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Identification of a synthetic lethal relationship between nucleotide excision repair (NER) deficiency and irifolven sensitivity in urothelial cancer

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Author contributions

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Conflict of interest

J. Börcsök, M. Diossy, Z. Sztupinszki, K.W. Mouw and Z. Szallasi are listed as co-inventors on a provisional patent owned by Children's Hospital Boston to quantify the *ERCC2* mutational signature in cancer biopsies. K.W. Mouw - Consulting or Advisory Role: EMD Serono, Pfizer. Research Funding: Pfizer. J.E. Rosenberg - Honoraria: UpToDate, Bristol-Myers Squibb, AstraZeneca, Medscape, Vindico, Peerview, Chugai Pharma, Research To Practice, Intellisphere, Clinical Care Options, Clinical Mind. Consulting or Advisory Role: Lilly, Merck, Agensys, Genentech, Sanofi, AstraZeneca/MedImmune, Bristol-Myers Squibb, EMD Serono, Seattle Genetics, Bayer, Inovio Pharmaceuticals, BioClin Therapeutics, QED Therapeutics, Adicet Bio, Sensei Biotherapeutics, Fortress Biotech, Pharmacyclics, Western Oncolytics, GlaxoSmithKline, Janssen Oncology, Astellas Pharma. Research Funding: Oncogenex (Inst), Agensys (Inst), Mirati Therapeutics (Inst), Novartis (Inst), Viralytics (Inst), Genentech (Inst), Incyte (Inst), Seattle Genetics (Inst), Bayer (Inst), AstraZeneca (Inst), QED Therapeutics (Inst), Astellas Pharma (Inst), Jounce Therapeutics (Inst). Patents, Royalties, Other Intellectual Property: Predictor of platinum sensitivity (Inst). Travel, Accommodations, Expenses: Genentech, Bristol-Myers Squibb. E.M. Van Allen - Advisory/Consulting: Tango Therapeutics, Genome Medical, Invitae, Enara Bio, Manifold Bio, Monte Rosa, Janssen. Research support: Novartis, BMS. Equity: Tango Therapeutics, Genome Medical, Syapse, Enara Bio, Manifold Bio, Monte Rosa, Microsoft. Travel reimbursement: Roche/Genentech. Patents: Institutional patents filed on chromatin mutations and immunotherapy response, and methods for clinical interpretation. E.R. Plimack - Clinical Research Support/Data Safety Monitoring Board: Astellas Pharma Inc., AstraZeneca Pharmaceuticals LP, Bristol-Myers Squibb Company, Genentech Inc., Infinity Pharmaceuticals Inc., Merck & Co. Inc., Pfizer Inc. Scientific Advisory Boards, Consultant, or Expert Witness: Genentech Inc., Merck & Co. Inc., Seattle Genetics Inc. D.B. Solit has consulted with/received honoraria from Pfizer, Loxo Oncology, Lilly Oncology, Illumina, Vivideon Oncology and Q.E.D. Therapeutics. J.H. Hoffman-Censits - Medical writing services: Roche Genentech. Consultant: AstraZeneca. Research support: Foundation Medicine.

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Abstract

Purpose: Cisplatin-based chemotherapy is a first-line treatment for muscle-invasive and metastatic urothelial cancer. Approximately 10% of bladder urothelial tumors have a somatic missense mutation in the nucleotide excision repair (NER) gene, *ERCC2*, which confers increased sensitivity to cisplatin-based chemotherapy. However, a significant subset of patients is ineligible to receive cisplatin-based therapy due to medical contraindications, and no NER-targeted approaches are available for platinum-ineligible or platinum-refractory *ERCC2*-mutant cases.

Experimental Design: We used a series of NER-proficient and NER-deficient preclinical tumor models to test sensitivity to irifolven, an abandoned anticancer agent. In addition, we used available clinical and sequencing data from multiple urothelial tumor cohorts to develop and validate a composite mutational signature of *ERCC2* deficiency and cisplatin sensitivity.

Results: We identified a novel synthetic lethal relationship between tumor NER deficiency and sensitivity to irifolven. Irifolven specifically targets cells with inactivation of the transcription-coupled NER (TC-NER) pathway and leads to robust responses *in vitro* and *in vivo*, including in models with acquired cisplatin resistance, while having minimal effect on cells with intact NER. We also found that a composite mutational signature of *ERCC2* deficiency was strongly associated

with cisplatin response in patients and was also associated with cisplatin and irifolven sensitivity in preclinical models.

Conclusions: Tumor NER deficiency confers sensitivity to irifolven, a previously abandoned anticancer agent, with minimal activity in NER-proficient cells. A composite mutational signature of NER deficiency may be useful in identifying patients likely to respond to NER-targeting agents, including cisplatin and irifolven.

Keywords

DNA repair; nucleotide excision repair; cancer genomics; biomarker discovery; synthetic lethality; irifolven; urothelial cancer

Introduction

Therapeutic approaches based on the principle of synthetic lethality are an attractive strategy for cancer treatment. Because DNA repair pathway aberrations are common in tumor cells, but are largely absent in normal cells, agents that target DNA repair-deficient cells may have a clinically exploitable therapeutic window. A successful example stemming from this principle is the development of PARP inhibitors for treatment of tumors with homologous recombination (HR) repair deficiency (1,2).

Nucleotide excision repair (NER) is a highly conserved DNA repair pathway that recognizes and repairs bulky intrastrand DNA adducts formed by genotoxic agents, such as UV radiation and platinum chemotherapies (3). NER is initiated through two separate branches of lesion recognition: transcription-coupled repair (TC-NER) is activated by RNA polymerase stalling at lesions in transcribed regions, while global genome repair (GG-NER) is able to recognize lesions throughout the genome. Following lesion recognition, TC-NER and GG-NER converge on a common NER pathway that excises and replaces the damaged DNA strand in an error-free manner.

Recently, sequencing and functional studies have revealed that NER pathway deficiency is present in a subset of tumors. Somatic missense mutations in *ERCC2*, a key NER gene that encodes the DNA helicase XPD, are present in approximately 10% of muscle-invasive bladder tumors (4). Although not strongly prognostic in patients treated without chemotherapy, *ERCC2* mutations confer increased sensitivity to platinum-based chemotherapy (5–7), and mutations in *ERCC2* and several other DNA repair genes (such as *ATM*, *FANCC*, and *BRCA1/2*) are being used as predictive biomarkers in several on-going clinical trials (8–10). Mutations in NER genes beyond *ERCC2* also occur sporadically in bladder cancer and other tumor types, and it is possible that these events may also confer a therapeutically exploitable NER deficiency.

Cisplatin-based chemotherapy is a first-line treatment for muscle-invasive and metastatic urothelial cancer. Although 60–70% of patients have an initial response, resistance develops in the majority of patients (11). In addition, nearly half of all patients with urothelial cancer are ineligible for cisplatin-based chemotherapy due to medical comorbidities (12). Anti-PD-1/PD-L1 agents are approved for cisplatin-ineligible or cisplatin-resistant patients;

however, only approximately 20%–30% of patients respond (13). Therefore, novel agents with activity in post-cisplatin and cisplatin-ineligible patient populations are needed.

Irofulven is a semisynthetic DNA alkylating agent that is a derivative of the fungal product, illudin S (14). Irofulven-mediated DNA damage is not recognized by mismatch repair (MMR) or GG-NER, but instead activates TC-NER when the damage is encountered by RNA polymerase. Therefore, irofulven has been shown to have approximately 100-fold increased cytotoxic activity in nontumor cell lines with a TC-NER or common NER pathway gene defect compared with the isogenic NER-proficient line (15,16). Although well-tolerated, irofulven showed only modest clinical benefit as a single agent in phase I/II clinical trials across a variety of tumor types (17–20). This apparent lack of efficacy may have occurred because NER deficiency is present in only a fraction of tumors, and these trials were conducted in biomarker-unselected populations.

Despite the potential clinical actionability of tumor NER deficiency, there are currently no functional or IHC assays available to reliably identify NER deficiency from clinical specimens. An alternative approach to identify tumor NER deficiency is through the use of next-generation sequencing (NGS)-based mutational signatures. Because DNA repair deficiency is frequently associated with specific mutational patterns (signatures), the presence of a mutational signature can indicate a specific DNA repair deficiency, even in the absence of an obvious DNA repair gene alteration (21,22). Such a strategy has been successfully employed to identify HR-deficient cancer cases for the prioritization of PARP inhibitor and platinum-based therapy in breast and ovarian cancer (23–25). For NER, a specific single-nucleotide variation (SNV)-based mutation signature (signature 5*) has been associated with *ERCC2* mutations in bladder tumors (22), but the signature was not associated with platinum response in tumors that lacked an *ERCC2* mutation. To improve predictive utility, SNV signatures can be combined with other non-SNV-based mutational features, such as short insertions/deletions (indels) or chromosomal rearrangements. These “composite” signatures may represent a more accurate measure of DNA repair deficiency, as has been demonstrated for HR deficiency (26).

Here, we identify a novel synthetic lethal relationship between NER deficiency and irofulven sensitivity. We show that inactivating mutations in genes of the TC-NER or common NER pathway are sufficient to drive irofulven sensitivity *in vitro* and *in vivo*, and we demonstrate that acquired cisplatin resistance does not induce cross-resistance to irofulven. We also define a composite mutational signature of *ERCC2* deficiency in bladder cancer that is strongly associated with cisplatin sensitivity, including in cases that lack an *ERCC2* mutation, and may, therefore, be a useful tool to predict sensitivity to NER-targeting agents, such as cisplatin and irofulven.

Materials and Methods

Patients and cohorts

In this study, 632 whole-genome (WGS) and whole-exome sequenced (WES) pretreatment samples were analyzed from four urothelial bladder tumor cohorts (Supp. Table 1).

The Cancer Genome Atlas (TCGA) cohort—The WGS normal and tumor bam files were downloaded from the International Cancer Genome Consortium data portal (<https://dcc.icgc.org/>). The WES normal and tumor bam files, as well as the vcf files generated by MuTect2, were downloaded from The Cancer Genome Atlas data portal (<https://portal.gdc.cancer.gov/>).

Dana-Farber Cancer Institute (DFCI)/Memorial Sloan Kettering Cancer Center (MSKCC) and Philadelphia cohorts—The normal and tumor bam files were downloaded from The Database of Genotypes and Phenotypes upon request (<https://www.ncbi.nlm.nih.gov/gap/>) using the phs000771 accession code.

Beijing Genomics Institute (BGI) cohort—The normal and tumor fastq files were downloaded from the Sequence Read Archive database (<https://www.ncbi.nlm.nih.gov/sra>) using the [SRA063495](https://www.ncbi.nlm.nih.gov/sra) accession code. The details of the alignment process are described in the Supplementary Methods.

The average coverage of the analyzed WGS and WES samples in each cohort is shown in Supp. Fig. 1–2.

Mutation, copy number, and structural variant calling

Germline variants were called with HaplotypeCaller in key NER-related genes, while somatic point mutations and indels were called with MuTect2 (GATK, v3.8). Germline and somatic mutations in key NER-related genes in each cohort are presented in Supp. Fig. 3–7. The high fidelity of the reported variants was ensured by the application of additional hard filters in addition to the tools' default filters (Supp. Table 2–3). Allele-specific copy-number profiles were estimated using Sequenza (27) (Supp. Fig. 8–9). Germline and somatic mutations were annotated using InterVar (28) and the genotypes of the samples were determined (Supp. Fig. 10–11). Structural variants were detected by BRASS (v6.0.0. <http://github.com/cancerit/BRASS>). Further details are available in the Supplementary Methods.

Mutational signatures

Somatic single-base substitution signatures were determined with the help of the deconstructSigs R package (29), using the Catalogue of Somatic Mutations in Cancer (COSMIC) signatures as a mutational process matrix (cancer.sanger.ac.uk/cosmic/signatures_v2). Doublet base substitutions and indels in each sample were classified into a 78-dimensional doublet base substitution and an 83-dimensional indel catalog, respectively, with the help of the ICAMS R package (30), and the previously described matrices of doublet base substitution and indel signatures were used (31) in a nonnegative least-squares problem to estimate the matrix of exposures to mutational processes. The extraction of rearrangement signatures was executed as described previously (32). The extracted single-base substitution signatures, doublet base substitution signatures, indel signatures and rearrangement signatures are shown in Supp. Fig. 12–19, and the cosine similarity between the single-base substitution signature profiles of each sample is presented in Supp. Fig. 20–21. Further details are provided in the Supplementary Methods.

Transcriptional strand bias

Transcriptional strand bias analysis was carried out using the MutationalPatterns R package (33), which identifies strand asymmetry in the reference frame of DNA transcription (34).

Cell lines and reagents

Bladder cancer cell lines were purchased from ATCC. The KE1 bladder cancer cell line is a derivative of KU19–19, in which an *ERCC2*T484 mutation has been introduced by CRISPR/Cas9 gene editing as described previously (6). The MDA-MB-468 breast cancer cell line and its *ERCC4*-complemented derivative have been described previously (35). siRNAs targeting specific NER genes, as well as a nontargeting siRNA, were purchased from Integrated DNA Technologies Inc. (see Supp. Table 5 for sequences). Cells were seeded in 6-well plates and grown to 50% confluency. A transfection mixture containing siRNA diluted to a final concentration of 30 nmol/L in Opti-MEM containing Lipofectamine 3000 (Life Technologies) was then added to cells. After 48 hours, cells were trypsinized and realiquoted to separate wells and transfected with 15 nmol/L siRNA for 24 hours immediately, prior to performing viability assays, as described below. To create the SW1710 *ERCC2*P463L-mutant cell line, a Cas9-encoding lentiviral vector, as well as a single-guide RNA (sgRNA) targeting the P463 codon were electroporated into SW1710 along with a donor template plasmid encoding the desired P463L codon change, as well as a GFP reporter cassette. After 48 hours, GFP-positive cells were isolated by cell sorting and expanded. The presence of the P463L mutation was validated by Sanger sequencing followed by RT-PCR and targeted NGS using the MSK-IMPACT assay. The sequences of sgRNA and PCR primers are listed in Supp. Table 6. Early-passage cells (6 passages) were used for all experiments and cell lines were tested monthly to confirm absence of *Mycoplasma*. For additional details, see Supplementary Methods.

Cell line mutational signatures

KU19–19 and KE1 cell populations were single-cell sorted, and individual cells were expanded to create multiple populations of cells. Each population of KU19–19 or KE1 cells was propagated separately in culture for 30 days while maintaining the population above 1×10^6 cells at all times. After 30 days, each population was single-cell sorted and individual clones were grown to approximately 1×10^6 cells. Cells were frozen, lysed, and genomic DNA was isolated. WGS was performed at the Broad Institute to an average depth of 30x. Somatic mutations were called using IsoMut (36), single-base substitution signatures, doublet base substitution signatures, and indel signatures were extracted as described in the Supplementary Methods.

In vitro drug sensitivity assays

Cells were seeded in either 96-well (5,000 cells/well) or 24-well (20,000 cells/well) plates. The following day, irifolven and cisplatin stock solutions were serially diluted in media and added to cells. After 48–72 hours, media were removed and CellTiter-Glo Reagent (Promega) was added. Plates were scanned using a Luminescence Microplate Reader (BioTek). Survival at each drug concentration was plotted as a percentage of survival in drug-free media with error bars representing the SD of at least three experiments.

For crystal violet imaging experiments, cells were seeded in 6-well plates (100,000–200,000 cells/well) with 15 nmol/L siRNA transfection mixture (as detailed above). At 24 hours, fresh media containing irifolven or PBS were added and the cells were incubated for an additional 72 hours. Cells were then fixed in formalin solution for 30 minutes and stained with crystal violet, prepared in equal volumes of methanol and water. Excess crystal violet was removed by washing with PBS, and plates were then dried and imaged.

Immunoblotting

Cells were lysed with ice-cold RIPA buffer supplemented with Protease and Phosphatase Inhibitors (Roche). Samples were then sonicated and protein concentrations were determined using the Bradford assay. Sample Buffer (Bio-Rad) was added and samples were then denatured at 90°C for 10 minutes. Samples were then loaded in NuPAGE Protein Gels (Thermo Fisher Scientific) and run at 90 V for 2–3 hours. The gels were then transferred to nitrocellulose membranes at 30 V overnight. Membranes were blocked for 30 minutes in 5% milk in TBS buffer. Sections of the membrane corresponding to the appropriate molecular weights were stained overnight in primary antibodies, prepared in 1% milk in TBST: XPF (1:700, clone D3G8C rabbit mAB, Cell Signaling Technology); cleaved caspase 3 (1:1000, clone D175, rabbit mAB, Cell Signaling Technology); total and cleaved PARP (1:1000, rabbit mAB, Cell Signaling Technology); phospho-H2AX Ser139 (1:1000, clone JBW301 mouse mAB, Millipore); β -tubulin (1:1000, mouse mAB, Santa Cruz Biotechnology); Rpb1 CTD (1:1000, clone 4H8 Mouse mAb, Cell Signaling Technology). A Li-COR Odyssey Infrared Imaging System was used for signal detection using IRDYE-conjugated secondary antibodies (LI-COR Biosciences).

Xenograft studies

Six-week-old female athymic nude mice, NU/J (stock no., 002019) were purchased from The Jackson Laboratory and housed at the Dana-Farber Cancer Institute Animal Resources Facility (Boston, MA). All animal experiments were performed in accordance with an Institutional Animal Care and Use Committee-approved protocol. At 7–10 weeks of age, mice were anesthetized with isoflurane and subcutaneously injected on the left flank with 3 million KE1 or 1 million KU19–19 cells mixed 1:1 with Matrigel (BD Biosciences) in PBS. Tumor size was measured with a digital caliper twice weekly and calculated using the formula: $(L \times W^2) \times 1/2$. Drug treatments were administered when the average tumor volume reached a minimum of 100 mm³. Irifolven was prepared in PBS to a stock concentration of 200 μ g/mL and was delivered intraperitoneally twice weekly at doses of 250 μ g/kg, 500 μ g/kg or 1 mg/kg for a total of 5 injections or until the tumor reached a prespecified protocol endpoint. Control mice were injected with PBS alone. At the end of the experiment, mice were sacrificed and tumors were excised for tumor weight measurements and imaging.

ERCC2mut composite mutational signature

We set out to build a statistical model for detecting mutational features associated with *ERCC2* mutation status. The classification model was trained on the TCGA BLCA WES data. The information gained from the extraction of signatures of single-base substitutions, signatures of doublet base substitutions, signatures of indels, and transcriptional strand bias

was used to build a logistic regression model. The training set consisted of 28 *ERCC2* somatic mutants and 367 wild-type (WT) samples (Supp. Table 7). The data were log-transformed and standardized. The detailed description of the model-building process is available in the Supplementary Methods.

Survival analysis

Survival analysis is described in the Supplementary Methods.

Data availability

The cell line WGS bam files were deposited at the European Nucleotide Archive under the accession number PRJEB36417.

Code availability

There are no restrictions to access the custom code used for the analyses presented in this study. Information is available from the authors on request.

Results

Irofulven preferentially targets NER-deficient tumors

Given that tumor NER deficiency has been shown to drive sensitivity to platinum-based agents in several tumor contexts (6,37), we sought to determine if NER deficiency was also sufficient to confer sensitivity to other agents that create DNA damage typically repaired by the NER pathway. Irofulven is a DNA alkylating agent that creates lesions which do not distort the DNA helix and are thus not recognized by most DNA repair pathways, including the GG-NER pathway (Fig. 1A) (15). However, when present in transcribed regions of the genome, irofulven lesions block RNA polymerase and activate TC-NER (15).

Given the activity of irofulven in TC-NER-deficient nontumor cells, we hypothesized that irofulven would also be toxic to tumor cells with loss of function of a TC-NER or common NER gene. KU19–19 is a bladder cancer cell line with WT *ERCC2* and no other known NER pathway defects. We used CRISPR/Cas9 gene editing to introduce an inactivating mutation at the endogenous *ERCC2* locus, and we have previously shown that the engineered *ERCC2*-mutated cell line (KE1) is NER deficient and has increased sensitivity to cisplatin *in vitro* and *in vivo* (6). To determine whether NER deficiency is also sufficient to drive sensitivity to irofulven, we compared irofulven sensitivity of KU19–19 and KE1 and found that the *ERCC2*-mutated, NER-deficient KE1 line exhibited significantly increased sensitivity to irofulven compared with the parental NER-proficient KU19–19 line (Fig. 1B, top).

To further investigate the relationship between *ERCC2* mutations and irofulven sensitivity, we used similar CRISPR/Cas9 techniques to introduce a clinically observed *ERCC2* mutation (P463L) in SW1710, another bladder cancer cell with no baseline NER dysfunction. The presence of the *ERCC2* P463L mutation in the derivative cell line (SC14) was confirmed by NGS, and immunoblotting demonstrated the presence of a full-length *ERCC2* gene product in the SC14 line (Supp. Fig. 22). The *ERCC2*-mutant SC14 line failed

to resolve UV-induced DDB2 foci in an immunofluorescence NER reporter assay (6,38) (Supp. Fig. 23A), consistent with loss of NER capacity. As was observed for the NER-deficient KE1 bladder cancer line, the SC14 line also displayed significantly increased sensitivity to irifolven (Fig. 1B, middle) as well as cisplatin (Supp. Fig. 23B). Together, these data demonstrate that introduction of distinct, clinically relevant *ERCC2* mutations in bladder cancer cell lines is sufficient to confer NER deficiency and drive marked sensitivity to irifolven.

Finally, we wished to determine whether NER deficiency conferred by alterations in genes other than *ERCC2* is also sufficient to drive irifolven sensitivity. We recently showed that the breast cancer cell line, MDA-MB-468, is NER deficient due to epigenetic silencing of *ERCC4* (XPF), and that NER activity and cisplatin sensitivity of the cell line could be rescued by reexpression of WT *ERCC4* (35). We tested irifolven sensitivity of the NER-deficient parental MDA-MB-468 cell line, as well as its WT *ERCC4*-complemented counterpart, and again observed dramatically higher irifolven sensitivity in the NER-deficient compared with NER-proficient cell line (Fig. 1B, bottom), suggesting that NER loss mediated by dysfunction of an NER gene beyond *ERCC2* can also drive irifolven sensitivity.

NER deficiency is sufficient to drive irifolven sensitivity *in vivo*

We next wished to test whether NER deficiency was sufficient to drive bladder tumor sensitivity to irifolven *in vivo*. We established NER-deficient KE1 xenografts and treated mice with increasing doses of irifolven twice weekly for 2.5 weeks (total of five doses). Irifolven was well-tolerated at all doses, and we observed a strong irifolven dose-response with near complete tumor regression observed at an irifolven dose of 1 mg/kg (Fig. 1C; Supp. Fig. 24). Post-treatment tumor weights were significantly lower in mice receiving 0.5 or 1 mg/kg irifolven compared with untreated mice (Fig. 1D). Conversely, as was observed *in vitro*, there was no response of the NER-proficient KU19–19 xenografts to irifolven (Supp. Fig. 25), confirming that irifolven preferentially targets NER-deficient tumor cells.

Irifolven specifically targets cancer cells with TC-NER or common NER pathway defects

To further investigate the specificity of the relationship between NER deficiency and irifolven sensitivity, we used siRNA to deplete genes in the TC-NER, GG-NER, or common NER pathways in the NER-proficient bladder cancer cell line KU19–19. Whereas depletion of the TC-NER gene, *ERCC6*, or the common NER gene, *ERCC3*, was sufficient to increase irifolven sensitivity, depletion of the GG-NER gene, *DDB2*, had minimal impact on irifolven sensitivity (Fig. 2A). These results are consistent with irifolven creating DNA lesions that are only recognized by the TC-NER pathway.

The TC-NER pathway is normally activated by RNA polymerase stalling at DNA lesions. However, in cells with loss or dysfunction of a TC-NER or common NER gene, stalled RNA polymerase can undergo ubiquitination and proteasomal degradation to prevent toxic accumulation of stalled polymerase complexes (39). Following irifolven treatment, we observed a time-dependent loss of RNA polymerase in NER-deficient KE1 cells, but not in NER-proficient KU19–19 cells, consistent with an inability of KE1 cells to repair irifolven-

mediated DNA damage via TC-NER (Fig. 2B, top). We also observed accumulation of the DNA damage marker, phospho-H2AX (γ H2AX), as well cleaved PARP and cleaved caspase-3, in irofulven-treated NER-deficient cells, consistent with apoptotic cell death (Fig. 2B, bottom).

Cisplatin resistance does not confer cross-resistance to irofulven

Platinum-based chemotherapy is a first-line treatment for numerous solid malignancies, including bladder cancer, but platinum resistance frequently arises and represents a challenging clinical problem. Although a variety of platinum resistance mechanisms have been characterized, restoration or upregulation of DNA repair is a common mechanism of resistance, particularly in HR-deficient breast and ovarian tumors (40). Mechanisms of platinum resistance in NER-deficient tumors are poorly understood, and we wished to determine whether acquired resistance to cisplatin would confer cross-resistance to irofulven through restoration of NER. To test this, we derived cisplatin resistance in the NER-deficient MDA-MB-468 cell line by gradual exposure to escalating doses of cisplatin. Although the cisplatin IC₅₀ value was approximately 3.5-fold higher in the cisplatin resistant cell line, the cell line remained highly sensitive to irofulven (Fig. 2C). Immunoblotting showed persistent lack of *ERCC4* expression in the cisplatin-resistant cell line, suggesting that cisplatin resistance in this model is driven by a mechanism other than restoration of NER (Fig. 2D). Taken together, these data suggest that irofulven may remain active in NER-deficient tumors that have become resistant to platinum-based therapy.

A composite mutational signature is strongly associated with *ERCC2* mutations in multiple bladder cancer cohorts

Given the robust association between NER gene defects and irofulven sensitivity in our preclinical models, we wished to determine whether we could identify mutational features of bladder tumors that are associated with NER deficiency and sensitivity to NER-targeting agents, such as cisplatin and irofulven. Characteristic SNV-based mutational signatures are often present in tumors harboring specific DNA repair defects, such as HR- or MMR deficiency (21), and we previously identified an SNV-based mutational signature ("signature 5*") that is enriched in *ERCC2*-mutated bladder tumors (22). However, DNA repair pathway alterations are also known to induce other types of genomic alterations, such as doublet base substitutions, short indels, and chromosomal rearrangements.

To further characterize the mutational features associated with *ERCC2* mutations in treatment-naïve bladder tumors, we analyzed TCGA bladder cancer WGS and WES datasets (41) to identify multiple types of mutational features catalogued in cancer (31) (Supp. Fig. 12–19). TCGA database contains only 23 WGS cases; therefore, we used mutational features extracted from 412 WES samples to train a logistic regression-based classifier of *ERCC2* mutation status (Supplementary Methods). In total, nine features contributed to the logistic regression-based *ERCC2*mut classifier: signature 2, signature 5, DBS4, ID2, ID8, ID10, TSB_ratio_CtoG, TSB_ratio_TtoA, and TSB_ratio_TtoG. (Fig. 3A; Supp. Table 8 and Supp. Fig. 26). Signature 2 and signature 5 were described previously to be associated with *ERCC2* mutation status (22). DBS4 is dominated by GC>AA or TC>AA dinucleotide substitutions. ID2 is predominantly composed of deletions of T at long (> 5 bp)

mononucleotide repeats and is found in many cancer types. ID8 is characterized by 5 bp deletions with short (2 bp) or no microhomologies and no repeats at deletion boundaries. ID10 predominantly consists of 5 bp insertions at one-unit repeats.

ERCC2 mutants were highly enriched among cases with high *ERCC2*mut classifier scores (Fig. 3B; $p < 2.2 \times 10^{-16}$, Fisher exact test) in the training data set. The composite *ERCC2*mut classifier more accurately identified *ERCC2*-mutant cases than was previously possible using COSMIC 5 signature alone (22): the AUC_{ROC} and AUC_{PRC} were 0.97 and 0.77, respectively, compared with 0.88 and 0.37 with the COSMIC 5 signature (Supp. Fig. 27). We identified a threshold score of 0.70 that minimized the cost of misclassification between *ERCC2*-mutant and WT cases in TCGA WES cohort (Supp. Fig. 28). The *ERCC2*mut classifier was also applied to the subset of TCGA cases with WGS data available ($n=23$) and there was a strong correlation between scores calculated using WGS and WES ($r=0.87$, Supp. Fig. 29).

To validate the ability of the composite *ERCC2*mut classifier to discriminate between *ERCC2*-mutant and WT bladder tumors, we analyzed WES data from three additional independent bladder cancer cohorts (5,7,42). The DFCI/MSKCC (5) ($n=50$) and Philadelphia (7) ($n=48$) cohorts are comprised of pretreatment tumors from patients with muscle-invasive bladder cancer who received neoadjuvant cisplatin-based chemotherapy followed by radical cystectomy, while the BGI cohort (42) ($n=98$) included patients with muscle-invasive bladder cancer or nonmuscle-invasive bladder cancer. In each cohort, *ERCC2*-mutant cases were significantly associated with an *ERCC2*mut classifier score 0.70 ($p = 3.3 \times 10^{-4}$ in the DFCI/MSKCC cohort, $p = 7.5 \times 10^{-5}$ in the BGI cohort, and $p = 7.9 \times 10^{-4}$ in the Philadelphia cohort; Fisher exact test; Fig. 3C–E, Supp. Fig. 30).

The composite mutational signature score correlates with cisplatin response in cases that lack an *ERCC2* mutation

ERCC2 mutations are associated with complete pathologic response and improved survival in patients with muscle-invasive bladder cancer treated with neoadjuvant cisplatin-based chemotherapy (5,7). However, not all patients with complete response harbor an *ERCC2* mutation; therefore, a method to identify potential cisplatin responders among patients who lack an *ERCC2* mutation could inform therapy selection. We hypothesized that tumors that are cisplatin sensitive, but lack an *ERCC2* mutation, may harbor other genetic or epigenetic alterations that confer functional NER deficiency and increased activity of the *ERCC2*mut composite signature.

Patients in the DFCI/MSKCC and Philadelphia cohorts were treated with cisplatin-based chemotherapy followed by radical cystectomy, and cisplatin responders were defined as patients with no residual invasive disease on pathologic examination of the cystectomy specimen. We found that the *ERCC2*mut signature scores were strongly associated with cisplatin response in both the DFCI/MSKCC and Philadelphia cohorts ($p = 2.1 \times 10^{-4}$ and $p = 0.003$, respectively; Fisher exact test; Fig. 4A, B). For example, in the DFCI/MSKCC cohort, 15 of 17 cases (88%) with an *ERCC2*mut signature score 0.70 had a complete response versus only 10 of 33 (30%) cases with a score <0.70 (Fig. 4A). In the Philadelphia cohort, all six cases (100%) with an *ERCC2*mut signature score 0.70 had a complete

response versus only 14 of 42 (33%) cases with score <0.70 (Fig. 4B). In both cohorts, overall survival (OS) was significantly longer in patients with a score ≥ 0.70 (Supp. Fig. 31–32).

We next restricted our analysis to patients without an *ERCC2* mutation because this is the subset of patients for whom cisplatin response is currently most difficult to predict. Among the 41 WT *ERCC2* cases in the DFCI/MSKCC cohort, only 16 (39%) were cisplatin responders (Fig. 4C). However, there was a significant enrichment of cisplatin responders among the cases with high ERCC2mut signature scores, 7 of the 9 (78%) WT *ERCC2* patients with a score ≥ 0.70 were responders versus only 9 of the 32 (28%) WT *ERCC2* patients with an ERCC2mut score <0.70 ($p = 0.02$). Therefore, WT *ERCC2* cases with a high ERCC2mut signature score were nearly three times as likely to have complete response to cisplatin as WT *ERCC2* patients with a low score. In addition, WT *ERCC2* patients with ERCC2mut score ≥ 0.70 had significantly better survival than WT *ERCC2* patients with lower scores ($p=0.046$; Fig. 4D). ERCC2mut scores were lower in the formalin-fixed, paraffin-embedded (FFPE)-derived Philadelphia cohort than in the other cohorts (which were derived from fresh frozen tissue), and there was only one WT *ERCC2* case with a score ≥ 0.70 (Fig. 4B; Supp. Fig. 33). Together, these data demonstrate that the composite ERCC2mut signature is associated with cisplatin response, including in bladder tumors that lack an *ERCC2* mutation. This result suggests that the ERCC2mut composite signature may reflect tumor NER deficiency conferred by mechanisms beyond *ERCC2* mutation, and may, therefore, provide additional predictive power to prioritize patients for NER-targeting agents, such as cisplatin or irifolven.

An NER gene defect is sufficient to induce the composite mutational signature

To further investigate the relationship among NER pathway function, sensitivity to NER-directed therapy, and the ERCC2mut composite mutational signature, we tested if *ERCC2* inactivation was sufficient to generate the composite mutational signature. KU19–19 is a bladder cancer cell line with no known NER pathway alterations (Supp. Fig. 34), while KE1 is an *ERCC2*-mutated derivative of KU19–19 that is NER deficient and displays increased sensitivity to cisplatin and irifolven (Fig. 1B) (6). For each cell line, separate clonal populations were propagated in parallel for 30 days and single cells were isolated, expanded to approximately 1×10^6 cells, and harvested for genomic DNA isolation (Fig. 5A). WGS was performed from clonal ‘parental’ (P0) populations, as well as from two independent ‘postpropagation’ clonal populations. Mutations were called as described previously (36) and ERCC2mut scores were determined for each sample (Supplementary Methods). Among the postpropagation populations, the NER-deficient KE1 cell line clones had significantly higher composite mutational signature scores than NER-proficient KU19–19 clones (Fig. 5B). These data demonstrate that NER deficiency created by loss of an NER gene is sufficient to induce the composite mutational signature and further supports a direct link among NER deficiency, the composite mutational signature, and sensitivity to cisplatin and irifolven.

Discussion

Tumor DNA repair deficiency is of particular interest in clinical oncology because it can potentially be targeted using a synthetic lethal-based strategy. However, the success of the synthetic lethal approach is dependent on accurate identification of the relevant DNA repair pathway deficiency in clinical tumor specimens, as well as on the availability of a therapeutic agent that is specifically active in DNA repair-deficient cells. Here, we characterize a novel synthetic lethal relationship between tumor NER deficiency and the abandoned anticancer drug, irifolven, and we show that NER-deficient tumors have a unique mutational signature that may expand identification of patients likely to respond to NER-targeted agents, such as cisplatin or irifolven.

Cisplatin-based chemotherapy is a preferred treatment approach for patients with muscle-invasive or metastatic bladder cancer. Mutations in *ERCC2* or other DNA repair genes are present in approximately 20% of bladder tumors and are associated with cisplatin response rates of 80–100% (7,9). However, despite the demonstrated activity of cisplatin in patients with tumor NER deficiency, 30–50% of patients with bladder cancer are unable to receive cisplatin due to renal dysfunction or other medical comorbidities (43). For these patients, other agents that target tumor NER deficiency represent an attractive therapeutic strategy. On the basis of its mechanism of action and on its reported activity in NER-deficient nontumor models, we elected to investigate irifolven, a semisynthetic alkylating agent previously tested as an anticancer agent in a number of phase I/II trials (17–20). Whereas irifolven response rates were very modest (10–15%) in unselected patient populations, our functional analyses showed that NER-deficient tumor cells are profoundly sensitive to irifolven both *in vitro* and *in vivo*. Similar NER-dependent irifolven sensitivity was also observed in additional cell line and patient-derived cancer models, including breast cancer models harboring *ERCC3* mutations (44). Because irifolven creates DNA lesions that are only recognized by the TC-NER pathway when transcribing RNA polymerase encounters the lesion, we found that inactivation of TC-NER or common NER genes, but not GG-NER-specific genes, was sufficient to induce irifolven sensitivity. These findings are consistent with a synthetic lethal mechanism of irifolven-induced tumor cell killing that is analogous to the synthetic lethal relationship that exists between HR deficiency and PARP inhibitor sensitivity in other tumor settings. Unfortunately, no tumors from irifolven-treated patients with bladder cancer are currently available to validate the relationship between NER gene mutation status and clinical irifolven sensitivity; however, a prospective clinical trial of irifolven is planned for patients with advanced urothelial tumors harboring predicted NER dysfunction as defined by NER gene defects (with or without LOH) or the presence of a high ERCC2mut signature score.

Despite initial responses in a subset of patients, most patients with metastatic bladder cancer who receive cisplatin-based chemotherapy ultimately develop cisplatin-resistant disease. Although several anti-PD-1/PD-L1 are approved in this setting, only a fraction of patients respond and median OS for platinum-refractory bladder cancer remains less than one year. Whereas mechanisms of resistance to platinum drugs and PARP inhibitors in HR-deficient tumors have been extensively studied, mechanisms of cisplatin resistance in NER-deficient tumors are not known. We found that acquired cisplatin resistance in an NER-deficient

tumor model failed to restore NER activity and, therefore, conferred minimal cross-resistance to irifolven, suggesting that irifolven may be an effective treatment for patients with tumor NER deficiency who progress on cisplatin-based chemotherapy. Given that upregulation of alternative DNA repair pathways is common in tumors with loss of a specific DNA repair pathway, such as NER or HR, combining irifolven with other DNA damage response–targeting agents, such as PARP inhibitors, may also be a rational strategy to explore (44).

In addition to identifying a drug that specifically targets a DNA repair–deficient state, successful implementation of a synthetic lethal therapeutic strategy also relies on accurate identification of tumors harboring the targetable DNA repair deficiency. Most commonly, this is accomplished by sequencing the DNA repair genes of interest, and putative damaging germline or somatic NER pathway alterations are present in up to 10% of patients with bladder cancer and other tumor types (44). However, relying exclusively on this approach to identify tumor DNA repair deficiency has several shortcomings, including (1) uncertainty regarding the biological impact of novel mutations (i.e., variants of unknown significance), (2) the potential for secondary genetic events to modulate the effect of an observed mutation (for example, *REV7* loss offsetting the impact of *BRCA1* loss (45)), and (3) loss of DNA repair pathway activity via an epigenetic mechanism or through alteration of a gene not previously implicated in the pathway. Mutational signature-based approaches have the potential to overcome many of these challenges because they detect the genomic consequences of DNA repair pathway deficiency, rather than the underlying cause. Furthermore, integrative analyses of multiple types of mutation events (SNVs, indels, rearrangements) may increase the ability of mutational signatures to identify clinically relevant DNA repair deficiency, as has been demonstrated for detection of HR deficiency in breast tumors (46). Here, we describe a composite mutational signature of *ERCC2* inactivation in bladder cancer. In multiple independent cohorts, this signature was strongly associated with *ERCC2* mutation status, and importantly, the signature was also associated with cisplatin response in cases that lack an *ERCC2* mutation. In fact, WT *ERCC2* cases with a high ERCC2mut score were nearly three times more likely to have complete response to cisplatin-based chemotherapy than WT *ERCC2* cases with a low signature score. Interestingly, several of the WT *ERCC2* cisplatin responders with a high ERCC2mut signature score harbored a predicted deleterious mutation in another NER gene, including two patients with truncating *ERCC6* mutations (Supp. Fig. 35). NER deficiency may also arise via epigenetic mechanisms, and further integrative genomic analyses will be required to understand the contribution of epigenetic processes to NER deficiency. We identified an optimal threshold signature score of 0.70 for bladder tumors sequenced from fresh frozen tissue; however, validation is required in prospectively collected cohorts, and additional work will be needed to define the optimal threshold for FFPE bladder tumor cohorts and to determine whether the signature can be reliably identified from less than WES data.

In summary, we identified a novel synthetic lethal relationship between tumor NER deficiency and sensitivity to the previously discarded anticancer agent, irifolven, and we show that NER deficiency is sufficient to drive sensitivity to irifolven in cisplatin-sensitive and cisplatin-resistant tumor models. Furthermore, we developed and validated a composite mutational signature of *ERCC2* deficiency that is strongly associated with NER deficiency

and irifolven sensitivity in preclinical models and also correlates with clinical cisplatin response, including in cases that lack an *ERCC2* mutation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational relevance

ERCC2 mutations are present in approximately 10% of bladder tumors and confer increased sensitivity to cisplatin-based chemotherapy. However, up to half of patients with advanced bladder cancer are ineligible to receive cisplatin-based chemotherapy due to medical comorbidities. We identified a synthetic lethal relationship between bladder tumor NER deficiency and sensitivity to irifolven, a discarded anticancer agent that creates DNA lesions which can only be repaired by the NER pathway. We show that NER-deficient tumors, including those with acquired cisplatin resistance, are sensitive to irifolven, suggesting that irifolven may provide a novel treatment approach for cisplatin-ineligible or cisplatin-resistant patients with tumor NER deficiency. In addition, we developed and validated a composite mutational signature of tumor *ERCC2* deficiency that is strongly associated with cisplatin response, including in cases that lack an *ERCC2* mutation. This signature may be useful in identifying patients likely to benefit from cisplatin or irifolven.

Figure 1:

NER-deficient cell lines are sensitive to irofulven *in vitro* and *in vivo*. **A.** Chemical structure of irofulven. **B.** Cell viability assays demonstrate increased irofulven sensitivity in NER-deficient cell lines compared with their isogenic NER-proficient counterparts. **C.** Irofulven dose-response for KE1 (*ERCC2* mutant, NER deficient) xenografts. The black arrow denotes the time of first irofulven injection. **D.** KE1 xenograft weights were significantly lower in 0.5 and 1.0 mg/kg irofulven-treated mice than in untreated mice. * p-value 0.01-0.05, ** p value 0.001 < p < 0.01, and *** p-value < 0.001.

Figure 2:

Irofulven sensitivity is conferred by TC-NER or common NER gene defects and persists in cisplatin-resistant models. **A.** SiRNA-mediated depletion of the TC-NER gene, *ERCC6*, or the common NER gene, *ERCC3*, results in significantly higher irofulven sensitivity compared with depletion of the GG-NER gene, *DDB2*, as measured by crystal violet staining (top) or viability assay (bottom). **B.** Immunoblot analysis showing RNA polymerase (RBP1) levels following irofulven treatment in NER-proficient KU19-19 and NER-deficient KE1 cells (top) and immunoblot analysis showing accumulation of phospho-H2AX, cleaved PARP, and cleaved caspase in irofulven-treated KE1 cells. **C.** A cisplatin-resistant derivative of the NER-deficient MDA-MB-468 cell line demonstrates decreased sensitivity to cisplatin, but remains sensitive to irofulven. **D.** Immunoblot analysis showing that *ERCC4* (XPF) protein expression remains absent in the cisplatin-resistant MDA-MB-468 line (lane 3), similar to the parental cisplatin-sensitive line (lane 2) and consistent with persistent NER deficiency. Lane 1 (positive control) shows an MDA-MB-468 line engineered to stably express WT *ERCC4*. * p-value 0.01-0.05, ** p value 0.001 < p < 0.01, and *** p-value < 0.001.

Figure 3:

Development of the ERCC2mut logistic regression-based classifier and validation of the association between ERCC2mut scores and *ERCC2* mutation status in three independent bladder cancer cohorts. Samples with an *ERCC2* mutation are shown in green and WT *ERCC2* samples are shown in gray. **A.** Nine mutational features were significantly associated with *ERCC2* mutation status in TCGA bladder cancer WES cohort and were used to develop the composite ERCC2mut score. **B.** ERCC2mut signature scores are strongly associated with *ERCC2* mutation status in TCGA bladder cancer cohort ($p < 2.2 \times 10^{-16}$). A value of 0.70 maximally separates *ERCC2*-mutant from WT cases and is denoted by the red horizontal dash-dotted line. In each validation cohort, *ERCC2* mutants are highly enriched among patients with a high ERCC2mut score (> 0.70). **C:** DFCI/MSK cohort (n=50), $p = 3.3 \times 10^{-4}$. **D:** BGI cohort (n=98), $p = 7.5 \times 10^{-5}$. **E:** Philadelphia cohort (n=48), $p = 7.9 \times 10^{-4}$. P-values were calculated using Fisher exact test.

Figure 4:

ERCC2mut signature scores are associated with cisplatin response, including among WT *ERCC2* cases. Cisplatin responders are colored in orange and nonresponders are in black. *ERCC2* mutant cases are denoted by green asterisks. **A.** ERCC2mut signature scores for all cases in the DFCI/MSKCC cohort (n=50). **B.** ERCC2mut signature scores for all cases in the Philadelphia cohort (n=48). **C.** ERCC2mut signature scores for WT *ERCC2* cases in the DFCI/MSKCC cohort (n=41); high ERCC2mut signature scores (≥ 0.70) are significantly associated with cisplatin response (p=0.02). **D.** OS for patients with WT *ERCC2* tumors in the DFCI/MSKCC cohort. OS was significantly longer for WT *ERCC2* patients with ERCC2mut signature scores ≥ 0.70 (p=0.046). Positive predictive value (PPV) and negative predictive value (NPV) of the ERCC2mut composite signature were calculated for each cohort. P-values were calculated by the Fisher exact test.

Figure 5:

NER deficiency drives the ERCC2mut composite mutational signature. **A.** Separate clonal populations of NER-proficient KU19–19 or NER-deficient KE1 cells were cultured in parallel for approximately 30 generations and single cells were then isolated, expanded, and harvested. **B.** WGS and mutational signature analysis revealed significantly higher ERCC2mut scores in the NER-deficient KE1 samples compared with the NER-proficient KU19–19 samples.