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European Association of Urology

Platinum Priority – Urothelial Cancer

Editorial by Rui Henrique, Sandra P. Nunes and Carmen Jeroónimo on pp. 251–252 of this issue

A Whole-genome CRISPR Screen Identifies a Role of MSH2 in Cisplatin-mediated Cell Death in Muscle-invasive Bladder Cancer

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Article info

Article history:
Accepted October 15, 2018

Associate Editor:
James Catto

Keywords:
MSH2
Mismatch repair
CRISPR screen
Bladder cancer
Cisplatin
Patient stratification
Chemotherapy

Abstract

Background: The response to first-line, platinum-based treatment of muscle-invasive bladder cancer has not improved in 3 decades.
Objective: To identify genes that influence cisplatin resistance in bladder cancer.
Design, setting, and participants: We performed a whole-genome CRISPR screen in a bladder cancer cell line to identify genes that mediate resistance to cisplatin.
Outcome measurements and statistical analysis: Targeted validation was performed in two bladder cancer cell lines. The top gene candidate was validated in a publicly available bladder cancer dataset.
Results and limitations: From the CRISPR screen, we identified *MSH2* as the most significantly enriched gene and mismatch repair as the most significantly enriched pathway that promoted resistance to cisplatin. Bladder cancer cells with knockdown of *MSH2* showed a reduction in cisplatin-mediated apoptosis. *MSH2* loss did not impact the sensitivity to other chemotherapies, including the cisplatin analog oxaliplatin. Bladder tumors with low MSH2 protein levels, quantified using reverse-phase protein array, showed poorer survival when treated with cisplatin- or carboplatin-based therapy; these results require future validation using immunohistochemistry. Additionally, results are retrospective from patients with primarily high-grade tumors; thus, validation in a controlled clinical trial is needed.
Conclusions: We generated in vitro evidence that bladder cancer cell lines depleted of MSH2 are more resistant to cisplatin. We additionally found an association between low MSH2 in bladder tumors and poorer patient survival when treated with platinum-based chemotherapy. If successfully validated prospectively, MSH2 protein level could assist in the selection of patients for chemotherapy.
Patient summary: We report the first evidence that MSH2 protein level may contribute to chemotherapy resistance observed in muscle-invasive bladder cancer. MSH2 has potential as a biomarker predictive of response to platinum-based therapy.

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https://doi.org/10.1016/j.eururo.2018.10.040
0302-2838/

1. Introduction

Bladder cancer is the ninth most common cancer type, with an estimated 430 000 new cases and 165 000 deaths worldwide in 2012 [1]. In addition to radical cystectomy, patients with muscle-invasive bladder cancer (MIBC) are typically treated with one of two chemotherapy regimens, methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) or gemcitabine and cisplatin (GC). The response rate to these cisplatin-based chemotherapies remains 50% in MIBC [2]. Identifying the molecular mechanisms that determine patient response to these treatments has a direct clinical impact, including as a biomarker to stratify patients according to response and potentially as an avenue to explore novel therapeutic targets.

Cancer cells that lack components of the mismatch repair (MMR) pathway, such as MLH1 and MSH2, are resistant to cisplatin in vitro [3,4]. Ovarian tumors with low MLH1 expression associate with poorer survival in platinum-treated patients [5–7]. Similarly, a study of 17 ovarian cancer patients treated with cisplatin-based chemotherapy found that patients with a poor response had significantly lower MSH2 protein levels [8]. Interestingly, low MMR protein expression does not always correlate with resistance to platinum-based therapies. In contrast to ovarian cancer, non–small cell lung cancer (NSCLC) patients with low MSH2 had improved survival when treated with platinum [9,10]. While the MMR pathway has not been associated with platinum resistance in bladder cancer, a subset of bladder cancers have reduced or absent protein expression of MSH2 as determined by immunohistochemistry (IHC) [11–15].

Here, we take an unbiased approach to investigate mediators of cisplatin resistance by performing, to the best of our knowledge, the first genome-wide CRISPR-Cas9 cisplatin resistance screen in bladder cancer cells. Our screen results show that cells with loss of *MSH2* are more resistant to cisplatin. We validated this finding by showing that bladder cancer cell lines with knockdown of *MSH2* had a reduction in cisplatin-mediated apoptosis. Consistent with our in vitro results, we found that MIBC tumors with low levels of MSH2 protein expression had poorer survival when treated with platinum-based chemotherapy compared with those with higher MSH2 expression. MSH2 levels did not associate with survival in patients who did not receive a pharmacologic or radiation treatment, suggesting that the association with survival is specific to platinum treatment.

2. Patients and methods

2.1. Cell culture, shRNA knockdown, and drug treatments

MGHU4 and 253J bladder cancer cell lines are representative of alterations found in MIBC [16,17]. Cells were cultured in Minimum Essential Medium media (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (MilliporeSigma, Burlington, MA, USA). PLKO.1 TRC shRNAs were transduced using psPAX2 (Addgene plasmid #12260) and pMD2.G (Addgene plasmid #12259; see Supplementary Table 1 for shRNAs) [18]. Transduced cells were selected with 2–5 μg/ml puromycin (MilliporeSigma). Cisplatin, gemcitabine (MilliporeSigma), methotrexate, doxorubicin, vinblastine, and oxaliplatin (ApexBio, Houston, TX, USA) were solubilized in water or dimethyl sulfoxide. For dose response experiments, cell viability was measured using the CellTiter-Glo luminescent assay (Promega, Madison, WI, USA).

2.2. Performing the CRISPR resistance screen

To generate sgRNA lentivirus, 12 μg of the human GeCKO (Genome-Scale CRISPR Knock-Out) lentiviral A library was transfected into 30 million HEK293 T cells for 24 h [19,20]. Viral supernatant was harvested, and MGHU4 cells were transduced at a calculated multiplicity of infection of 0.3 followed by selection with 2 μg/ml puromycin. Two million MGHU4 cells were plated in quadruplicate for each condition. Cells were treated with 3 μM cisplatin or vehicle for 30 h. Treatment media were removed and cells were allowed to grow to confluency prior to harvesting genomic DNA (Machery-Nagel, Bethlehem, PA, USA).

2.3. Sequencing of sgRNA

The sgRNA sequences of each sample were polymerase chain reaction (PCR) amplified from 4 μg of genomic DNA using primers containing adaptor and barcoding sequences. DNA fragments were size selected using agarose gel and sequenced using a 1×125 bp run on the HiSeq2500 (Illumina Inc., San Diego, CA, USA). Reads generated from each sample were aligned to the indexed sgRNA sequences with the Bowtie2 sequence aligner using the "very-sensitive-local" option [21]. The counts of sgRNA were summarized using htseq [22]; sgRNA counts across all samples were compiled, and differential sgRNA abundance was calculated using DESeq2 [23]. To map the sgRNA results to the gene level (approximately three gRNAs per gene), we calculated the mean fold change and combined individual sgRNA p values from DESeq2 using Fisher's method followed by multiple hypothesis testing correction using the Benjamini-Hochberg procedure [24].

2.4. Screen enrichment analysis

The top resistant genes were identified by having a q value of <0.01 and $\log_2$ fold change >2 in the cisplatin-treated group compared to the vehicle control group. The Database for Annotation, Visualization and Integrated Discovery (DAVID), which uses a modified Fisher's exact test followed by Benjamini-Hochberg multiple hypothesis testing correction [24], was used to determine the enriched gene ontology (GO) term biological processes within the top resistant genes [25]. In addition to GO term enrichment, Gene Set Enrichment Analysis (GSEA), which uses the Kolmogorov-Smirnov statistic, was used to determine the enrichment of the KEGG MMR pathway across the entire ranked gene list [26,27].

2.5. Western blots

For each sample, 25 μg of protein was loaded onto 10% SDS-PAGE and transferred to nitrocellulose membranes (BioRad, Hercules, CA, USA) followed by a Western blot (see Supplementary Table 2 for antibodies). Membranes were imaged using the Odyssey Fc Imaging System (LI-COR Biosciences, Lincoln, NE, USA) following application of ECL (MilliporeSigma).

2.6. Caspase activation

Cells were cotreated with the indicated concentration of cisplatin and 4 μM CellEvent Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) according to manufacturer instructions. Phase contrast and green fluorescence were imaged every 3 h following initial treatment using the Incucyte Zoom system (Essen Bioscience, Ann Arbor, USA) with a 4× objective. The displayed results are cumulative

green cell counts normalized to total cell confluency. We compared the induction in cisplatin-treated MSH2 knockdown cells with that in treated nontargeting cells using a repeated-measure one-way analysis of variance (ANOVA; independent variable is knockdown).

2.7. Quantitative reverse transcriptase PCR

Total RNA was extracted using the NucleoSpin RNA kit (Machery-Nagel). Synthesis of cDNA was performed with the iScript cDNA Synthesis Kit (BioRad). The SsoFast Evagreen supermix (BioRad) was used for quantitative reverse transcriptase PCR assays (see Supplementary Table 3 for primer sequences). Target genes were normalized to mean GAPDH expression and a two-way ANOVA was performed, where the two factors were knockdown and treatment.

2.8. Analysis of bladder tumors

All human MIBC data were from The Cancer Genome Atlas (TCGA) [16]. Clinical, treatment, and survival data were downloaded using the TCGAbiolinks R package [28]. One hundred patients were treated with platinum-based (cisplatin or carboplatin) chemotherapy, 12 of whom were in the neoadjuvant setting, but molecular characterization was performed

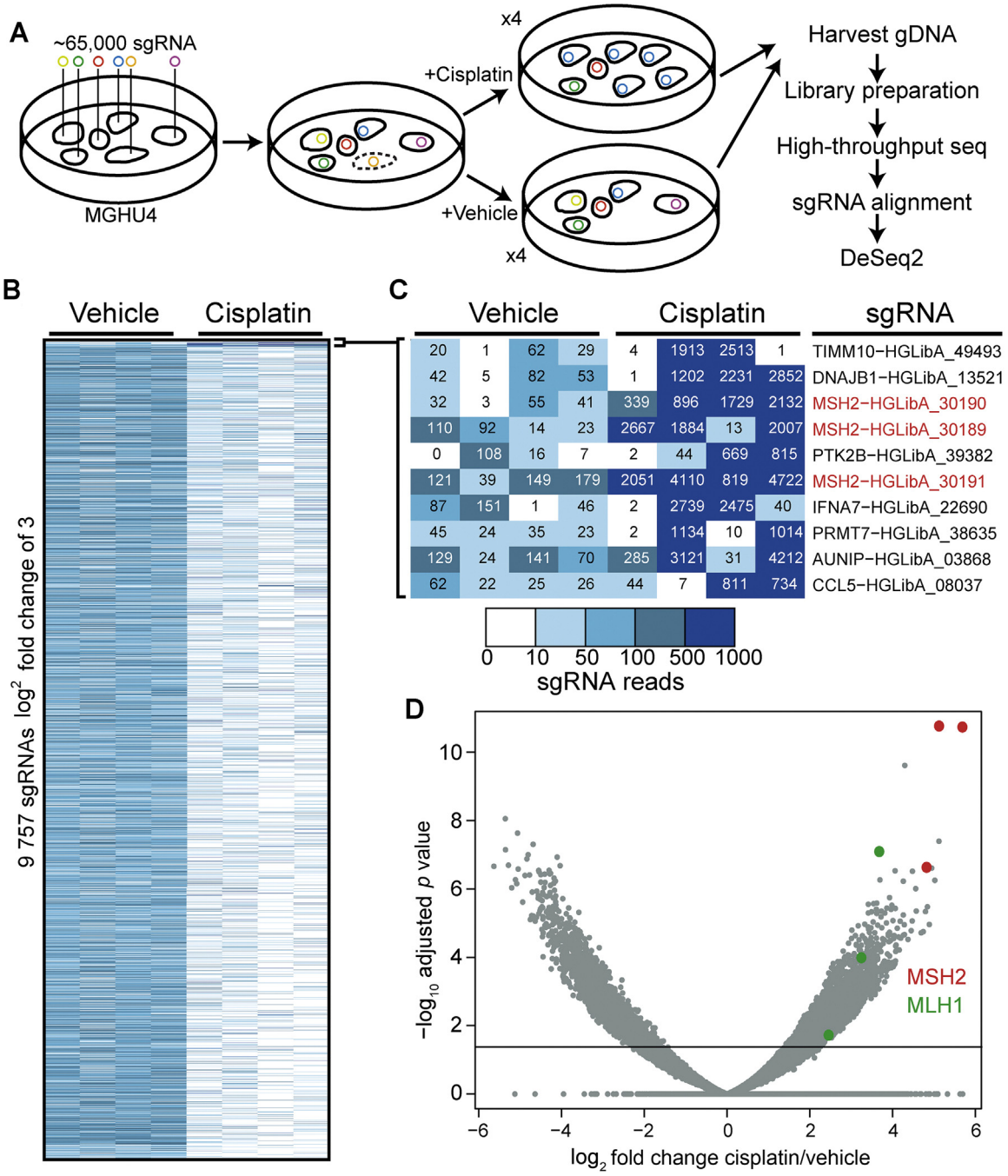

Fig. 1 – A whole-genome CRISPR screen to identify mediators of cisplatin resistance in a bladder cancer cell line. (A) Experimental outline of the screen and analysis. (B) A heatmap displaying median-centered counts for 9757 differentially abundant sgRNAs. (C) Counts from the screen for the top 10 resistant sgRNAs. (D) A volcano plot displaying the $\log_2$ fold change and adjusted *p* value for all sgRNAs identified in the screen. A threshold of adjusted $p < 0.05$ is indicated in the plot.

prior to any chemotherapy treatment [16]. Level 4, batch-normalized reverse-phase protein array (RPPA) was from the study by Li et al. [29] and *MSH2* mRNA (RNA Seq V2 RSEM) expression was from the studies by Gao et al. [30] and Cerami et al. [31]. TCGA bladder cancer patients receiving cystectomies were identified using data from the Broad GDAC Firehose [32]. We used neoantigens published by Robertson et al. [16]. Overall, 340 TCGA patients had RPPA and clinical data (Supplementary Table 4). Overall survival was analyzed using the survival, survminer, and ggplot2 R packages [33–35]. Patient characteristics were compared using a Student *t* test or Fisher's exact test. The primary endpoint is overall survival, which was defined as the time from diagnosis to death from any cause. Patients were censored at the point of their last follow-up (last known time the patient was alive). For all survival analyses, each arm was censored after the at-risk population had <10 patients. Statistical differences between two patient groups were evaluated with the log-rank test.

3. Results

3.1. Whole-genome CRISPR screen identifies mediators of cisplatin resistance

We executed an unbiased CRISPR screen (65 383 sgRNAs; approximately three sgRNAs per gene [19,20]) to identify sgRNA constructs that increased in the population of cisplatin-treated (3 μM for 30 h) compared with vehicle-treated cells (Fig. 1A). We identified 9757 sgRNA constructs with a $\log_2$ fold change of 3 between the two groups (Fig. 1B and 1C, and Supplementary Table 5). The top two gene candidates are *MSH2* and *MLH1* (Fig. 1D and Supplementary Table 6).

3.2. MMR pathway mediates cisplatin sensitivity

We performed pathway-level analysis to identify processes that mediate cisplatin resistance. GO term enrichment analysis [25] on the top 48 significantly resistant genes identified MMR as the top hit (Fig. 2A). To confirm this result, we tested MMR using GSEA across the 16 564 genes in our screen (ranked on mean fold change) and confirmed MMR pathway significance (Fig. 2B) [26]. *MSH2* and *MLH1* each had three significantly resistant sgRNAs, and several other genes in the MMR pathway had a single resistant sgRNA (Fig. 2C) [27].

3.3. Bladder cancer cell lines with knockdown of MSH2 are resistant to cisplatin

We moved forward with MSH2 because it was the top ranked and most statistically significant hit in our screen (Fig. 1D). Additionally, five studies found a subset of bladder cancers with lower expression of MSH2 [11–15]. Strong shRNA knockdowns of MSH2 resulted in 45–142% increases in resistance to cisplatin in MGHU4 and 253J bladder cancer cell lines (Fig. 3A–C). These cells had reduced caspase activation compared with nontargeting control, cisplatin-treated cells (Fig. 3D–G). These data show that the loss of MSH2 in cisplatin-treated bladder cancer cells increases survival and reduces apoptosis.

To investigate the role of the DNA-damage response, we treated MGHU4 and 253J MSH2 knockdown cells with cisplatin for 48 h, followed by measurements of genes involved in DNA-damage response (Supplementary Fig. 1). Cisplatin treatment in cells transduced with nontargeting shRNA led to an accumulation of p-ATM and p53. We observed reductions in the accumulation p-ATM and stabilization of p53 in MSH2 knockdown cells. Cisplatin increased the transcriptional response of BAX, MDM2, p21, and PUMA in nontargeting cells. However, in MSH2

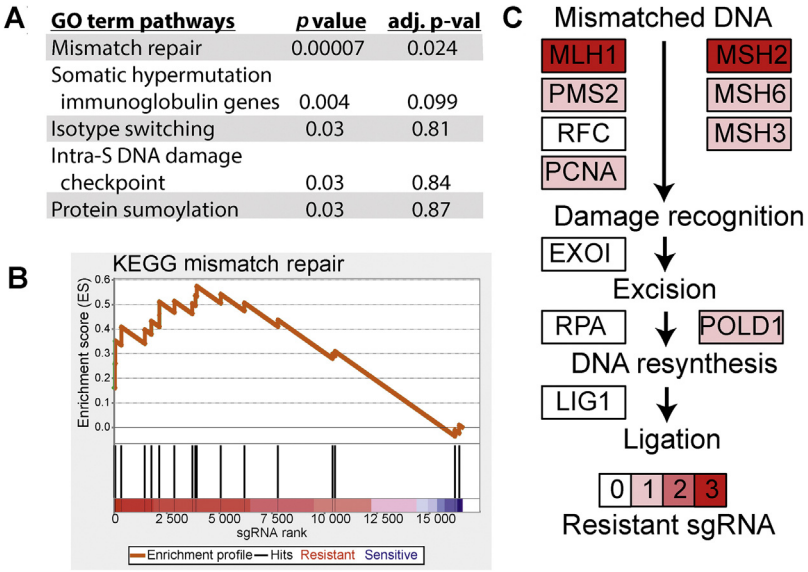

Fig. 2 – sgRNA constructs targeting genes of the mismatch repair pathway are enriched in the cisplatin-treated MGHU4 cells. The mean $\log_2$ fold change and combined *p* value were calculated using the three sgRNAs targeting each gene in the screen. (A) Top GO term biological process results from DAVID enrichment analysis using the top resistant genes in the screen. Enrichment was determined by a modified Fisher's exact test and corrected for multiple hypothesis testing (Benjamini-Hochberg). (B) GSEA results of the KEGG mismatch repair pathway on the ranked list of genes by mean $\log_2$ fold change ($p < 0.05$, Kolmogorov-Smirnov test). (C) Components and genes of the mismatch repair pathway are depicted with the number of significantly resistant sgRNAs from our synthetic lethal screen targeting each gene (out of three). adj. p-val = adjusted *p* value; DAVID = Database for Annotation, Visualization and Integrated Discovery; GO = gene ontology; GSEA = Gene Set Enrichment Analysis.

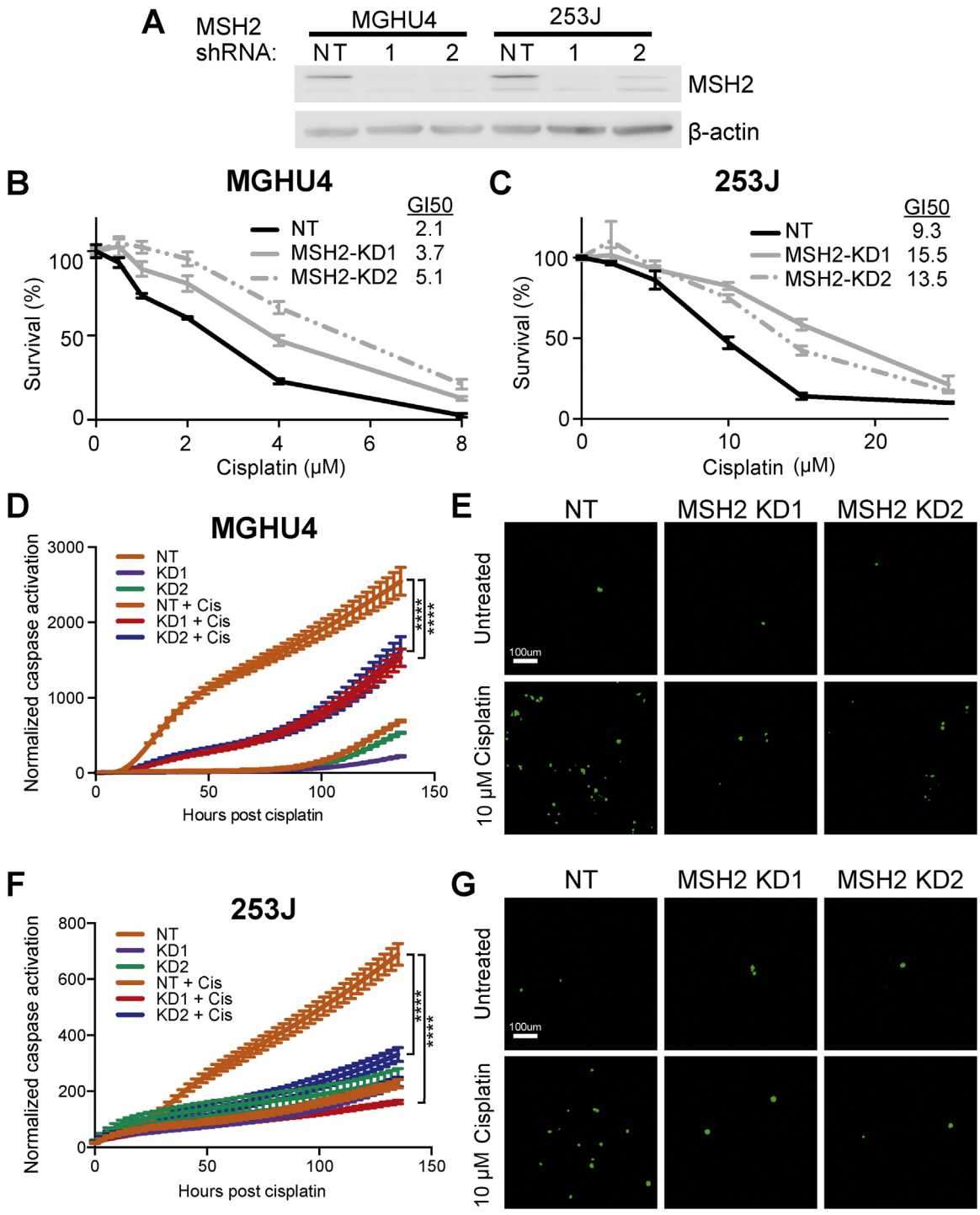

Fig. 3 – Knockdown of MSH2 increases cisplatin resistance in bladder cancer cell lines. Two shRNA constructs targeting MSH2 and one nontargeting shRNA control were transduced into MGHU4 and 253J bladder cancer cell lines. (A) Western blot showing the level of knockdown of MSH2. (B) MGHU4 and (C) 253J cell lines were treated with the indicated doses of cisplatin for 48 h, and cell viability was measured using an ATP-based assay (mean ± SEM of three independent experiments). The GI50 (μM) of cell line was calculated with using a four-parameter nonlinear regression fit using least squares. (D) MGHU4 and (F) 253J cells were treated with 10 and 15 μM cisplatin, respectively, or vehicle. Cumulative caspase activation (green cells) was measured and normalized to cell confluency. Representative images of (E) MGHU4 and (G) 253J cells are shown at the 24 h time point. Statistical significance of Figures 3D and 3F were determined using a repeated-measure one-way ANOVA (independent variable is knockdown). Data represents the mean ± SEM of a representative experiment (of three experiments). ANOVA = analysis of variance; SEM = standard error of the mean. **** $p < 0.0001$.

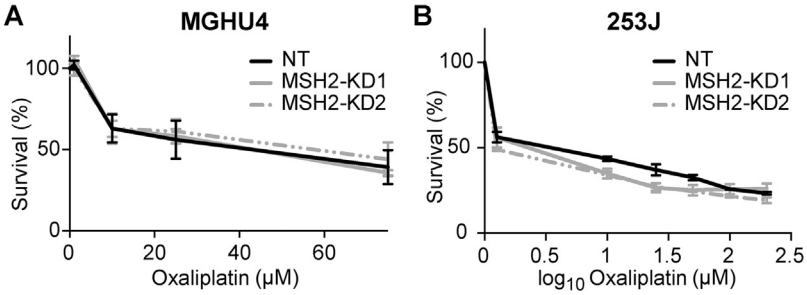

Fig. 4 – MSH2 knockdown bladder cancer cell lines are equally sensitive to oxaliplatin. (A) MGHU4 and (B) 253J bladder cancer cell lines were treated with the indicated doses of oxaliplatin for 48 h, and cell viability was measured using an ATP-based assay.

knockdowns, induction of MDM2 is reduced, and in the 253J cells, induction of BAX and p21 was also attenuated. While we observed a stronger DNA-damage response in MSH2 knockdown cells than was reported in past studies, there was a reduction in some of these factors [3,36,37].

3.4. *Cells with low MSH2 are equally sensitive to oxaliplatin and other chemotherapies*

Previous work has shown that the loss of MMR does not affect sensitivity to the cisplatin analog oxaliplatin [38]. We tested oxaliplatin response, as well as the other constituent components of the MVAC and GC regimens, in bladder cancer cells. Cells with reduced MSH2 were equally sensitive to all of the tested chemotherapies compared with control (Fig. 4 and Supplementary Fig. 2). These results indicate that MSH2 loss mediates resistance to cisplatin specifically, without affecting the other drugs in the standard MVAC and GC treatments or the cisplatin analog oxaliplatin.

3.5. *MSH2 protein levels do not correlate with clinicopathologic features in bladder cancer*

Studies have shown conflicting evidence that bladder cancers with reduced MSH2 protein expression associate with stage, grade, and survival [11–15]. To investigate the clinical impact of MSH2, we analyzed 340 bladder cancer patients from TCGA with both molecular and clinical data [16]. MSH2 and MSH6 are tightly linked as they are components of the MutSα MMR complex; their strong correlation strengthens our confidence in the TCGA RPPA

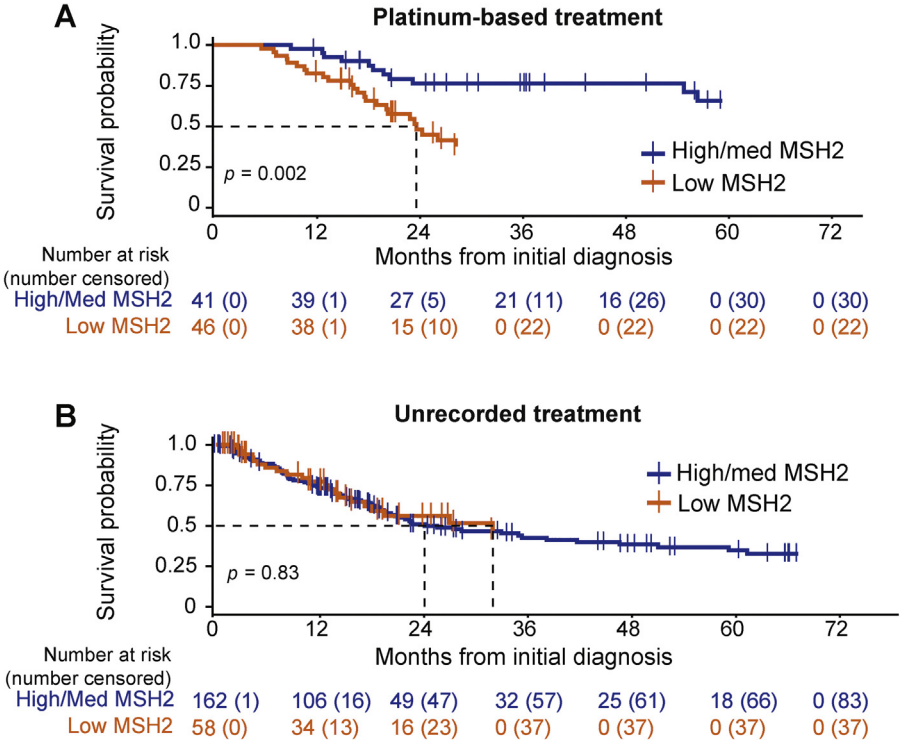

Fig. 5 – Platinum-treated bladder cancer patients with low levels of MSH2 protein correlate with poorer survival. Survival of patients was compared with MSH2 RPPA level in 340 bladder cancer patients. Overall survival is plotted for bladder cancer patients with a (A) platinum-based treatment or (B) nonpharmacologic or radiation treatment. Survival of patients with low MSH2 (red) is compared with those with medium or high levels of MSH2 (purple). The statistical difference in survival was calculated using a log-rank test. med = medium; RPPA = reverse-phase protein array.

data (Supplementary Fig. 3A, $\rho = 0.66$). We found a weak correlation between *MSH2* mRNA and protein levels, indicating that mRNA levels are a poor surrogate for MSH2 protein levels in bladder cancer (Supplementary Fig. 3B, $\rho = 0.29$). Surprisingly, low MSH2 protein levels did not have an impact on the number of neoantigens, stage, or grade, suggesting that MSH2 is independent of these features (Supplementary Fig. 3C–E) [16].

3.6. *MSH2 protein levels correlate with survival in platinum-treated bladder cancer patients*

We investigated whether MSH2 protein levels correlate with survival in platinum-treated (cisplatin or carboplatin) patients. We divided TCGA bladder cancer patients into three equal groups based on their MSH2 protein expression (Supplementary Fig. 4A), and following the work of prior biomarker studies [39,40], we compared the low MSH2 group with the combined medium/high groups. We found that for patients treated with platinum-based therapy, individuals with low MSH2 had significantly poorer survival compared with patients with medium or high protein levels (Supplementary Fig. 4B and Fig. 5A, $p = 0.002$, log-rank test). We next investigated low MSH2 protein levels in patients without a recorded pharmacologic or radiation treatment, and found that in these patients, MSH2 protein level did not have any association with survival (Supplementary Fig. 4C and Fig. 5B, $p = 0.83$, log-rank test). For these patients, we found no discernable differences in clinical characteristics (Supplementary Tables 7 and 8). These results suggest that low MSH2 protein identifies patients less responsive to platinum therapy and is not simply prognostic of overall survival.

4. Discussion

No clinically actionable biomarker of resistance to first-line chemotherapy in MIBC is currently available. The MMR pathway has been shown to impact the response to cisplatin-based chemotherapy in multiple cancers [3–6,8–10]; however, clinical responses vary across cancer type. For platinum-treated patients, low MSH2 correlates with poor survival in ovarian cancer [8] but improved survival in NSCLC [9,10]. Here, we report the first association between low MSH2 protein and poorer survival in bladder cancer patients treated with platinum-based therapy, and this biomarker is not simply prognostic of patient survival.

Consistent with in vitro studies in other cancer types [3,36,37], we observed a reduction in the DNA-damage response when cells had low levels of MSH2. It is unclear whether this reduction alone accounts for the cisplatin resistance that we observed. Future studies are needed to address additional mechanisms of response and also to determine whether the DNA-damage response can serve as a therapeutic target to address MMR-mediated cisplatin resistance.

Microsatellite instability has been linked to both MMR and chemotherapy response in other cancer types; yet, it is infrequent in bladder tumors [12,13,41] and thus cannot be used as an effective bladder cancer biomarker. It has previously been shown that a subset of bladder tumors have low to no expression of MSH2 [11–15]. While patients expressing low levels of MSH2 protein had an impaired response to platinum-based chemotherapy, they do not have microsatellite instability or an increase in neoantigens (Supplementary Fig. 3C). Future work will investigate the potential separation of MMR functions in regard to DNA repair activity versus the response to cisplatin.

The disease control rate of oxaliplatin-based therapy in patients who have failed cisplatin- or carboplatin-based chemotherapy is 36% [42]. We found that oxaliplatin and the noncisplatin components of the MVAC and GC chemotherapy regimens were equally effective in bladder cancer cell lines irrespective of MSH2 expression. Therefore, an unanswered question is whether bladder tumors with low MSH2 levels would respond well to MVAC and GC regimens with oxaliplatin being substituted for cisplatin.

Current protein diagnostics rely on IHC [43]. For this reason, it will be necessary to validate our findings using IHC on patient samples. Our results are based on retrospective patient data that were not collected as a standardized clinical trial [16]. The collected samples covered a broad range of patient characteristics in relation to clinicopathologic features. Similarly, some of these patients were treated with MVAC, while others received GC; 12% of platinum-treated patients were provided neoadjuvant treatment. The lack of standardized patient selection presents confounding factors that are better controlled within a clinical trial. However, using the patient and tumor characteristics available to us, we did not find notable differences by MSH2 expression that would influence our results (Supplementary Tables 7 and 8).

5. Conclusions

In this study, we found that MSH2 contributes to in vitro resistance to cisplatin in bladder cancer cell lines. We showed that patients with low levels of MSH2 had poorer survival when treated with platinum-based chemotherapy. Future studies will be necessary to validate MSH2 protein levels as a prospective biomarker of platinum-based therapeutic resistance in bladder cancer.

Author contributions: James C. Costello had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Goodspeed, Costello.
Acquisition of data: Goodspeed, Jean.
Analysis and interpretation of data: Goodspeed, Costello.
Drafting of the manuscript: Goodspeed, Costello.
Critical revision of the manuscript for important intellectual content: Goodspeed, Costello.
Statistical analysis: Goodspeed, Costello.
Obtaining funding: Goodspeed, Costello.
Administrative, technical, or material support: Costello.
Supervision: Costello.
Other: None.

Financial disclosures: James C. Costello certifies that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: None.

***Funding/Support and role of the sponsor*:** This work was supported by the Boettcher Foundation (J.C.C.), T32GM007635 (A.G.), the Front Range Cancer Challenge (A.G.), and in part by the Genomics, Functional Genomics, and the Biostatistics & Bioinformatics Shared Resource of the University of Colorado Cancer Center (P30CA046934).

Acknowledgments: We would like to thank Robert T. Jones, Kelly D. Sullivan, and Rani K. Powers for insightful discussions. We would also like to thank the Functional Genomics Facility for their genomic library expertise.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.eururo.2018.10.040.

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