells were plated in triplicate onto 100 mm dishes 4 hours prior to 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 methanol (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 to 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 microRNA expression

Total RNA was extracted using the mirVana™ kit (Ambion, TX). The expression of 357 miRs was determined using rtPCR with microfluidic cards (Human microRNA v1.0, Applied Biosystems, Warrington, UK) used as per manufacturers instructions and analysed on an ABI 7900HT real time PCR system. Relative expression was calculated from the ΔCt value for each microRNA, calculated by the subtraction of the plate mean Ct value from the Ct value for each specific microRNA. ΔΔCt values between resistant and sensitive lines were calculated, and used to generate fold changes in expression using the expression $2^{-\Delta\Delta Ct}$. Standalone taqman qPCR assays (Applied biosystems), used according to the manufacturers instructions were also used to confirm downregulation of microRNA-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 RNAse A (Sigma, UK) to remove cisplatin-damaged RNA. Exactly 1 μg DNA from each sample was loaded into a dotblotter (Biorad, CA), washed thoroughly with 20x SSPE buffer and baked (80°C, 30 mins). The DNA was then blocked with 5% milk and probed using R-C18 monoclonal antibody (Oncolyse, Germany) raised against 1,2 [GG] intrastrand adducts (14) and then with an HRP-conjugated anti-rat IgG (#7707 – NEB, US). Dots were developed using a chemiluminescent substrate (GE Healthcare, UK), 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 glutathione biosynthesis (SLC3A2(4F2HC), GCLC, GCLM, GSS, GSR, SLC7A11(xCT)) was measured using real time 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. mRNA quantification was normalized to the average of β -actin and U1 RNA expression and fold changes in resistant cells calculated using from 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) and NB300-318 (Novus).

Cellular glutathione concentration

Levels of intracellular GSH were measured using a GSH-Glo ™ glutathione assay kit (Promega, UK). In triplicate, 5000 cells were plated, left for 4hrs before measuring the luminescence with a SpectraMax luminometer (Molecular Devices, US), according to manufacturers instructions. Tris(2-carboxyethyl)phosphine (Thermo, UK) was added to reduce oxidized GSH and ascertain the levels of total GSH present in each sample.

Restoration of underexpressed microRNA expression

To modulate miR expression, cells were transfected with Pre-miR™ miRNA precursors or relevant scrambled negative control RNAs (Applied Biosystems, Warrington, UK) using Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA). Six well plates were loaded with 5 pM Pre-miR in 500 μl serum-free medium to which 5 μl RNAiMAX was added 20 mins before 100000 cells. Cells were used after 48-72 hrs and desired alteration of miR

expression level was confirmed using a taqman microRNA qPCR (Applied Biosystems, Warrington, UK).

Luciferase reporter assay

The first 1100 bp of the SLC7A11 3′UTR containing the microRNA-27a binding site with was cloned from EJ-R/EJ mixed cDNA and ligated into pMIR REPORT (Invitrogen, UK). The resulting pMIR REPORT + SLC7A11 3′UTR plasmid was subjected to a round of site directed mutagenesis in order to introduce two mutations in to the microRNA-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 microRNA-27a inhibitor, or scrambled RNA control (50 pMol) (Applied Biosystems, UK) and pRL-TK renilla luciferase plasmid (0.7 ng). 48 hours post transfection firefly and renilla luciferase were quantified sequentially using the Dual Luciferase Assay kit (Promega, UK) and luminescence was measured using the manufacturers 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, UK. Sulfasalazine was obtained from Sigma, UK. For clonogenic assays cells were pre-treated for 48 hours with siRNA / sulfasalazine prior to re-plating and treatment 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, S2). The first cohort included 139 primary tumors representing all disease phenotypes, collected prospectively from the Royal Hallamshire Hospital, Sheffield. We microdissected sections of freshly 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 3, multi-centre 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)) (15). For each patient we obtained archived FFPE tissue and extracted 3 × 1.5 mm cores (33-31A P/25, Miltex Inc, York, PA, USA) from cancerous regions to construct a tissue microarray as detailed elsewhere (16) (17). TMA content and construction were controlled by a certified uropathologist (AH).

Tissue Array

Protein expression was determined using automated immunohistochemistry (programme: SCC1, Benchmark XT, Ventana Medical Systems, Arizona). For SLC7A11 we used a polyclonal antibody (xCT, Thermo-Scientific (Cat.-No. PA1-16893)) at 1:400 dilution for 30 min. Sections were visualized with diaminobenzidine (DAB) chromogen and counterstained in a modified Mayer haematoxylin. 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 two fold difference (either gain or loss) in expression between resistant and sensitive cells, and selected those in which this reached statistical significance (defined as $p<0.05$, Students T Test). We compared these with miR expression profiles in primary BC (18) generated using the same array. Hierarchical clustering was performed using Cluster 3.0 and visualized in Tree view (Eisen Lab). MicroRNAs that were differentially expressed in resistant compared to 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's 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. Illinois)). 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 pathological, radiological 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's two tailed T test.

Results

microRNA expression is altered in cisplatin-resistant bladder cancer cells

To identify changes in microRNA 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_{50} concentrations for cisplatin were typically <1 μM for sensitive lines and 10 μM for resistant lines. MicroRNA expression profiling in these paired cell lines revealed that whilst the expression of the majority of microRNAs remained unchanged (Fig. 1B), a microRNA 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 microRNA profile (Fig. 1C).

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

Resistance to cisplatin can occur either prior to DNA adduct formation via changes in cellular metabolism of cisplatin or post-adduct 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 crosslinks 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 crosslinks compared with sensitive cells (Fig. 2A, $p<0.001$ for all concentrations of cisplatin above 1 μM). When cisplatin was removed from growth media following a two hour treatment of 12 μM, in both the sensitive and resistant cell lines adducts were repaired, as evidenced by the reduction in the number of adducts in genomic DNA over the following 48 hours (Fig. 2B, 2C). The rate at which these adducts were removed appeared broadly similar

between resistant and sensitive lines. This suggests a resistance mechanism related to cisplatin influx/efflux or to cytoplasmic detoxification of cisplatin prior to the cytotoxic interaction with DNA in the nucleus, rather than a difference in ability of resistant cells to tolerate or repair cisplatin-induced DNA damage.

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) (19) and influx (CTR1) (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 (Fig 3D). CTR1 mRNA was typically threefold upregulated in all resistant cell lines when compared to 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 glutathione leading to its sequestration and detoxification(22), and increased glutathione levels have been observed in other cisplatin-resistant cell lines (23). We found significantly increased concentrations of both reduced (GSH) and oxidized (GSSG) glutathione 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 glutathione 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 paired sensitive and resistant 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 tenfold 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_c- cystine/glutamate exchanger, and as cystine bioavailability is a critical factor in the rate of glutathione production it may represent the point at which the increased rate of glutathione production in resistant cells is regulated. This suggests that changes in the expression of genes involved in glutathione production, particularly SLC7A11 might be responsible for resistance in our resistant cell lines.

Overexpression of microRNA 27a reduces levels of SLC7A11 and intracellular glutathione, and resensitises resistant cells to cisplatin

To determine whether SLC7A11 was a candidate for regulation by microRNA, we examined the 3′UTR for sites of potential microRNA interaction using TargetScan (www.targetscan.org) and compared them to our expression profiles. Of the microRNAs predicted to target the 3′UTR of SLC7A11, we observed microRNAs-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. MicroRNA-32 was also shown to have the potential to interact with the 3′UTR of SLC7A11, and while 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.

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However transfection of resistant cells with a commercially available pre-miR precursor to microRNA-27a, but not microRNA-25 or -32, lowered the expression of SLC7A11 (Fig. 4A) and increased sensitivity by one hundred fold following a 12 μM treatment with cisplatin (Fig. 4B). This suggests reduced expression of microRNA-27a can induce cisplatin resistance in bladder cancer cells, by reducing the level of cellular SLC7A11. Downregulation of microRNA-27a in EJ-R cells was also further confirmed by standalone qPCR (Supplementary Figure 1). The restoration of cisplatin sensitivity following increased expression of microRNA-27a was associated with an increase in 1,2 (G,G) intrastrand adducts following cisplatin treatment (Fig. 4C) and a decrease in intracellular glutathione (Fig. 4D), supporting the hypothesis that microRNA-27a downregulation protects cells from the cytotoxic effect of cisplatin via alterations in glutathione biosynthesis. To determine whether the SLC7A11 3′UTR can be a direct target of microRNA-27a in living cells, we constructed a luciferase reporter assay using the wild type and a mutant SLC7A11 miR-27 seed sequence. Inhibition of microRNA-27a production in HEK293 cells using a commercially available inhibitory RNA significantly increased expression of luciferase reporter augmented with the relevant part of the SLC7A11 3′UTR. This effect was abolished by mutation of the microRNA-27a seed sequence (Fig. 4E).

The cisplatin sensitivity of a resistant ovarian cancer cell line can also be increased by modulation of microRNA27a 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 glutathione concentrations than its cisplatin-sensitive parent (7). Resistance could be induced with transfection of components of the X_c^- transporter (SLC7A11 and SLC3A2). We transfected A2780-cDDP with pre-miR-27a, and similar to EJ-R cells, transfection resulted in a tenfold increase in cisplatin sensitivity, suggesting that altered levels of microRNA-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 an siRNA against SLC7A11 (Fig 5A, B). Taken together these data suggest that direct targeting of the SLC7A11 mRNA 3′UTR by microRNA-27a, alters glutathione 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, resensitises cisplatin-resistant bladder cancer cells

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

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

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. While our *in vitro* data implicate microRNA-27a in cisplatin resistance, its similarity to microRNA-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 microRNAs 27a/27b were directly correlated ($r=0.75$ $p>0.001$) and inversely related to SLC7A11 mRNA expression ($r=-0.64$, $p>0.001$) (Fig. 6A, Supplementary Table 1). When outcome was analysed, tumors with high SLC7A11 and/or low microRNA-27a/27b expression progressed more frequently to advanced disease following treatment, compared to 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 two cisplatin-based regimens (15) and with gemcitabine-cisplatin. High membranous expression of SLC7A11 was identified in 22/215 (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% CI 1.1-3.5 $p=0.017$)) and extent of lymph node involvement (HR 4.4 (1.1-18.5) $p=0.04$)) were the only variables independently predicting progression and cancer-specific survival (Fig. 6D, Supplementary Table 2).

Discussion

Many previous studies have examined alterations in microRNA 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 germ line tumours (29). None of these studies identified microRNA-27a as a potential marker for or mediator of cisplatin resistance. The action of specific microRNAs tends to be highly specific to particular tissues, and consistent with this microRNA-27a has been reported as having both oncogenic and tumour suppressive roles in various tumours. In cancer types such as breast (30) and prostate (31) microRNA-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 microRNA-27a functions as a tumour suppressor (32). Consistent with our data suggesting microRNA-27a as a key mediator of drug resistance and prognosis in the context of bladder cancer, a recent paper found microRNA-27a to be consistently downregulated in fixed formalin paraffin embedded bladder tumour material from patients who experienced disease progression when compared to material from those patients who experienced a complete response (33).

MicroRNA-27a forms part of a larger signature of microRNA expression that stratifies cisplatin resistance in bladder cancer. While many of these microRNAs no doubt have functional roles in the mediation of cisplatin resistance, we have uncovered a critical role for microRNA-27a, namely its decreased expression in cisplatin resistant bladder cancer modulating SLC7A11 upregulation and therefore allowing for increased cystine import and increased glutathione synthesis. This linked our microRNA 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. Whilst 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, microRNA profiling revealed many other similarities between the resistant cell lines.

Our findings have direct clinical implications for patient care. Firstly, analysis of tumour material revealed that microRNA-27a downregulation and associated SLC7A11 upregulation is not unique to a cell culture model of cisplatin resistance and occurs in tumours *in vivo*. Secondly, we identified that cisplatin resistance could be reversed by either re-instatement of the microRNA, by siRNA-induced knockdown of SLC7A11, or by inhibition of SLC7A11 with a small molecule inhibitor. Since 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. Thirdly, the expression of microRNA-27a/27b and SLC7A11, or increased intracellular glutathione within tumors may be used to predict cisplatin response in individual patients. This could guide chemotherapy choice towards alternative agents not detoxified by glutathione in tumors likely to be resistant. Recent radiological advances have enabled the spatial and temporal resolution of glutamate (26) and glutathione (35) *in vivo* using magnetic resonance imaging spectroscopy. 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 mircoRNA-27a mediated regulation of intracellular glutathione. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of 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 during the development of cisplatin resistance in bladder cancer, and showed that one of these microRNAs (microRNA-27a) targets a cystine/glutamate exchanger, which mediates glutathione synthesis SLC7A11. Cancer samples from patients showed 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 FDA approved drug known to inhibit SLC7A11.

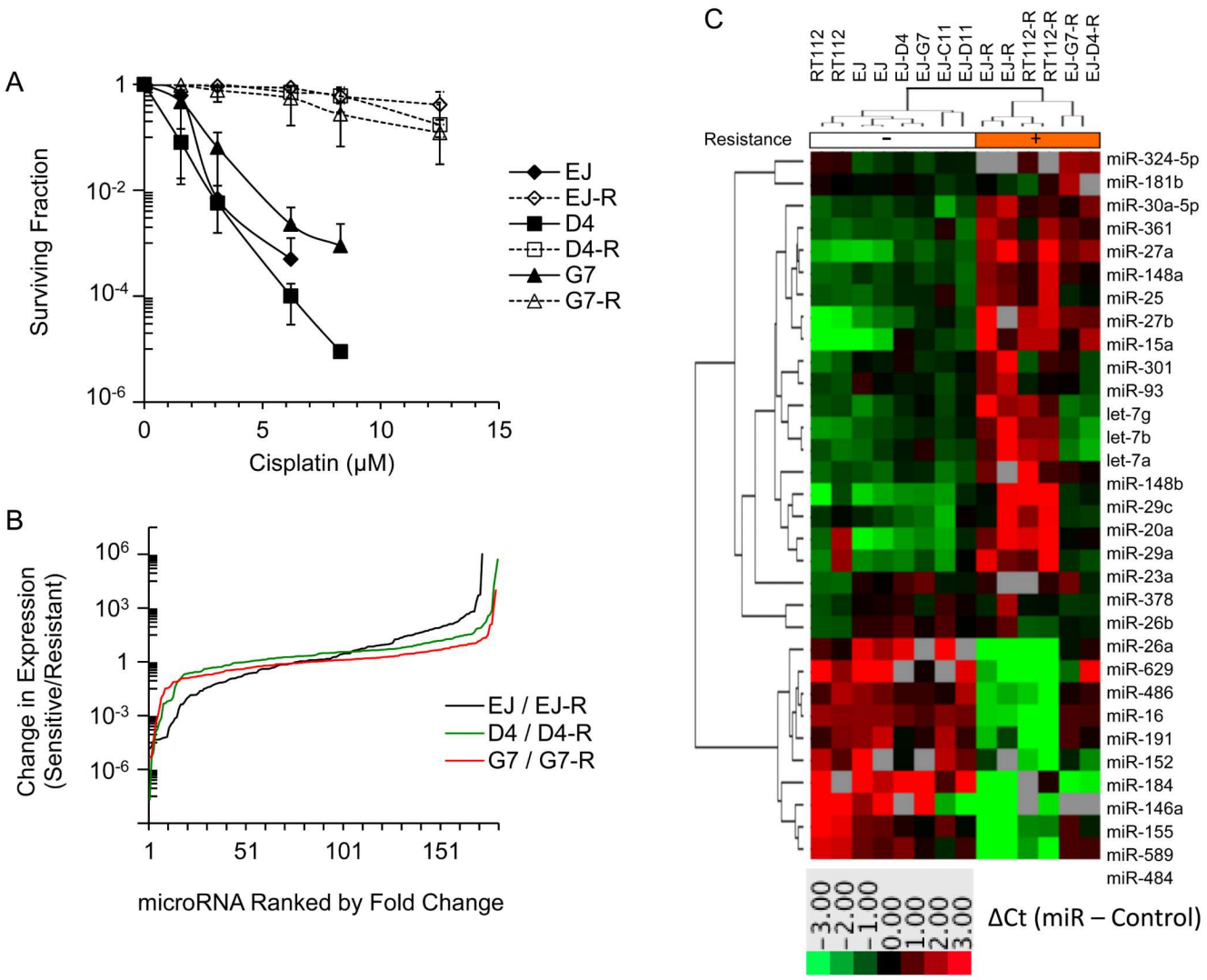

Figure 1. Characterization of cisplatin resistant cell lines and microRNA expression

(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 indicate SD, n=4 (b) Fold change in expression of c. 160 microRNAs in three paired sensitive and resistant lines. (c) Hierarchical clustering 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.

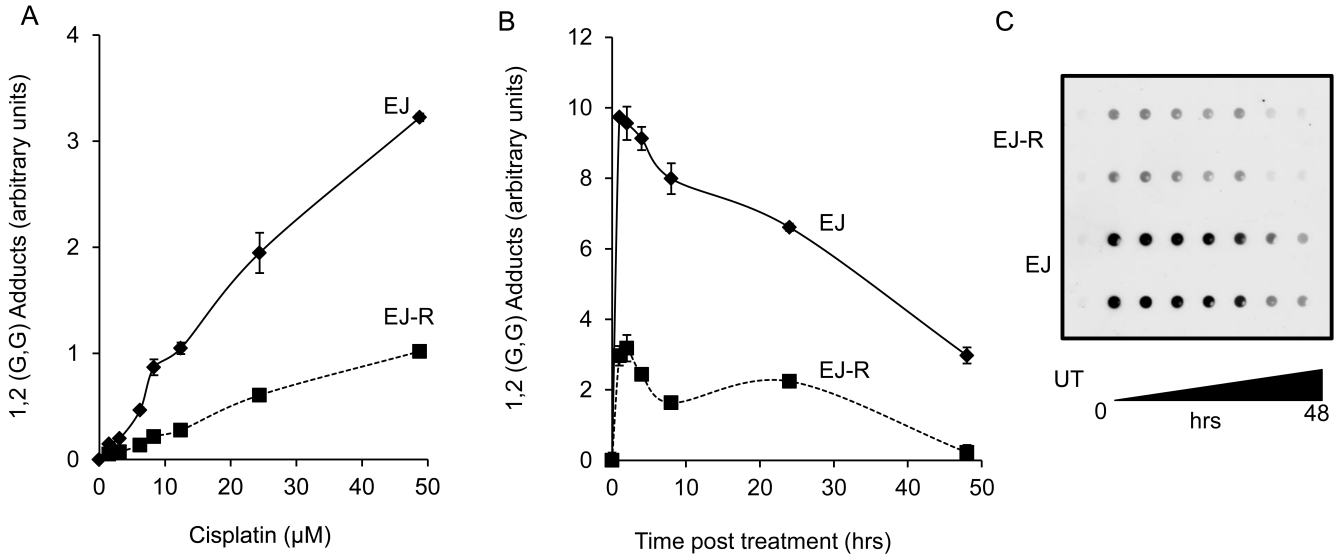

Figure 2. Genotoxicty of cisplatin and measurement of cisplatin/DNA adducts in sensitive and resistant cells

(a) Measurement of 1,2 (G,G) Intrastrand Cisplatin-DNA adducts in genomic DNA of EJ and EJ-R cells following a two hour treatment with a range of cisplatin concentrations. Error bars indicate SD, n=3. $p<0.001$ for all concentrations of cisplatin above 1 μM (b) Densitromic plot of extent of 1,2 (G,G) Intrastrand Cisplatin-DNA adduct repair in EJ / EJ-R for 48 hours following a two hour treatment with 12 μM cisplatin. Error bars indicate SD, n=3 (c) Example dot blot used to generate data visualized in Fig 3B.

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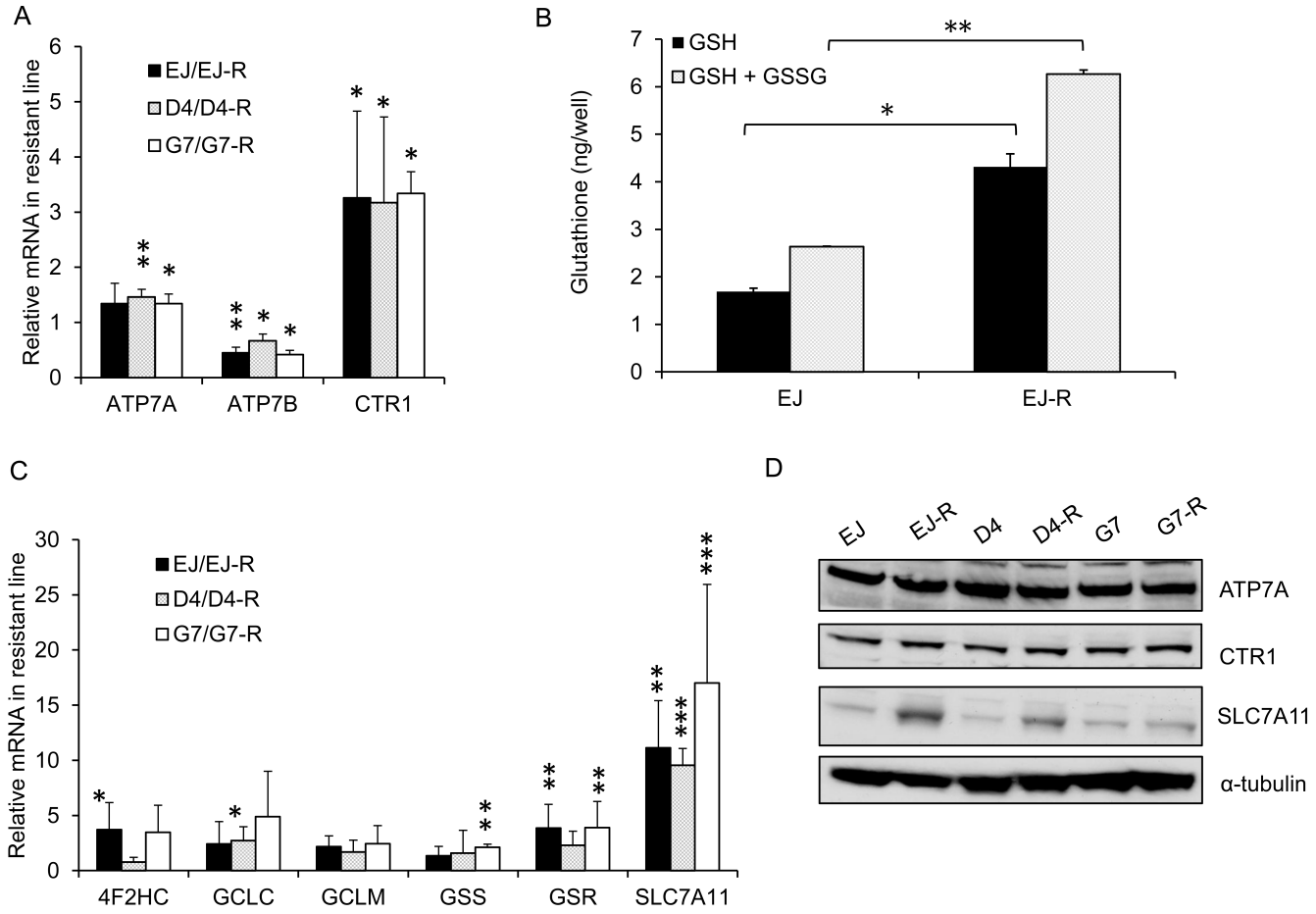

Fig 3. Analysis of candidate pathways mediating cisplatin resistance in sensitive and resistant cells

(a) Change in expression of three mRNAs concerned with cisplatin import (CTR1) and export (ATP7A, ATP7B) in three paired cisplatin sensitive (EJ, D4, G7) and resistant (EJ-R, D4-R, G7-R) cell lines. (b) Measurement of intracellular glutathione (Reduced glutathione/ GSH – black bars, Total glutathione GSH and GSSG, white bars) in EJ bladder cancer cells and their cisplatin-resistant derivative line EJ. Error bars indicate SD, n=3, * $p<0.01$ **$p<0.0005$. (c) Change in expression of six mRNAs concerned with glutathione biosynthesis in three paired cisplatin sensitive (EJ, D4, G7) and resistant (EJ-R, D4-R, G7-R) cell lines. (d) Western blot of proteins concerned with cisplatin import and export (ATP7A (163 kDa), CTR1 (35 kDa), SLC7A11 (55 kDa) against α-tubulin (55kDa)) in three paired cisplatin sensitive (EJ, D4, G7) and resistant (EJ-R, D4-R, G7-R) cell lines.

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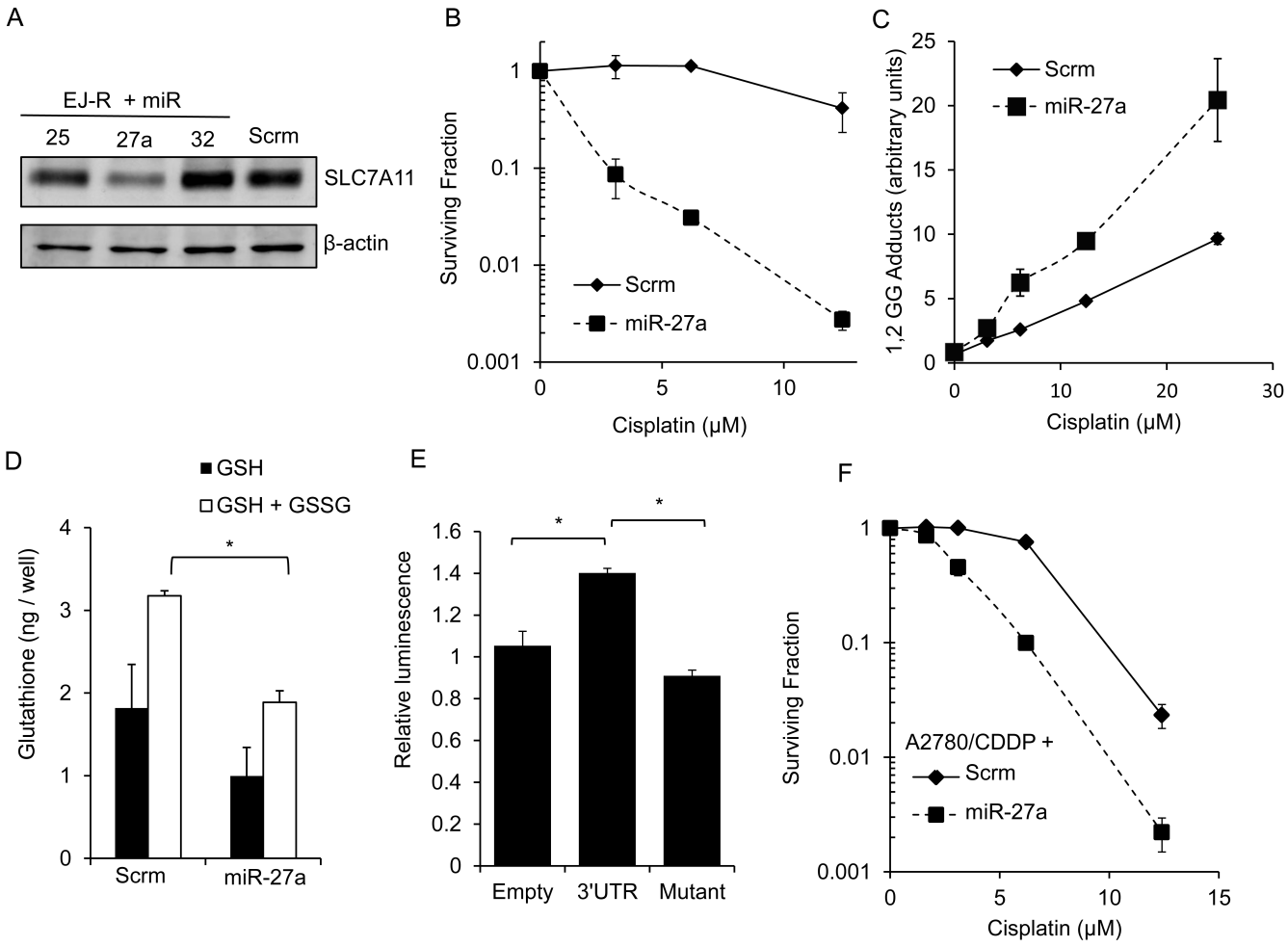

Figure 4. Effect of microRNA-27a manipulation on SLC7A11 expression, cisplatin sensitivity and cisplatin genotoxicity in resistant bladder and ovarian cancer cells

(a) Western blot of SLC7A11 expression following transfection of cisplatin-resistant cells with microRNA-27a, microRNA-25 or microRNA-32, (mIR-27a, -25, -32). (b) Effect of increased microRNA-27a expression on cisplatin sensitivity of EJ-R cells compared to a scrambled control oligo as measured by clonogenic assay. Error bars indicate SD, n=3 (c) Effect of increased microRNA-27a expression of level of cisplatin induced DNA damage in EJ-R cells compared to untreated and scrambled RNA controls. Error bars indicate SD, n=3 (d) Effect of increased microRNA-27a expression on levels of reduced (black bars) and total (white bars) glutathione *= $p<0.01$. Error bars indicate SD, n=3 (e) Effect on luciferase expression of a microRNA-27a inhibitor in HEK293 cells compared to scrambled control . The luciferase gene was augmented with a portion of the 3′UTR of SLC7A11 containing the microRNA-27a binding site, or a similar construct with the microRNA-27a abolished by site-directed mutagenesis (mutant). (f) Effect of increased microRNA-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 indicate SD, n=3

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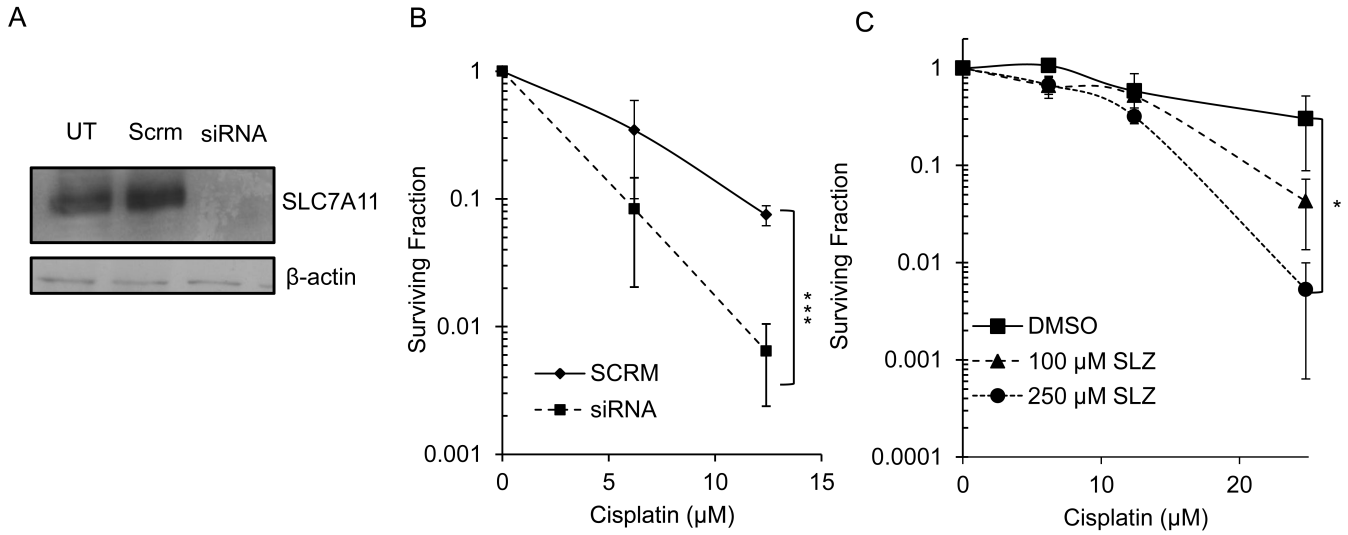

Figure 5. Effect of SLC7A11 knockdown and inhibition on cisplatin sensitivity

(a) Use of siRNA directed against SLC7A11 to reduce SLC7A11 protein levels in EJ-R cells 48 hours post transfection. (b) Effect of SLC7A11 knockdown compared with scrambled RNA control on cisplatin sensitivity of EJR cells as measured by clonogenic assay. Error bars indicate SD, n=3. *** = $p<0.005$ (e) Effect of sulfasalazine pretreatment on cisplatin sensitivity of EJ-R cells as measured by clonogenic assay. Error bars indicate SD, n=3 * $p<0.1$

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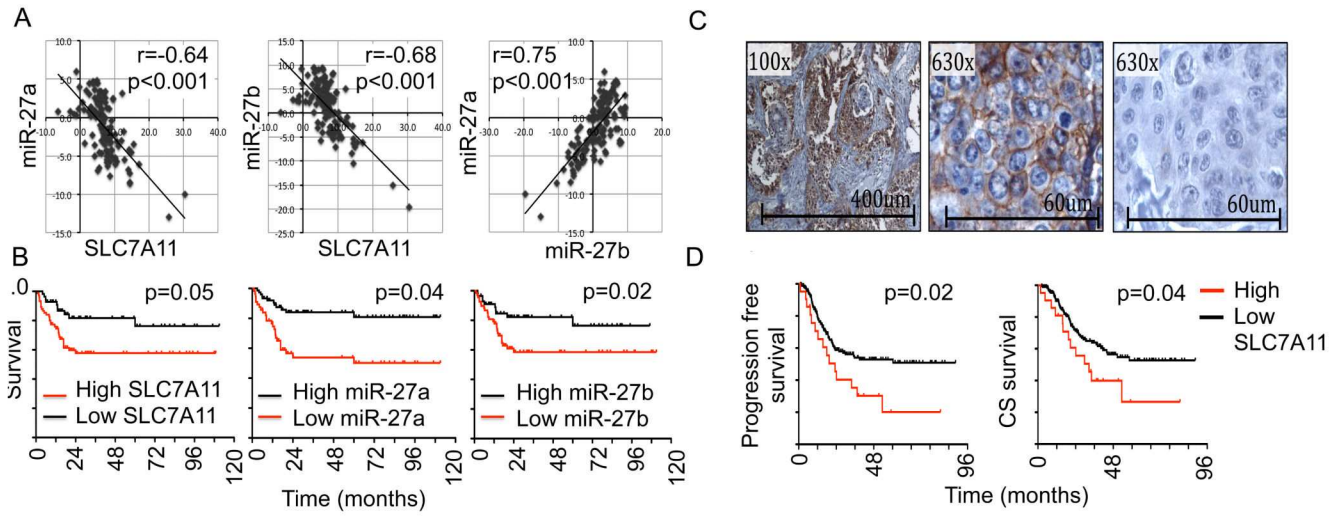

Fig 6. Clinical significance of decreased miR-27a/b expression and increased SLC7A11 expression in bladder tumours

(a) Expression of miR-27a/27b and SLC7A11 mRNA in bladder cancer samples as determined by real-time PCR. (b). Correlation between low microRNA-27a expression (red line) and an aggressive tumor phenotype as shown by progression to invasion and metastases following treatment in 139 freshly frozen tumors. (c). High membranous SLC7A11 protein expression was identified in 10.5% of tumors (examples shown of high (100x and 630x) and absent expression (630x)) in a tissue array of tumors from patients treated with adjuvant chemotherapy for invasive bladder cancer. (d) SLC7A11 protein expression stratifies progression free and cancer specific survival (CSS) in patients treated with this agent within an RCT of cisplatin-based regimens. (*P<0.05, ** P<0.001)

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