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An oncofetal glycosaminoglycan modification provides therapeutic access to cisplatin-resistant bladder cancer

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Running head: Targeting oncofetal glycosaminoglycan in bladder cancer

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ABSTRACT

Background: Although cisplatin-based neo-adjuvant chemotherapy (NAC) improves survival of unselected patients with muscle-invasive bladder cancer (MIBC), only a minority responds to therapy and chemoresistance remains a major unmet need in this disease setting.

Objective: To investigate the clinical significance of oncofetal chondroitin sulfate (ofCS) in cisplatin-resistant MIBC and to evaluate these as targets for second line therapy.

Design, Setting, and Participants: An ofCS-binding recombinant VAR2CSA protein derived from the malaria parasite *Plasmodium falciparum* (rVAR2) was used as an *in situ*, *in vitro* and *in vivo* ofCS-targeting reagent in cisplatin-resistant MIBC. The ofCS expression landscape was analyzed in two independent cohorts of matched pre- and post-NAC treated MIBC patients.

Intervention: An rVAR2 protein armed with cytotoxic hemiasterlin compounds (VDC886) was evaluated as a novel therapeutic strategy in a xenograft model of cisplatin-resistant MIBC.

Outcome Measurements and Statistical Analysis: Anti-neoplastic effects of targeting ofCS.

Results and Limitation: *In situ,* ofCS was significantly overexpressed in residual tumors after NAC in two independent patient cohorts ($p<0.02$). Global gene-expression profiling and biochemical analysis of primary tumors and cell lines revealed syndican-1 (SDC1) and chondroitin sulfate proteoglycan 4 (CSPG4) as ofCS-modified proteoglycans in MIBC. *In-vitro*, ofCS was expressed on all MIBC cell lines tested and VDC886 eliminated these cells in the low-nanomolar IC_{50} concentration range. *In-vivo*, VDC886 effectively retarded growth of chemoresistant orthotopic bladder cancer xenografts and prolonged survival ($p=0.005$). The number of mice and the use of cisplatin only for the generation of chemoresistant xenografts are limitations of our animal model design.

Conclusions: Targeting ofCS provides a promising second line treatment strategy in cisplatin-resistant MIBC.

Patient Summary: Cisplatin-resistant bladder cancer overexpresses particular sugar chains compared to chemotherapy naïve bladder cancer. Using a recombinant protein from the malaria *Plasmodium falciparum* parasite, we can target these sugar chains and our results showed a significant antitumor effect in cisplatin-resistant bladder cancer. This novel treatment paradigm provides a therapeutic access as second line therapy for bladder cancer not responding to cisplatin.

INTRODUCTION

Bladder cancer is the 5th most common cancer in the world and the most costly cancer to treat on a per patient basis due to required clinical surveillance and multiple therapeutic interventions[1]. Muscle-invasive bladder cancer (MIBC) is a highly aggressive flavor of the disease with a 5-year survival probability of ~50%[2]. Despite efforts in refining surgical techniques and optimizing systemic therapy, the prognosis has remained unchanged for more than 20 years[3]. Clinical improvement can be ascribed to Cisplatin-based neo-adjuvant chemotherapy (NAC), which offers an overall survival benefit of 5-6%[4] and standard-of-care in MIBC[5]. However, only approximately 40% of patients demonstrate a major response to NAC, while adverse treatment effects and a delay in surgery potentially harm 60%. Therefore, second line treatment options for MIBC are currently in great demand[6]. Although 60% of patients with metastatic MIBC demonstrate an objective response to cisplatin-based chemotherapy this response is rarely durable, and almost all of these patients succumb to their disease[7]. Atezolizumab has recently become the first FDA-approved agent for the second line treatment of metastatic bladder cancer[8], but there remains a great need for additional second and additional line agents in all states of MIBC.

Glycosaminoglycans (GAGs) are carbohydrate modifications attached to to proteins in the cell membrane. Changes in expression and composition of GAGs have been reported in bladder cancer over the past three decades[9-11]. Chondroitin sulfate (CS) is a major cancer-associated GAG that also has a key role in malaria pathogenesis[12]. The *Plasmodium falciparum* malaria parasite has evolved a protein, called VAR2CSA that mediates attachment of infected erythrocytes to a distinct type of CS in the placental syncytium[13]. CS chains are comprised of repeated disaccharides units, made up of glucoronic acid (GlcA) and N-acetylgalactosamine (GalNAc) residues. CS chains are highly heterogeneous showing variations in both chain length and disaccharide modifications, such as sulfation. Placental-type CS is highly sulfated on

carbon-4 of the GalNAc hexose and this particular modification is required for exclusive sequestration of VAR2CSA positive erythrocytes in the placenta[14]. Placenta and cancer share obvious phenotypic features such as highly proliferating cells, the ability to invade adjacent tissue, and promoting angiogenesis[15]. In the placenta, these features are partly facilitated by CS and many tumors re-express placental-type CS as an oncofetal GAG[16]. Therefore, recombinant malarial VAR2CSA (rVAR2) proteins can be conveniently utilized to detect oncofetal chondroitin sulfate (ofCS) in human cancer[16,17]. Finally, rVAR2 conjugated with the hemiasterlin toxin analog KT886 derived from the marine sponge *Hemiasterella minor* (VDC886), show anti tumor activity in non-Hodgkin's lymphoma, prostate cancer, and metastatic breast cancer with no adverse treatment effects in non-pregnant mice[16].

We noticed that several cancer-associated proteins can be modified with an ofCS chain, including CD44[16], SDC1[18], and CSPG4[19], and that CD44 and SDC1 have previously been suggested as a candidate biomarker in bladder cancer[20-25]. Accordingly we hypothesized that human MIBC might display ofCS chains that could be exploited for rVAR2-based targeted therapy. In particular we aimed to investigate the role of ofCS expression in cisplatin-resistant MIBC with the intention of developing an additional line treatment option for this disease state.

MATERIAL AND METHODS

Bladder cancer patient cohorts

Bladder tumor tissue was collected at three institutions (University of Bern, Switzerland, at Vancouver General Hospital, Canada and University Hospital of Southampton, UK) from patients receiving at least 3 cycles of cisplatin-based neoadjuvant chemotherapy prior to radical cystectomy with pelvic lymph node dissection (**Supplementary Table S1**). Tissue was harvested from pre-chemotherapy TURBT specimens in all patients and from post-

chemotherapy radical cystectomy specimens in patients with residual MIBC (ypT). The Bern cohort was used as the discovery cohort, and the Vancouver and Southampton cohorts were amalgamated as the validation cohort.

A complete and detailed description of Materials and Methods used in this study can be found in in the **Supplementary Information**.

RESULTS

To analyze the ofCS expression in bladder cancer pre- and post-treatment with cisplatin-based chemotherapy, we performed immunohistochemical (IHC) analysis on two independent cohorts of matched primary chemotherapy-naïve and cisplatin-resistant bladder tumors using rVAR2 as the ofCS detection reagent (**Fig. 1A, 1B** and **Supplementary Fig. 1A-E**). The ofCS expression is absent in urothelium of the adjacent normal bladder (**Fig. 1A, left**) and is strongly restricted to the bladder tumors (**Fig. 1A, right**). To differentiate ofCS expression between tumor environment and the cancer cell compartments, IHC analyses of an epithelial marker, E-cadherin and a mesenchymal marker, Vimentin were performed (**Fig. 1A**). The pre-chemotherapy bladder tumors were sampled by transurethral resection (TURBT) and the matched cisplatin-resistant tumors after neo-adjuvant chemotherapy were sampled from subsequent radical cystectomy specimens. The discovery cohort was comprised of more advanced tumors from a single institution as compared to the validation cohort combined from two institutions (**Supplementary Table S1**). Overall, high ofCS expression is seen in approximately 92% (n=110/120) of chemotherapy-naïve bladder tumors in both cohorts. Among which, 17% (n=8/47) of the tumors in the discovery cohort (**Fig. 1C, left**) and 33% (n=24/73) of the validation cohort (**Fig. 1C, right**) showed high membranous expression of ofCS (ofCS^{High}) in the cancer cell compartment.

In chemotherapy-naïve tumors, ofCS expression failed to predict response to chemotherapy (p=0.4, OR= 2.28, 95%CI=0.35-25.87) and inform on survival in univariable and multivariable analyses. In cisplatin-resistant disease (ypT) ofCS expression increased to 57% (n=16/28; p=0.001; vs. 29%, n=8/28 in TURBT) of tumors showed ofCS^{High} in cancer cells in the discovery cohort (**Fig. 1D, left**) and 70% (n=23/33; p=0.01; vs. 33%, n=11/33 in TURBT) in the validation cohort (**Fig. 1D, right**). In cisplatin-resistant MIBC, ofCS^{High} was associated with extra-vesical extension in the cystectomy specimen in the discovery cohort (p=0.005) (**Fig. 1E, left** and **Fig. 1F**) but was not significant in the validation cohort (p=0.081) (**Fig. 1E right**). This shift in ofCS expression was associated with poor overall survival (OS) in the discovery cohort (p=0.045) (**Fig. 1G, upper**) but not in the validation cohort (p=0.5), which had a shorter clinical follow-up of 3.3 years (95%CI: 2.52-3.5) (**Fig. 1G, lower**). In multivariable analysis, only pathological tumor stage added independent prognostic information (**Supplementary Table S2)**. Accordingly, these data indicate that ofCS is upregulated in cisplatin-resistant bladder cancer cells and this event may be associated with poor outcome.

Several proteoglycans can carry ofCS GAG modifications[16]. To search for ofCS-modified proteoglycans in bladder cancer, we analyzed gene expression data of known CS-modified proteoglycans[16,28] in two independent bladder cancer cohorts. In both datasets, luminal type tumors were enriched for SDC1, SDC4, and APLP2, while more basal tumors were enriched for a number of genes including CD44 and CSPG4 (**Fig. 2A**). SDC1, CSPG4, and CD44 have been widely associated with multiple types of cancer[16,18,19], and CD44 and SDC1 have previously been suggested as a candidate biomarker in bladder cancer[20-25]. Indeed, protein expression of CD44 (**Fig. 2B**), SDC1 (**Fig. 2C**), and CSPG4 (**Fig. 2D**) showed a strong correlation with mRNA expression (**Figs. 2E-G**) and was strongly correlated with ofCS^{High} cases (**Fig. 2H-J**). Together, these data suggest that CD44, SDC1, and CSPG4 are expressed in human bladder cancer and associated with ofCS.

We next analyzed a panel of 7 bladder cancer cell lines derived from MIBC patients for ofCS-expression by flow cytometry using rVAR2 as the ofCS detection reagent. All MIBC cell lines analyzed expressed ofCS as detected by rVAR2 in a concentration and CS-dependent manner (**Fig. 3A**). This was supported by sensitivity to the ofCS targeting rVAR2 drug conjugate (VDC) VDC886[16] in the low-nanomolar IC_{50} concentration range (**Fig. 3B**). Interestingly, 4 out of 7 cell lines expressed CSPG4; 7 out of 7 expressed CD44; and 5 out of 7 expressed SDC1 (**Fig. 3C**), but this expression pattern was not obviously related to VDC886 sensitivity. This might indicate that not all of the selected proteoglycans (CD44, SDC1, and CSPG4) are modified in the individual cell lines and/or that additional proteoglycans contribute to the ofCS presentation. To investigate this, we subjected UM-UC13 cells to biochemical analysis to directly assess whether or not CD44, SDC1, and CSPG4 were modified with ofCS. ofCS-modified proteins and proteins in complex with ofCS-modified proteins, were purified from cell lysate using immobilized rVAR2. The resulting fractions were treated with chondroitinase ABC to reduce the CSPGs to their component core proteins. A shift in gel mobility towards the predicted protein size on SDS-PAGE, upon Chondroitinase ABC treatment therefore implies CS substitution. Surprisingly, while CD44, SDC1, and CSPG4 were expressed in UM-UC13 cells, only SDC1 and CSPG4 produced a shift in molecular weight after chondroitin treatment (**Fig. 3D**). Chondroitinase ABC treatment did not reduce the size of SDC1 to its predicted core protein size, but merely produced an intermediary sized protein. This is in line with the heparan sulfate (HS) substitution status of SDC1, a GAG that will not be digested with Chondroitinase ABC. This indicates that SDC1 and CSPG4, but not CD44, are ofCS-modified in these cells. To validate this result, we treated UM-UC13 cells with validated siRNAs targeting CD44, SDC1, and CSPG4, and assayed for binding to rVAR2 as an indication of ofCS expression. While the CD44 siRNA had no effect on rVAR2 binding, SDC1 and CSPG4 siRNAs reduced rVAR2 binding to UM-UC13 cells with 50% and 20% respectively (**Fig. 3E**). This corroborates the biochemical analysis showing that SDC1 and

CSPG4, but not CD44, are ofCS-modified in UM-UC13 cells, reflecting that ofCS modification of eligible proteins is not mandatory and likely a dynamic event.

To assess the expression of ofCS-modified proteoglycans in cells treated with cisplatin *in vivo* we inoculated UM-UC13 cells into the bladder wall of nude mice with ultrasound guidance (S**upplementary Fig. S3A-B**). As the UM-UC13 tumors developed, the mice were subjected cisplatin treatment and resistant tumors were allowed to recover. To analyze the ofCS modification status of proteoglycans in UM-UC13 tumors and to potentially identify novel proteoglycans, we purified ofCS-associated proteoglycans from *ex-vivo* cells and analyzed those by mass spectrometry. This analysis identified 8 ofCS-modified proteoglycans including SDC1 and CSPG4 (**Supplementary Table S3**). Similar to the *in vitro* cell line study, this analysis showed that CD44 was not modified with ofCS in UM-UC13 cells, and that ofCS-modification of SDC1 and CSPG4 is retained *in vivo*. Proximity-ligation assay (PLA) analysis of ofCS chains and CD44, SDC1 and CSPG4, confirmed the mass spectrometry result (**Supplementary Fig. S3C**) but surprisingly, CD44 was also in proximity to ofCS. This suggests that while CD44 is not itself modified with ofCS in UM-UC13 cells, it is indeed expressed in close proximity to ofCS-modified proteoglycans.

Capitalizing on our findings that human cisplatin-resistant MIBC upregulates ofCS (**Fig. 1A-F**) and that MIBC cells are sensitive to VDCs *in vitro* (**Fig. 3B**), we next tested whether VDC886 had efficacy against cisplatin-resistant MIBC *in vivo*. Again, UM-UC13 cells were inoculated into the bladder wall of nude mice with ultrasound guidance (**Supplementary Fig. S3A-B**) and tumor growth was subsequently monitored using ultrasound over 40 to 60-day period (**Supplementary Fig. S4A**). As the UM-UC13 tumors developed, the mice were subjected to repeating cycles of cisplatin treatment and the resultant tumors were passaged through 6 generations of mice (G1-G6) to create highly stable cisplatin-resistant xenografts (**Fig. 4A**). Importantly, the *ex-vivo* tumor cells retained their rVAR2 binding, in an concentration and CS-

dependent manner (**Fig. 4B**). They also showed the same rVAR2 internalization capacity, as the parental line, G0 (**Fig. 4C**).

Importantly, the completely cisplatin-resistant G6 and cisplatin-sensitive G0 cells showed equal sensitivity to VDC886 *ex-vivo* (**Fig. 4D** and **Supplementary Fig. S4B**).

Next, mice with established G6-initiated cisplatin-resistant tumors were randomized into 4 groups and treated biweekly (4 treatments in total) with vehicle, rVAR2, KT886, or VDC886 (**Fig. 4E**) in combination with cisplatin. The G6 cisplatin-resistant tumors expressed SDC1, CSPG4, and ofCS (**Supplementary Fig. S4C**). Remarkably, VDC886 treatment strongly retarded growth of cisplatin-resistant tumors (**Fig. 4F** and **4G**) and significantly prolonged survival of the mice (**Fig. 4H**). Importantly, clinicopathological examination of VDC886 treated mice demonstrated no organ toxicity (**Supplementary Fig. S4D**) and the weight of the mice was stable in all groups (**Supplementary Fig. S4E**). In the VDC886 treated group, one mouse had complete response; one mouse presented with significant tumor regression (**Supplementary Fig. S4F**); and four mice had stable tumor volume without progression during the experiment, with viable tumor cells in histology. In summary, our work demonstrates that ofCS-modified proteoglycans can provide therapeutic access to cisplatin-resistant MIBC.

DISCUSSION

We have tested an unconventional approach for treating cisplatin-resistant MIBC based on the evolutionarily refined parasite-host anchor protein VAR2CSA derived from the *P. falciparum* malaria parasite. We found that ofCS was present at high levels in cisplatin-resistant MIBC promoting ofCS as a potential access point for targeted therapy. Supporting this notion, VDC886 was able to suppress growth of cisplatin-resistant MIBC. High ofCS expression was associated with advanced tumor stage, cisplatin-resistance and poor overall survival of MIBC patients.

These associations were more robust in the discovery cohort, which contained more advanced disease and a longer follow-up. In the validation cohort, presentation of ofCS was not prognostic. While this suggests that high ofCS may only relate to survival in more advanced MIBC, this association requires further investigation.

Several different proteoglycans can be modified with ofCS chains[16]. As a result, intra-tumoral heterogeneity in proteoglycan expression does not necessarily translate into varying levels of ofCS modifications. CD44 has previously been shown to carry ofCS chains in melanoma[16,29,30]. We found that CD44 mRNA and protein expression correlated with high ofCS presentation in MIBC but biochemical interrogation showed that CD44 was not modified with ofCS, at least not in UM-UC13 cells. Contrary to CD44, the CSPGs SDC1 and CSPG4 were modified in UM-UC13 cells and correlated with ofCS expression in primary tumors. Mass spectrometry of rVAR2-purified proteoglycans identified another 6 ofCS-modified candidates. It is likely that additional proteoglycans contribute to ofCS presentation in MIBC.

The most important finding of our study was the increased cellular ofCS expression in cisplatin-resistant MIBC when compared to paired chemotherapy-naïve MIBC. These findings suggest that targeting ofCS with VDCs provide an attractive approach particularly in cisplatin-resistant MIBC. The 1st generation VDC compound VDC886 is comprised of the DBL1X-ID2a domains of the malarial VAR2CSA protein loaded with an average of three KT886 hemiasterlin toxin analogs derived from the marine sponge *Hemiasterella minor*[16]. VDC886 eliminated all MIBC cell lines in the low nanomolar IC_{50} concentration range. As the MIBC cell lines investigated all express high levels of ofCS, the small differences in IC_{50} are most likely due to differences in internalization kinetics. In an animal model of cisplatin-resistant MIBC, VDC886 was able to efficiently target established cisplatin-resistant tumors and rescue the mice from tumor-associated morbidity and death. The completely cisplatin-resistant G6 and cisplatin-sensitive G0 cells isolated from mice showed equal sensitivity to VDC886 *ex-vivo*. This is likely due to high

baseline levels of ofCS in MIBC cell lines, and suggests that the ofCS modification is a passenger, rather than a driver, of cisplatin resistance. Nevertheless, ofCS glycosaminoglycan-modifications constitute a therapeutic access point in cisplatin-resistant MIBC.

CONCLUSIONS

We have found an oncofetal glycosaminoglycan antigen in cisplatin-resistant MIBC that can be targeted by a malarial host-cell anchor protein armed with hemiasterlin compounds. This discovery potentially offers a new treatment paradigm for human MIBC patients not responding to cisplatin.

DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

M. Daugaard, A. Salanti, M. Ø. Agerbæk, and T. M. Clausen are shareholders in VAR2 Pharmaceuticals Aps. VAR2 Pharmaceuticals is a biotechnology company that develops cancer therapeutics and diagnostic tools. J. S. Babcook and J. Rich are employees of Zymeworks Inc.

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FIGURE LEGENDS

FIGURE 1

ofCS expression in chemotherapy-naïve (TURBT) and cisplatin-resistant (ypT) bladder cancer

(A) Representative H&E and IHC images in matched adjacent normal bladder (left panel) and bladder cancer cases (right panel) with an epithelial marker, E-cadherin and a mesenchymal marker, Vimentin expression in parallel with ofCS expression. Scale bar represents 200 μm.

(B) Representative IHC images of ofCS expression in matched bladder cancer cases showing cellular ofCS expression in paired TURBT (left panel) and cisplatin resistant tumors at cystectomy (ypT) (right panel). Scale bar represents 50 μm.

(C) ofCS expression was examined in chemotherapy-naïve bladder cancers (TURBT) in two independent cohorts (discovery: left, validation: right). "Tumor" represents ofCS expression in overall bladder tumor including the surrounding microenvironment, and "cancer cells" represent membranous ofCS expression only in cancer cells.

(D) Plots indicating paired analysis of ofCS expression of cancer cells in chemotherapy-naïve (TURBT) and cisplatin-resistant (ypT) tumors in discovery (left) and validation (right) cohorts. Each box indicates the tumor of a given patient and the lines indicates the pairs between TURBT to ypT.

(E) Barplots indicating the relation of cellular ofCS expression in ypT compared to tumor stage in discovery (left) and validation (right) cohorts.

(F) Representative IHC images of ofCS expression according to ypT stages of MIBC in 8 different patients from a validation cohort. Scale bar represents 50 μm.

(G) Kaplan-Meier plots for overall survival (OS) stratified according to high and low cellular ofCS expression in ypT (discovery: upper, validation: lower).

FIGURE 2

ofCS-carrying proteoglycans in bladder cancer

(A) Heatmap of gene expression of proteoglycans that have been shown to be ofCS modified. The TURBT samples from both cohorts have been selected. Virtually all samples show high expression of at least one validated proteoglycan.

(B) Representative IHC images showing high and low CD44 protein expression of bladder cancer. For statistical analysis in the subsequent panel (E), low expressing tumors (1^{st}-3^{rd} quartile) were compared to high expression tumors (4^{th} quartile), respectively. Scale bar represents 100 µm.

(C) Representative IHC images showing high and low SDC1 protein expression of bladder cancer. For statistical analysis in the subsequent panel (F), low expressing tumors (1^{st}-3^{rd} quartile) were compared to high expression tumors (4^{th} quartile), respectively. Scale bar represents 100 µm.

(D) Representative IHC images showing high and low CSPG4 protein expression of bladder cancer. For statistical analysis in the subsequent panel (G), low expressing tumors (1^{st}-3^{rd} quartile) were compared to high expression tumors (4^{th} quartile), respectively. Scale bar represents 100 µm.

(E) Boxplot indicates the CD44 mRNA expression (y-axis) of samples with low and high CD44 protein expression, respectively.

(F) Boxplot indicates the SDC1 mRNA expression (y-axis) of samples with low and high SDC1 protein expression, respectively.

(G) Boxplot indicates the CSPG4 mRNA expression (y-axis) of samples with low and high CSPG4 protein expression, respectively.

(H) Boxplot indicating CD44 protein expression levels in ofCS^{High} and ofCS^{Low} bladder tumors.

(I) Boxplot indicating SDC1 protein expression levels in ofCS^{High} and ofCS^{Low} bladder tumors.

(J) Barplot indicating CSPG4 protein expression levels in ofCS^{High} and ofCS^{Low} bladder tumors.

FIGURE 3

rVAR2 binds human bladder cancer cells

(A) Relative geometric mean fluorescence intensity (MFI) of a panel of bladder cancer cell lines incubated with recombinant V5-tagged control protein (rContr) or VAR2CSA (rVAR2) as indicated and detected by flow cytometry using anti-V5-FITC.

(B) Indicated human bladder cancer cell lines were treated with rVAR2 drug conjugate (VDC886). The column graph displays IC50 kill-values of VDC886.

(C) CD44, CSPG4 and SDC1 protein expression in the same bladder cancer cell line panel is shown, with GAPDH as a loading control.

(D) Westen blot of rVAR2 column pulldown of CD44, CSPG4, and SDC1. Blots shows input, run through (RT), wash, and elution with and without Chondroitinase ABC treatment.

(E) UM-UC13 cells were treated with validated siRNAs targeting CD44, SDC1, and CSPG4 and assayed for binding to rVAR2.

FIGURE 4

Potential of VDC886 for targeted therapy of cisplatin-resistant MIBC

(A) Diagram illustrating ultrasound-guided xenograft animal model for cisplatin-resistant human bladder cancer. After initial injection of UM-UC13 cells into the bladder wall, mice were constantly treated with cisplatin (3mg/kg weekly). Tumors were passaged into the next generation for 6 successive cycles. The 6^{th} generation was used for the final animal model. During every passage, tumors were stored (FFPE, Frozen) and an *ex-vivo* cell line was created for each generation.

(B) Binding of rVAR2 to UM-UC13 parental and *ex-vivo* cells (generation 6) are indicated. Relative mean fluorescence intensity (MFI) incubated with recombinant control protein (rContr) or rVAR2 as indicated and detected by flow cytometry using anti-V5-FITC.

(C) Internalization of Alexa488-labelled rVAR2 in parental (upper panel) and *ex-vivo* (lower panel) UM-UC13 bladder cancer cells detected by confocal microscopy 30 minutes after addition of rVAR2-Alexa488 (green) and DAPI (blue).

(D) *Ex-vivo* bladder cancer cell lines were seeded in 96-well plates and treated with VDC886 in concentrations ranging from 0.01 pM to 200 nM. The column graph displays IC50 kill-values of VDC886 performance in G0 and G6. Kill-values for the other generations were virtually the same (data not shown).

(E) Schematic illustration of the assignment of the mice to the respective treatment groups. VDC886 was tested against the vehicle, rVAR2 and KT886, respectively.

(F) Representative ultrasound images of each group at day 17, 31 and 45. The tumor growth was highlighted in red circle for each group.

(G) Comparing tumor growth between VDC886 and control groups. Treatment was administered intravenously twice per week (red arrow heads) as indicated.

(H) Survival curve of VDC886 and Vehicle treated mice from (G).

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TAKE HOME MESSAGES

A secondary glycosaminoglycan modification, ofCSA, is overexpressed in MIBC after chemotherapy and can be targeted by malarial-derived VAR2CSA drug-conjugates. This novel treatment paradigm promotes ofCSA as a target in MIBC not responding to cisplatin.

***Revision notes**

Comments to Author:

Reviewer #6: Attention to the following issues would improve the manuscript:

2. Line 141: say "multivariate" rather than "multivariable"
Response: We apologize for this misunderstanding. We changed now “multivariate” to “multivariable” as suggested by the reviewer in the manuscript as well as in Supp Table 2.

5. Line 146: report p values close to 0.05 to two significant figures (e.g. (Fig. 1E, left and Fig. 1F) but was not significant in the validation cohort (p=0.08) (Fig. 1E right). This shift in ofCS expression was associated with poor overall survival (OS) in the discovery cohort (p=0.04).
Response: We added additional decimal point on the p-values close to 0.05 in the entire manuscript as well as in the figures.

Figure 1

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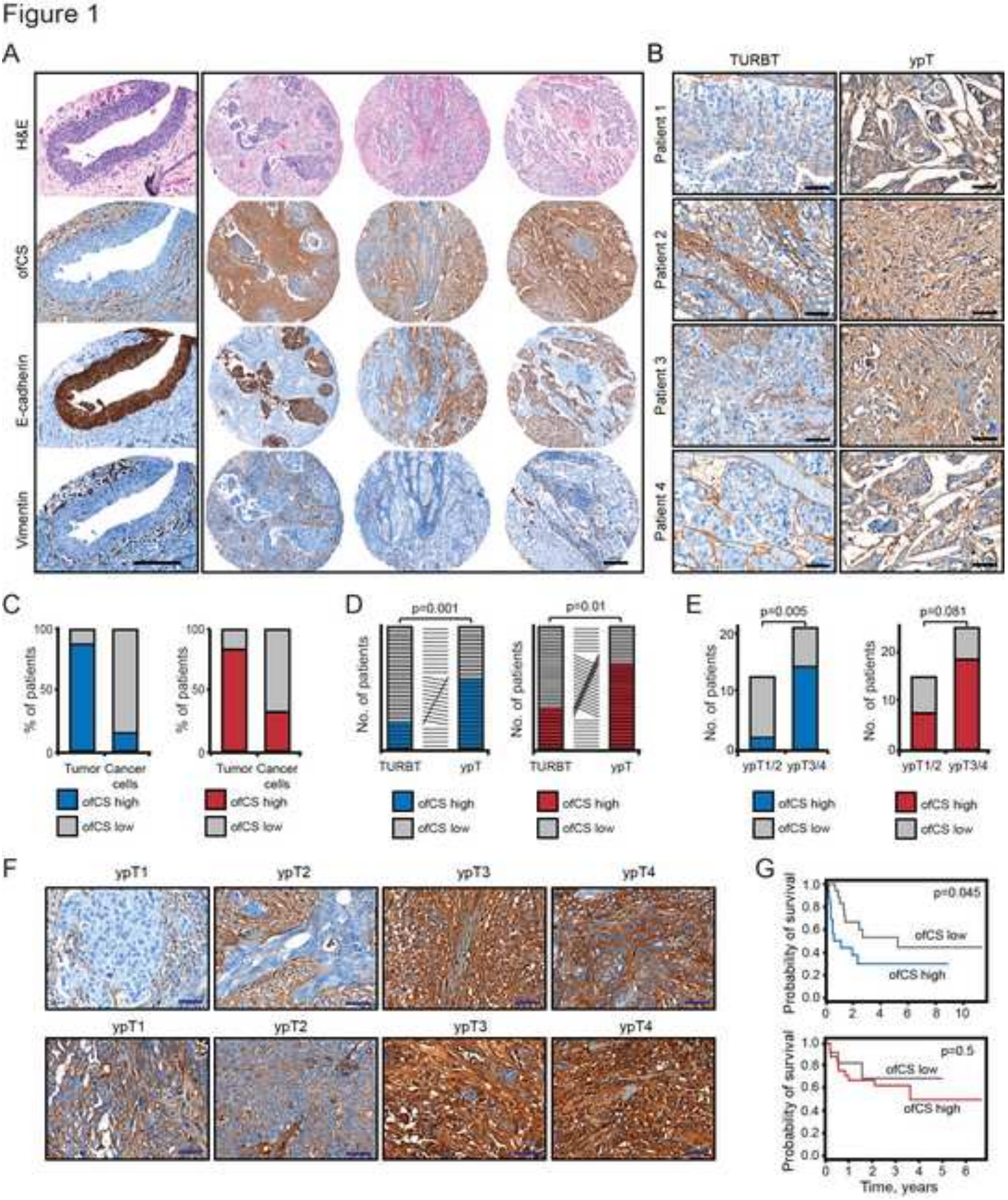

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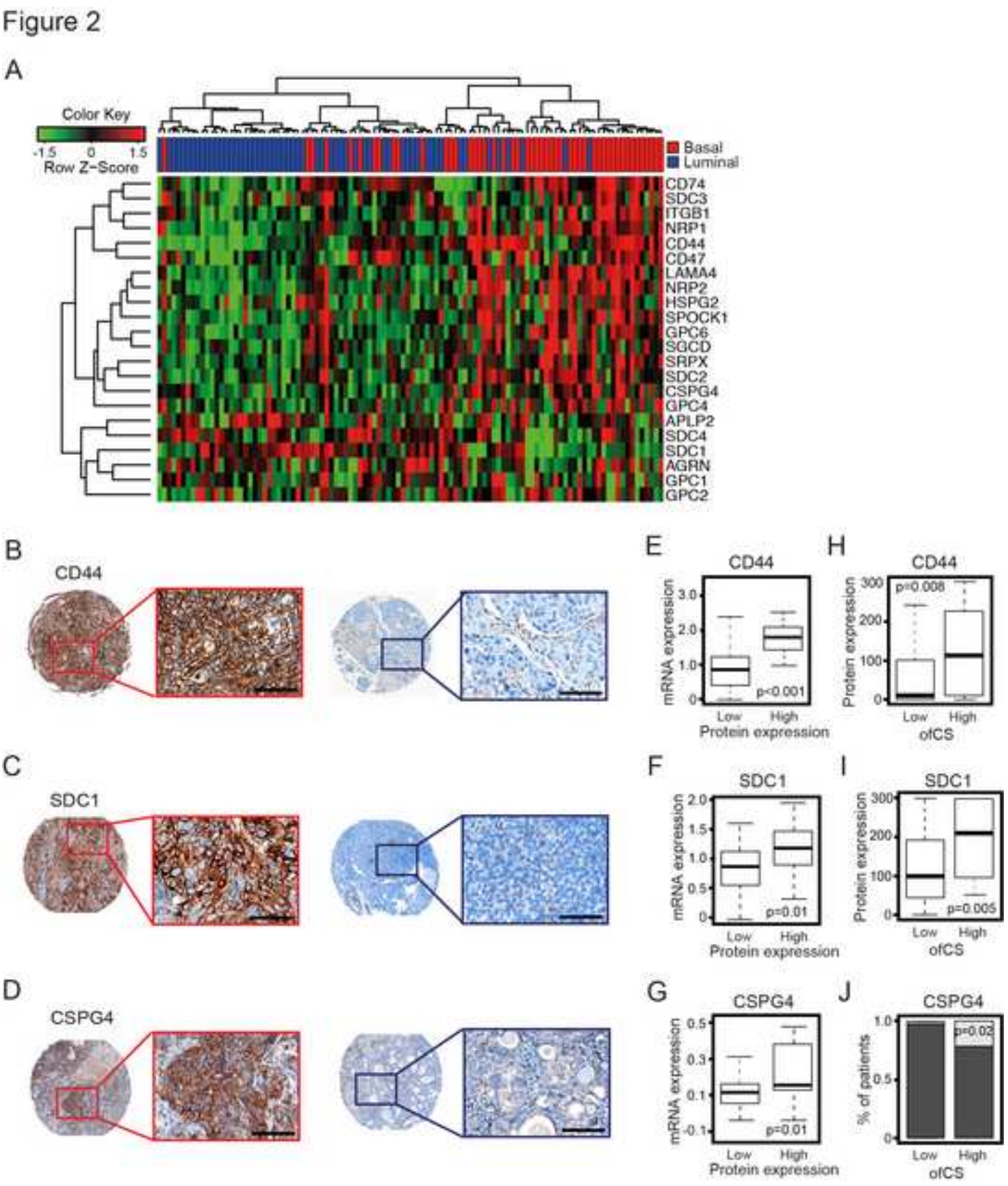

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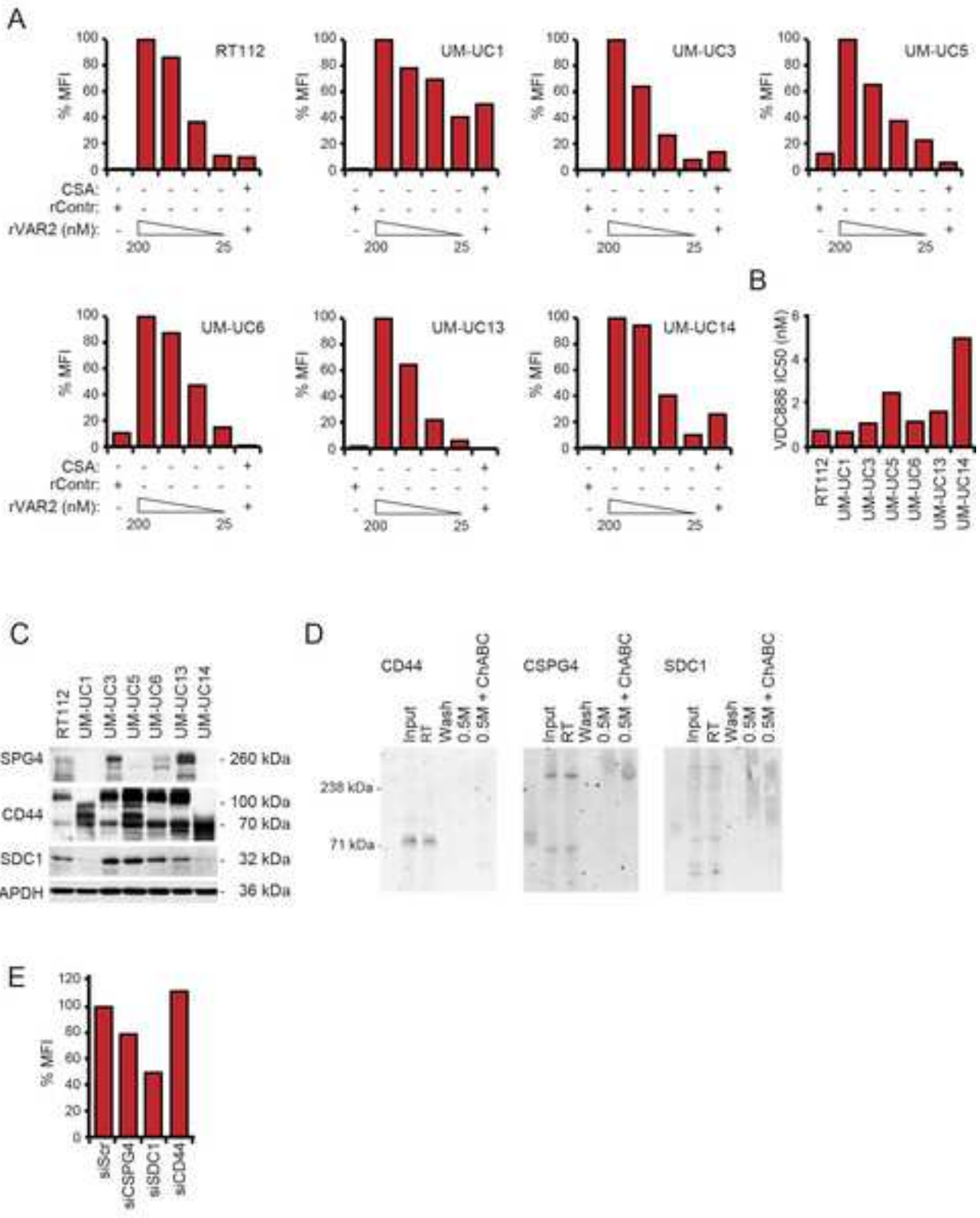

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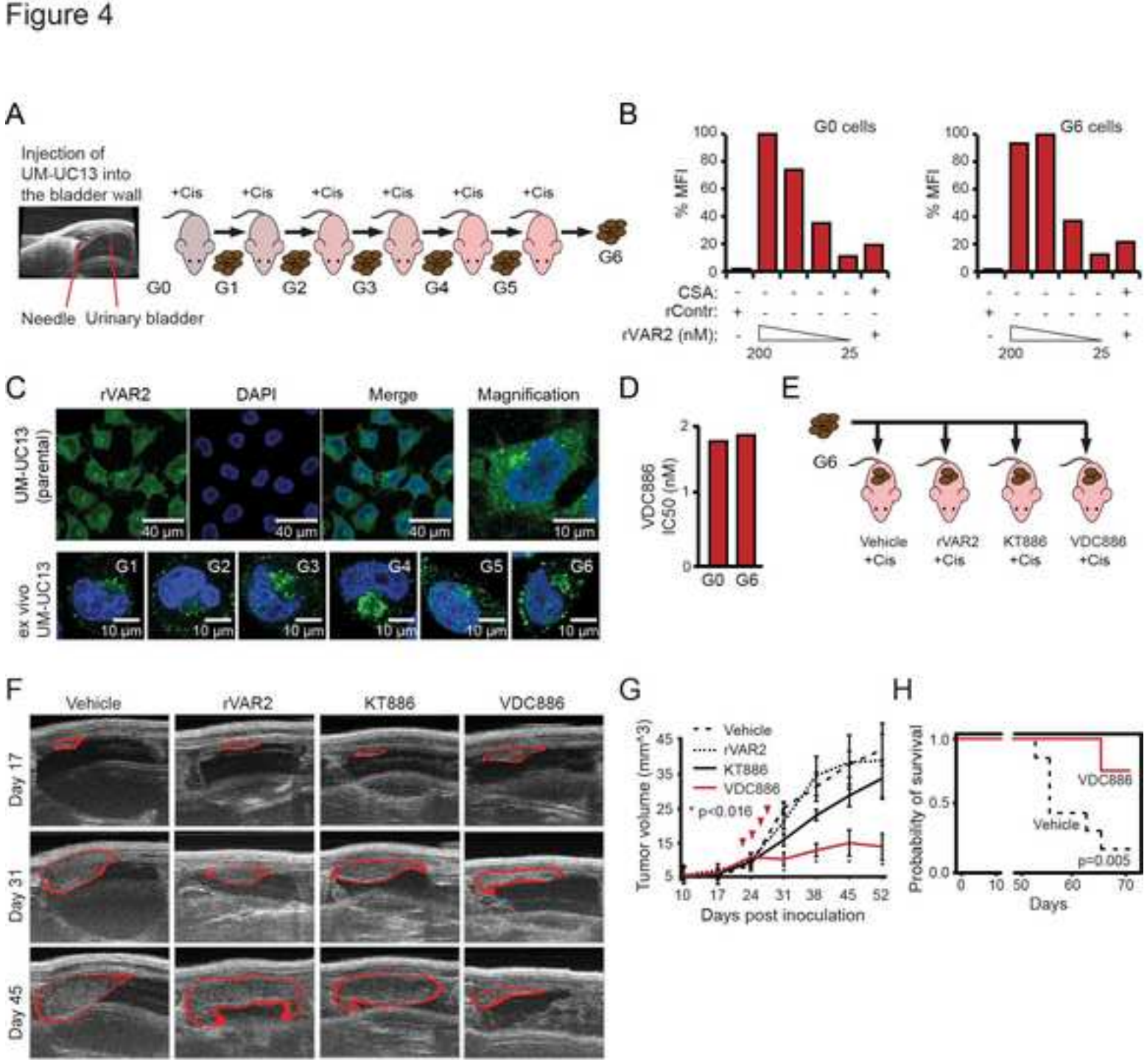

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An oncofetal glycosaminoglycan modification provides therapeutic access to cisplatin-resistant bladder cancer

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Running head: Targeting oncofetal glycosaminoglycan in bladder cancer

Key words: Bladder cancer; cisplatin-resistance; malaria protein; second-line therapy; targeted therapy

ABSTRACT

Background: Although cisplatin-based neo-adjuvant chemotherapy (NAC) improves survival of unselected patients with muscle-invasive bladder cancer (MIBC), only a minority responds to therapy and chemoresistance remains a major unmet need in this disease setting.

Objective: To investigate the clinical significance of oncofetal chondroitin sulfate (ofCS) in cisplatin-resistant MIBC and to evaluate these as targets for second line therapy.

Design, Setting, and Participants: An ofCS-binding recombinant VAR2CSA protein derived from the malaria parasite *Plasmodium falciparum* (rVAR2) was used as an *in situ*, *in vitro* and *in vivo* ofCS-targeting reagent in cisplatin-resistant MIBC. The ofCS expression landscape was analyzed in two independent cohorts of matched pre- and post-NAC treated MIBC patients.

Intervention: An rVAR2 protein armed with cytotoxic hemiasterlin compounds (VDC886) was evaluated as a novel therapeutic strategy in a xenograft model of cisplatin-resistant MIBC.

Outcome Measurements and Statistical Analysis: Anti-neoplastic effects of targeting ofCS.

Results and Limitation: *In situ,* ofCS was significantly overexpressed in residual tumors after NAC in two independent patient cohorts ($p<0.02$). Global gene-expression profiling and biochemical analysis of primary tumors and cell lines revealed syndican-1 (SDC1) and chondroitin sulfate proteoglycan 4 (CSPG4) as ofCS-modified proteoglycans in MIBC. *In-vitro*, ofCS was expressed on all MIBC cell lines tested and VDC886 eliminated these cells in the low-nanomolar IC_{50} concentration range. *In-vivo*, VDC886 effectively retarded growth of chemoresistant orthotopic bladder cancer xenografts and prolonged survival ($p=0.005$). The number of mice and the use of cisplatin only for the generation of chemoresistant xenografts are limitations of our animal model design.

Conclusions: Targeting ofCS provides a promising second line treatment strategy in cisplatin-resistant MIBC.

Patient Summary: Cisplatin-resistant bladder cancer overexpresses particular sugar chains compared to chemotherapy naïve bladder cancer. Using a recombinant protein from the malaria *Plasmodium falciparum* parasite, we can target these sugar chains and our results showed a significant antitumor effect in cisplatin-resistant bladder cancer. This novel treatment paradigm provides a therapeutic access as second line therapy for bladder cancer not responding to cisplatin.

INTRODUCTION

Bladder cancer is the 5th most common cancer in the world and the most costly cancer to treat on a per patient basis due to required clinical surveillance and multiple therapeutic interventions[1]. Muscle-invasive bladder cancer (MIBC) is a highly aggressive flavor of the disease with a 5-year survival probability of ~50%[2]. Despite efforts in refining surgical techniques and optimizing systemic therapy, the prognosis has remained unchanged for more than 20 years[3]. Clinical improvement can be ascribed to Cisplatin-based neo-adjuvant chemotherapy (NAC), which offers an overall survival benefit of 5-6%[4] and standard-of-care in MIBC[5]. However, only approximately 40% of patients demonstrate a major response to NAC, while adverse treatment effects and a delay in surgery potentially harm 60%. Therefore, second line treatment options for MIBC are currently in great demand[6]. Although 60% of patients with metastatic MIBC demonstrate an objective response to cisplatin-based chemotherapy this response is rarely durable, and almost all of these patients succumb to their disease[7]. Atezolizumab has recently become the first FDA-approved agent for the second line treatment of metastatic bladder cancer[8], but there remains a great need for additional second and additional line agents in all states of MIBC.

Glycosaminoglycans (GAGs) are carbohydrate modifications attached to to proteins in the cell membrane. Changes in expression and composition of GAGs have been reported in bladder cancer over the past three decades[9-11]. Chondroitin sulfate (CS) is a major cancer-associated GAG that also has a key role in malaria pathogenesis[12]. The *Plasmodium falciparum* malaria parasite has evolved a protein, called VAR2CSA that mediates attachment of infected erythrocytes to a distinct type of CS in the placental syncytium[13]. CS chains are comprised of repeated disaccharides units, made up of glucoronic acid (GlcA) and N-acetylgalactosamine (GalNAc) residues. CS chains are highly heterogeneous showing variations in both chain length and disaccharide modifications, such as sulfation. Placental-type CS is highly sulfated on

carbon-4 of the GalNAc hexose and this particular modification is required for exclusive sequestration of VAR2CSA positive erythrocytes in the placenta[14]. Placenta and cancer share obvious phenotypic features such as highly proliferating cells, the ability to invade adjacent tissue, and promoting angiogenesis[15]. In the placenta, these features are partly facilitated by CS and many tumors re-express placental-type CS as an oncofetal GAG[16]. Therefore, recombinant malarial VAR2CSA (rVAR2) proteins can be conveniently utilized to detect oncofetal chondroitin sulfate (ofCS) in human cancer[16,17]. Finally, rVAR2 conjugated with the hemiasterlin toxin analog KT886 derived from the marine sponge *Hemiasterella minor* (VDC886), show anti tumor activity in non-Hodgkin's lymphoma, prostate cancer, and metastatic breast cancer with no adverse treatment effects in non-pregnant mice[16].

We noticed that several cancer-associated proteins can be modified with an ofCS chain, including CD44[16], SDC1[18], and CSPG4[19], and that CD44 and SDC1 have previously been suggested as a candidate biomarker in bladder cancer[20-25]. Accordingly we hypothesized that human MIBC might display ofCS chains that could be exploited for rVAR2-based targeted therapy. In particular we aimed to investigate the role of ofCS expression in cisplatin-resistant MIBC with the intention of developing an additional line treatment option for this disease state.

MATERIAL AND METHODS

Bladder cancer patient cohorts

Bladder tumor tissue was collected at three institutions (University of Bern, Switzerland, at Vancouver General Hospital, Canada and University Hospital of Southampton, UK) from patients receiving at least 3 cycles of cisplatin-based neoadjuvant chemotherapy prior to radical cystectomy with pelvic lymph node dissection (**Supplementary Table S1**). Tissue was harvested from pre-chemotherapy TURBT specimens in all patients and from post-

chemotherapy radical cystectomy specimens in patients with residual MIBC (ypT). The Bern cohort was used as the discovery cohort, and the Vancouver and Southampton cohorts were amalgamated as the validation cohort.

A complete and detailed description of Materials and Methods used in this study can be found in in the **Supplementary Information**.

RESULTS

To analyze the ofCS expression in bladder cancer pre- and post-treatment with cisplatin-based chemotherapy, we performed immunohistochemical (IHC) analysis on two independent cohorts of matched primary chemotherapy-naïve and cisplatin-resistant bladder tumors using rVAR2 as the ofCS detection reagent (**Fig. 1A, 1B** and **Supplementary Fig. 1A-E**). The ofCS expression is absent in urothelium of the adjacent normal bladder (**Fig. 1A, left**) and is strongly restricted to the bladder tumors (**Fig. 1A, right**). To differentiate ofCS expression between tumor environment and the cancer cell compartments, IHC analyses of an epithelial marker, E-cadherin and a mesenchymal marker, Vimentin were performed (**Fig. 1A**). The pre-chemotherapy bladder tumors were sampled by transurethral resection (TURBT) and the matched cisplatin-resistant tumors after neo-adjuvant chemotherapy were sampled from subsequent radical cystectomy specimens. The discovery cohort was comprised of more advanced tumors from a single institution as compared to the validation cohort combined from two institutions (**Supplementary Table S1**). Overall, high ofCS expression is seen in approximately 92% (n=110/120) of chemotherapy-naïve bladder tumors in both cohorts. Among which, 17% (n=8/47) of the tumors in the discovery cohort (**Fig. 1C, left**) and 33% (n=24/73) of the validation cohort (**Fig. 1C, right**) showed high membranous expression of ofCS (ofCS^{High}) in the cancer cell compartment.

In chemotherapy-naïve tumors, ofCS expression failed to predict response to chemotherapy (p=0.4, OR= 2.28, 95%CI=0.35-25.87) and inform on survival in univariate and multivariate analyses. In cisplatin-resistant disease (ypT) ofCS expression increased to 57% (n=16/28; p=0.001; vs. 29%, n=8/28 in TURBT) of tumors showed ofCS^{High} in cancer cells in the discovery cohort (**Fig. 1D, left**) and 70% (n=23/33; p=0.01; vs. 33%, n=11/33 in TURBT) in the validation cohort (**Fig. 1D, right**). In cisplatin-resistant MIBC, ofCS^{High} was associated with extra-vesical extension in the cystectomy specimen in the discovery cohort (p=0.005) (**Fig. 1E, left** and **Fig. 1F**) but was not significant in the validation cohort (p=0.08) (**Fig. 1E right**). This shift in ofCS expression was associated with poor overall survival (OS) in the discovery cohort (p=0.04) (**Fig. 1G, upper**) but not in the validation cohort (p=0.5), which had a shorter clinical follow-up of 3.3 years (95%CI: 2.52-3.5) (**Fig. 1G, lower**). In multivariate analysis, only pathological tumor stage added independent prognostic information (**Supplementary Table S2)**. Accordingly, these data indicate that ofCS is upregulated in cisplatin-resistant bladder cancer cells and this event may be associated with poor outcome.

Several proteoglycans can carry ofCS GAG modifications[16]. To search for ofCS-modified proteoglycans in bladder cancer, we analyzed gene expression data of known CS-modified proteoglycans[16,28] in two independent bladder cancer cohorts. In both datasets, luminal type tumors were enriched for SDC1, SDC4, and APLP2, while more basal tumors were enriched for a number of genes including CD44 and CSPG4 (**Fig. 2A**). SDC1, CSPG4, and CD44 have been widely associated with multiple types of cancer[16,18,19], and CD44 and SDC1 have previously been suggested as a candidate biomarker in bladder cancer[20-25]. Indeed, protein expression of CD44 (**Fig. 2B**), SDC1 (**Fig. 2C**), and CSPG4 (**Fig. 2D**) showed a strong correlation with mRNA expression (**Figs. 2E-G**) and was strongly correlated with ofCS^{High} cases (**Fig. 2H-J**). Together, these data suggest that CD44, SDC1, and CSPG4 are expressed in human bladder cancer and associated with ofCS.

We next analyzed a panel of 7 bladder cancer cell lines derived from MIBC patients for ofCS-expression by flow cytometry using rVAR2 as the ofCS detection reagent. All MIBC cell lines analyzed expressed ofCS as detected by rVAR2 in a concentration and CS-dependent manner (**Fig. 3A**). This was supported by sensitivity to the ofCS targeting rVAR2 drug conjugate (VDC) VDC886[16] in the low-nanomolar IC_{50} concentration range (**Fig. 3B**). Interestingly, 4 out of 7 cell lines expressed CSPG4; 7 out of 7 expressed CD44; and 5 out of 7 expressed SDC1 (**Fig. 3C**), but this expression pattern was not obviously related to VDC886 sensitivity. This might indicate that not all of the selected proteoglycans (CD44, SDC1, and CSPG4) are modified in the individual cell lines and/or that additional proteoglycans contribute to the ofCS presentation. To investigate this, we subjected UM-UC13 cells to biochemical analysis to directly assess whether or not CD44, SDC1, and CSPG4 were modified with ofCS. ofCS-modified proteins and proteins in complex with ofCS-modified proteins, were purified from cell lysate using immobilized rVAR2. The resulting fractions were treated with chondroitinase ABC to reduce the CSPGs to their component core proteins. A shift in gel mobility towards the predicted protein size on SDS-PAGE, upon Chondroitinase ABC treatment therefore implies CS substitution. Surprisingly, while CD44, SDC1, and CSPG4 were expressed in UM-UC13 cells, only SDC1 and CSPG4 produced a shift in molecular weight after chondroitin treatment (**Fig. 3D**). Chondroitinase ABC treatment did not reduce the size of SDC1 to its predicted core protein size, but merely produced an intermediary sized protein. This is in line with the heparan sulfate (HS) substitution status of SDC1, a GAG that will not be digested with Chondroitinase ABC. This indicates that SDC1 and CSPG4, but not CD44, are ofCS-modified in these cells. To validate this result, we treated UM-UC13 cells with validated siRNAs targeting CD44, SDC1, and CSPG4, and assayed for binding to rVAR2 as an indication of ofCS expression. While the CD44 siRNA had no effect on rVAR2 binding, SDC1 and CSPG4 siRNAs reduced rVAR2 binding to UM-UC13 cells with 50% and 20% respectively (**Fig. 3E**). This corroborates the biochemical analysis showing that SDC1 and

CSPG4, but not CD44, are ofCS-modified in UM-UC13 cells, reflecting that ofCS modification of eligible proteins is not mandatory and likely a dynamic event.

To assess the expression of ofCS-modified proteoglycans in cells treated with cisplatin *in vivo* we inoculated UM-UC13 cells into the bladder wall of nude mice with ultrasound guidance (S**upplementary Fig. S3A-B**). As the UM-UC13 tumors developed, the mice were subjected cisplatin treatment and resistant tumors were allowed to recover. To analyze the ofCS modification status of proteoglycans in UM-UC13 tumors and to potentially identify novel proteoglycans, we purified ofCS-associated proteoglycans from *ex-vivo* cells and analyzed those by mass spectrometry. This analysis identified 8 ofCS-modified proteoglycans including SDC1 and CSPG4 (**Supplementary Table S3**). Similar to the *in vitro* cell line study, this analysis showed that CD44 was not modified with ofCS in UM-UC13 cells, and that ofCS-modification of SDC1 and CSPG4 is retained *in vivo*. Proximity-ligation assay (PLA) analysis of ofCS chains and CD44, SDC1 and CSPG4, confirmed the mass spectrometry result (**Supplementary Fig. S3C**) but surprisingly, CD44 was also in proximity to ofCS. This suggests that while CD44 is not itself modified with ofCS in UM-UC13 cells, it is indeed expressed in close proximity to ofCS-modified proteoglycans.

Capitalizing on our findings that human cisplatin-resistant MIBC upregulates ofCS (**Fig. 1A-F**) and that MIBC cells are sensitive to VDCs *in vitro* (**Fig. 3B**), we next tested whether VDC886 had efficacy against cisplatin-resistant MIBC *in vivo*. Again, UM-UC13 cells were inoculated into the bladder wall of nude mice with ultrasound guidance (**Supplementary Fig. S3A-B**) and tumor growth was subsequently monitored using ultrasound over 40 to 60-day period (**Supplementary Fig. S4A**). As the UM-UC13 tumors developed, the mice were subjected to repeating cycles of cisplatin treatment and the resultant tumors were passaged through 6 generations of mice (G1-G6) to create highly stable cisplatin-resistant xenografts (**Fig. 4A**). Importantly, the *ex-vivo* tumor cells retained their rVAR2 binding, in an concentration and CS-

dependent manner (**Fig. 4B**). They also showed the same rVAR2 internalization capacity, as the parental line, G0 (**Fig. 4C**).

Importantly, the completely cisplatin-resistant G6 and cisplatin-sensitive G0 cells showed equal sensitivity to VDC886 *ex-vivo* (**Fig. 4D** and **Supplementary Fig. S4B**).

Next, mice with established G6-initiated cisplatin-resistant tumors were randomized into 4 groups and treated biweekly (4 treatments in total) with vehicle, rVAR2, KT886, or VDC886 (**Fig. 4E**) in combination with cisplatin. The G6 cisplatin-resistant tumors expressed SDC1, CSPG4, and ofCS (**Supplementary Fig. S4C**). Remarkably, VDC886 treatment strongly retarded growth of cisplatin-resistant tumors (**Fig. 4F** and **4G**) and significantly prolonged survival of the mice (**Fig. 4H**). Importantly, clinicopathological examination of VDC886 treated mice demonstrated no organ toxicity (**Supplementary Fig. S4D**) and the weight of the mice was stable in all groups (**Supplementary Fig. S4E**). In the VDC886 treated group, one mouse had complete response; one mouse presented with significant tumor regression (**Supplementary Fig. S4F**); and four mice had stable tumor volume without progression during the experiment, with viable tumor cells in histology. In summary, our work demonstrates that ofCS-modified proteoglycans can provide therapeutic access to cisplatin-resistant MIBC.

DISCUSSION

We have tested an unconventional approach for treating cisplatin-resistant MIBC based on the evolutionarily refined parasite-host anchor protein VAR2CSA derived from the *P. falciparum* malaria parasite. We found that ofCS was present at high levels in cisplatin-resistant MIBC promoting ofCS as a potential access point for targeted therapy. Supporting this notion, VDC886 was able to suppress growth of cisplatin-resistant MIBC. High ofCS expression was associated with advanced tumor stage, cisplatin-resistance and poor overall survival of MIBC patients.

These associations were more robust in the discovery cohort, which contained more advanced disease and a longer follow-up. In the validation cohort, presentation of ofCS was not prognostic. While this suggests that high ofCS may only relate to survival in more advanced MIBC, this association requires further investigation.

Several different proteoglycans can be modified with ofCS chains[16]. As a result, intra-tumoral heterogeneity in proteoglycan expression does not necessarily translate into varying levels of ofCS modifications. CD44 has previously been shown to carry ofCS chains in melanoma[16,29,30]. We found that CD44 mRNA and protein expression correlated with high ofCS presentation in MIBC but biochemical interrogation showed that CD44 was not modified with ofCS, at least not in UM-UC13 cells. Contrary to CD44, the CSPGs SDC1 and CSPG4 were modified in UM-UC13 cells and correlated with ofCS expression in primary tumors. Mass spectrometry of rVAR2-purified proteoglycans identified another 6 ofCS-modified candidates. It is likely that additional proteoglycans contribute to ofCS presentation in MIBC.

The most important finding of our study was the increased cellular ofCS expression in cisplatin-resistant MIBC when compared to paired chemotherapy-naïve MIBC. These findings suggest that targeting ofCS with VDCs provide an attractive approach particularly in cisplatin-resistant MIBC. The 1st generation VDC compound VDC886 is comprised of the DBL1X-ID2a domains of the malarial VAR2CSA protein loaded with an average of three KT886 hemiasterlin toxin analogs derived from the marine sponge *Hemiasterella minor*[16]. VDC886 eliminated all MIBC cell lines in the low nanomolar IC_{50} concentration range. As the MIBC cell lines investigated all express high levels of ofCS, the small differences in IC_{50} are most likely due to differences in internalization kinetics. In an animal model of cisplatin-resistant MIBC, VDC886 was able to efficiently target established cisplatin-resistant tumors and rescue the mice from tumor-associated morbidity and death. The completely cisplatin-resistant G6 and cisplatin-sensitive G0 cells isolated from mice showed equal sensitivity to VDC886 *ex-vivo*. This is likely due to high

baseline levels of ofCS in MIBC cell lines, and suggests that the ofCS modification is a passenger, rather than a driver, of cisplatin resistance. Nevertheless, ofCS glycosaminoglycan-modifications constitute a therapeutic access point in cisplatin-resistant MIBC.

CONCLUSIONS

We have found an oncofetal glycosaminoglycan antigen in cisplatin-resistant MIBC that can be targeted by a malarial host-cell anchor protein armed with hemiasterlin compounds. This discovery potentially offers a new treatment paradigm for human MIBC patients not responding to cisplatin.

DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

M. Daugaard, A. Salanti, M. Ø. Agerbæk, and T. M. Clausen are shareholders in VAR2 Pharmaceuticals Aps. VAR2 Pharmaceuticals is a biotechnology company that develops cancer therapeutics and diagnostic tools. J. S. Babcook and J. Rich are employees of Zymeworks Inc.

AUTHORS' CONTRIBUTIONS

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Seiler, HZ. Oo, D. Tortora, T. M. Clausen, C. K. Wang, G. Kumar, M.S. Ørum-Madsen, S. S. Lee, S. Esfandnia, M.A. Nordmaj, D. Battsogt, N. Al-Nakouzi, P.C. Black, A. Salanti, M. Daugaard

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Other (provided experimental data): none

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FIGURE LEGENDS

FIGURE 1

ofCS expression in chemotherapy-naïve (TURBT) and cisplatin-resistant (ypT) bladder cancer

(A) Representative H&E and IHC images in matched adjacent normal bladder (left panel) and bladder cancer cases (right panel) with an epithelial marker, E-cadherin and a mesenchymal marker, Vimentin expression in parallel with ofCS expression. Scale bar represents 200 μm.

(B) Representative IHC images of ofCS expression in matched bladder cancer cases showing cellular ofCS expression in paired TURBT (left panel) and cisplatin resistant tumors at cystectomy (ypT) (right panel). Scale bar represents 50 μm.

(C) ofCS expression was examined in chemotherapy-naïve bladder cancers (TURBT) in two independent cohorts (discovery: left, validation: right). "Tumor" represents ofCS expression in overall bladder tumor including the surrounding microenvironment, and "cancer cells" represent membranous ofCS expression only in cancer cells.

(D) Plots indicating paired analysis of ofCS expression of cancer cells in chemotherapy-naïve (TURBT) and cisplatin-resistant (ypT) tumors in discovery (left) and validation (right) cohorts. Each box indicates the tumor of a given patient and the lines indicates the pairs between TURBT to ypT.

(E) Barplots indicating the relation of cellular ofCS expression in ypT compared to tumor stage in discovery (left) and validation (right) cohorts.

(F) Representative IHC images of ofCS expression according to ypT stages of MIBC in 8 different patients from a validation cohort. Scale bar represents 50 μm.

(G) Kaplan-Meier plots for overall survival (OS) stratified according to high and low cellular ofCS expression in ypT (discovery: upper, validation: lower).

FIGURE 2

ofCS-carrying proteoglycans in bladder cancer

(A) Heatmap of gene expression of proteoglycans that have been shown to be ofCS modified. The TURBT samples from both cohorts have been selected. Virtually all samples show high expression of at least one validated proteoglycan.

(B) Representative IHC images showing high and low CD44 protein expression of bladder cancer. For statistical analysis in the subsequent panel (E), low expressing tumors (1^{st}-3^{rd} quartile) were compared to high expression tumors (4^{th} quartile), respectively. Scale bar represents 100 µm.

(C) Representative IHC images showing high and low SDC1 protein expression of bladder cancer. For statistical analysis in the subsequent panel (F), low expressing tumors (1^{st}-3^{rd} quartile) were compared to high expression tumors (4^{th} quartile), respectively. Scale bar represents 100 µm.

(D) Representative IHC images showing high and low CSPG4 protein expression of bladder cancer. For statistical analysis in the subsequent panel (G), low expressing tumors (1^{st}-3^{rd} quartile) were compared to high expression tumors (4^{th} quartile), respectively. Scale bar represents 100 µm.

(E) Boxplot indicates the CD44 mRNA expression (y-axis) of samples with low and high CD44 protein expression, respectively.

(F) Boxplot indicates the SDC1 mRNA expression (y-axis) of samples with low and high SDC1 protein expression, respectively.

(G) Boxplot indicates the CSPG4 mRNA expression (y-axis) of samples with low and high CSPG4 protein expression, respectively.

(H) Boxplot indicating CD44 protein expression levels in ofCS^{High} and ofCS^{Low} bladder tumors.

(I) Boxplot indicating SDC1 protein expression levels in ofCS^{High} and ofCS^{Low} bladder tumors.

(J) Barplot indicating CSPG4 protein expression levels in ofCS^{High} and ofCS^{Low} bladder tumors.

FIGURE 3

rVAR2 binds human bladder cancer cells

(A) Relative geometric mean fluorescence intensity (MFI) of a panel of bladder cancer cell lines incubated with recombinant V5-tagged control protein (rContr) or VAR2CSA (rVAR2) as indicated and detected by flow cytometry using anti-V5-FITC.

(B) Indicated human bladder cancer cell lines were treated with rVAR2 drug conjugate (VDC886). The column graph displays IC50 kill-values of VDC886.

(C) CD44, CSPG4 and SDC1 protein expression in the same bladder cancer cell line panel is shown, with GAPDH as a loading control.

(D) Westen blot of rVAR2 column pulldown of CD44, CSPG4, and SDC1. Blots shows input, run through (RT), wash, and elution with and without Chondroitinase ABC treatment.

(E) UM-UC13 cells were treated with validated siRNAs targeting CD44, SDC1, and CSPG4 and assayed for binding to rVAR2.

FIGURE 4

Potential of VDC886 for targeted therapy of cisplatin-resistant MIBC

(A) Diagram illustrating ultrasound-guided xenograft animal model for cisplatin-resistant human bladder cancer. After initial injection of UM-UC13 cells into the bladder wall, mice were constantly treated with cisplatin (3mg/kg weekly). Tumors were passaged into the next generation for 6 successive cycles. The 6^{th} generation was used for the final animal model. During every passage, tumors were stored (FFPE, Frozen) and an *ex-vivo* cell line was created for each generation.

(B) Binding of rVAR2 to UM-UC13 parental and *ex-vivo* cells (generation 6) are indicated. Relative mean fluorescence intensity (MFI) incubated with recombinant control protein (rContr) or rVAR2 as indicated and detected by flow cytometry using anti-V5-FITC.

(C) Internalization of Alexa488-labelled rVAR2 in parental (upper panel) and *ex-vivo* (lower panel) UM-UC13 bladder cancer cells detected by confocal microscopy 30 minutes after addition of rVAR2-Alexa488 (green) and DAPI (blue).

(D) *Ex-vivo* bladder cancer cell lines were seeded in 96-well plates and treated with VDC886 in concentrations ranging from 0.01 pM to 200 nM. The column graph displays IC50 kill-values of VDC886 performance in G0 and G6. Kill-values for the other generations were virtually the same (data not shown).

(E) Schematic illustration of the assignment of the mice to the respective treatment groups. VDC886 was tested against the vehicle, rVAR2 and KT886, respectively.

(F) Representative ultrasound images of each group at day 17, 31 and 45. The tumor growth was highlighted in red circle for each group.

(G) Comparing tumor growth between VDC886 and control groups. Treatment was administered intravenously twice per week (red arrow heads) as indicated.

(H) Survival curve of VDC886 and Vehicle treated mice from (G).

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***Revision notes**

Comments to Author:

Reviewer #6: Attention to the following issues would improve the manuscript:

1. Line 140: Don't just say "did not predict", provide some data (e.g. OR with 95% C.I. plus a p value)
Response: We added the data of this test to the manuscript (p=0.4, OR= 2.28, 95%CI=0.35-25.87).

2. Line 141: say "multivariate" rather than "multivariable"
Response: The wording was changed according to the suggestion of the reviewer.

3. Line 142: A p value is reported of 0.001. It is unclear what the null hypothesis is and what statistical test was used.
Response: We appreciate this comment of the reviewer. This analysis is a comparison of the tumors with high membranous expression of ofCS ($ofCS^{High}$) in chemotherapy naïve TURBT specimens (6/28) and cisplatin resistant post chemotherapy RC specimens (16/28). The test used was a two-sided exact Fisher's test and the null hypothesis was, that the rate of $ofCS^{High}$ is the same in TURBT and RC. We have modified the text to: "…increased to 57% (n=16/28; p=0.001; vs. 29%, n=8/28 in TURBT) of tumors showed…".

4. Line 145: don't use the word "trend" for a non-significant p value.
Response: We modified this sentence to: "but was not significant in the validation cohort (p=0.08)".

5. Line 146: report p values close to 0.05 to two significant figures
Response: This p-values refers to the analysis made in Figure 1E left. The panel in Figure 1F is a representation of IHC pictures that indicate a higher expression in more advanced tumor stages and supports the data in Figure 1E right. We therefore, moved the link to Figure 1F to the indication of Figure 1E right (one line above).

6. Line 149: Please see the European Urology guidelines for presentation of statistics: http://www.europeanurology.com/article/S0302-2838(14)00598-3/pdf/guidelines-for-reporting-of-statistics-in-european-urology with respect to the summary statistics needed for time to event analyses.
Response: We would like to thank the reviewer for this comment. We have added more data to this number (clinical follow-up of 3.3 years, 95%CI: 2.52-3.5)

7. What happened to graph J on figure 2?
Response: The IHC expression of CSPG4 was very homogeneous in the entire tumor. A determination of the CSPG4 protein expression using an H-Score was not meaningful. We determined this expression by intensity. Therefore, the expression of both CSPG4 and ofCS is categorical for this analysis and shown as a barplot of the contingency table analyzed using a two sided exact Fisher's test. In the revised manuscript we have indicated the evaluation of CSPG4 protein expression as a categorical parameter in the supplemental text.