Somatic ERCC2 Mutations Correlate with Cisplatin Sensitivity in Muscle-Invasive Urothelial Carcinoma

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ABSTRACT
Cisplatin-based chemotherapy is the standard of care for patients with muscle-invasive urothelial carcinoma. Pathologic downstaging to pT0/pTis after neoadjuvant cisplatin-based chemotherapy is associated with improved survival, although molecular determinants of cisplatin response are incompletely understood. We performed whole-exome sequencing on pretreatment tumor and germline DNA from 50 patients with muscle-invasive urothelial carcinoma who received neoadjuvant cisplatin-based chemotherapy followed by cystectomy (25 pT0/pTis “responders”; 25 pT2+ “nonresponders”) to identify somatic mutations that occurred preferentially in responders. ERCC2, a nucleotide excision repair gene, was the only significantly mutated gene enriched in the cisplatin responders compared with nonresponders (q < 0.01). Expression of representative ERCC2 mutants in an ERCC2-deficient cell line failed to rescue cisplatin and UV sensitivity compared with wild-type ERCC2. The lack of normal ERCC2 function may contribute to cisplatin sensitivity in urothelial cancer, and somatic ERCC2 mutation status may inform cisplatin-containing regimen usage in muscle-invasive urothelial carcinoma.

SIGNIFICANCE: Somatic ERCC2 mutations correlate with complete response to cisplatin-based chemosensitivity in muscle-invasive urothelial carcinoma, and clinically identified mutations lead to cisplatin sensitivity in vitro. Nucleotide excision repair pathway defects may drive exceptional response to conventional chemotherapy. Cancer Discov; 4(10); 1140–53. © 2014 AACR.

See related commentary by Turchi et al., p. 1118.
INTRODUCTION

Platinum-based chemotherapy has been the standard of care for patients with muscle-invasive and metastatic urothelial carcinoma for more than 20 years (1–3), and neoadjuvant cisplatin-based chemotherapy leads to a 14% to 25% relative risk reduction for death from muscle-invasive urothelial carcinoma (cT2-T4aN0M0; refs. 3–5). Pathologic downstaging to complete response (pT0) or carcinoma in situ (pTis) at cystectomy occurs in 26% to 38% of patients treated with neoadjuvant chemotherapy compared with 12.3% to 15% for patients undergoing cystectomy alone (3, 4, 6), and the 5-year survival for pT0/pTis patients is 85% after neoadjuvant chemotherapy (3) compared with 43% for patients with persistent muscle-invasive disease (≥pT2; ref. 7). Therefore, the benefit of neoadjuvant chemotherapy seems to be most dramatic in patients who are found to have pathologic complete responses at the time of surgical resection. However, the inability to predict which patients will derive clinical benefit has limited the use of this toxic approach in the urologic community (8–10).

Cisplatin causes accumulation of DNA cross-links, which interfere with DNA replication and gene transcription, and eventually promotes cell death. Repair of cisplatin-induced DNA damage occurs primarily through DNA repair pathways such as nucleotide excision repair (NER; ref. 11) and homologous recombination (which includes BRCA1 and BRCA2; ref. 12). The NER pathway involves multiple genes, including ERCC1–5, CDK7, DDB1–2, XPA, and XPC (13). Germline alterations in NER genes result in multiple recessive inherited disorders, including xeroderma pigmentosum (XP; ref. 13). Because of a deficiency in NER, patients with XP have significantly increased risk of developing skin cancers and other malignancies (14).

Germline or expression-based changes in several NER genes have been suggested to modulate clinical response to cisplatin-based chemotherapy (15, 16). However, prospective studies have not confirmed these observations (17, 18). ERCC2 (an NER helicase) loss-of-function correlates with cisplatin sensitivity in preclinical models (19), whereas ERCC2 overexpression leads to cisplatin resistance (20). These data suggest that tumors with loss of NER function may exhibit increased cisplatin sensitivity, and recent data have identified somatic ERCC2 mutations in 7% to 12% of urothelial carcinomas (21, 22). We hypothesized that somatic mutations in the NER pathway may correlate with response to cisplatin-based neoadjuvant chemotherapy in patients with urothelial carcinoma. To test this, and more generally to define genomic correlates of chemotherapy response, we performed whole-exome sequencing (WES) of urothelial
cancerous tumors from patients with extreme responses to neoadjuvant cisplatin-based combination chemotherapy.

RESULTS
Somatic Genetic Alterations in Muscle-Invasive Urothelial Carcinoma

We sequenced pretreatment tumor and germline DNA from 50 patients treated with neoadjuvant cisplatin-based chemotherapy for muscle-invasive urothelial carcinoma; 25 “responders” had no residual invasive disease (pT0/pTis) on pathologic examination following cystectomy, and 25 “nonresponders” had residual muscle-invasive (pT2) disease (Fig. 1A; Methods). Although multiple chemotherapeutic regimens were used, the only common agent among all patients was cisplatin (Table 1 and Supplementary Table S1). No significant differences in clinical characteristics were identified between responders and nonresponders at baseline (P > 0.05; Mann-Whitney test).

The mean target coverage from WES was 121× for tumors and 130× for paired germline samples (Supplementary Table S1). The median mutation rate was 9.7 mutations per megabase (mutations/Mb) for responders and 4.4 mutations/Mb for nonresponders (P = 0.0003; Mann-Whitney test; Fig. 1B; Supplementary Fig. S1A and S1B; Supplementary Table S1), raising the possibility of reduced DNA repair fidelity among cisplatin responders. All observed somatic mutations and short insertion/deletions across both responders and nonresponders were reported in Supplementary Table S2.

A statistical assessment (23) of the base mutations and genomic correlates (Fig. 1; Methods) of the aggregate cohort (n = 50 patients) demonstrates a set of statistically significant genes that are altered in urothelial carcinoma (TPS3, RB1, KDM6A, and ARID1A). The negative log of the q values for the significance level of mutated genes is shown (for all genes with q < 0.1) on the right. ERCC2 mutation status is also shown below the other genes, although ERCC2 was not significantly mutated across the combined cohort. Additional data about allelic fraction ranges for each case (bottom), mutation rates (top), and mutational frequency (left) are also summarized.
nonresponders nominated four significantly altered genes previously implicated in urothelial carcinoma (21, 22, 24): TP53, RB1, KDM6A, and ARID1A (Fig. 1B and Supplementary Tables S3 and S4). In addition, nine nonsynonymous somatic mutations were observed in ERCC2 (Fig. 1B; Supplementary Fig. S2; Supplementary Table S2). Although ERCC2 did not reach cohort-wide statistical significance, its known role in DNA repair and report of being recurrently mutated in bladder cancer (21, 22) raised the possibility that ERCC2 mutations might associate with cisplatin response.

### Table 1. Patient characteristics

<table>
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<th>Total (N = 50)</th>
<th>Responders (n = 25)</th>
<th>Nonresponders (n = 25)</th>
<th>P &gt; 0.05</th>
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<tr>
<td>Age at TUR, y</td>
<td>62.5 ± 8.9</td>
<td>61 ± 10.1</td>
<td>64 ± 7.3</td>
<td>P &gt; 0.05</td>
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<tr>
<td>Female sex, n (%)</td>
<td>13 (26)</td>
<td>6 (24)</td>
<td>7 (28)</td>
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<td>Ethnicity, n (%)</td>
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<td>1 (4)</td>
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<td>24 (96)</td>
<td>24 (96)</td>
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<tr>
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<td>46 ± 26.0</td>
<td>47 ± 33.8</td>
<td>P &gt; 0.05</td>
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<tr>
<td>Median length of follow-up (days ± SD)</td>
<td>351 ± 363.2</td>
<td>372.5 ± 416.2</td>
<td>329.5 ± 287.1</td>
<td>P &gt; 0.05</td>
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<td>Patients with recurrence, n (%)</td>
<td>13 (26)</td>
<td>2 (8)</td>
<td>11 (44)</td>
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</table>

**NOTE:** Clinical characteristics of the total patient cohort, as well as data stratified by responder or nonresponder status. Plus–minus values are medians ± SD. Abbreviations: ddGC, dose-dense gemcitabine and cisplatin; ddMVAC, dose-dense methotrexate, vinblastine, doxorubicin, and cisplatin; GC, gemcitabine and cisplatin; TUR, transurethral resection. For only patients alive at the time of this study. P < 0.05 is considered significant (Mann–Whitney test).

### Somatic ERCC2 Mutations in Cisplatin-Based Chemotherapy Responders

We performed an enrichment analysis to identify genes that were selectively mutated in the responders compared with nonresponders (Supplementary Methods). Among 3,277 genes with at least one possibly damaging somatic alteration (Supplementary Methods), ERCC2 was the only gene significantly enriched in the responder cohort (Fig. 2A and Supplementary Tables S5 and S6). Indeed, all ERCC2 nonsynonymous somatic
mutations occurred in the cisplatin-sensitive tumors (P < 0.001; Fisher exact test). ERCC2 remained significantly enriched in responders following false discovery analysis performed on genes in which the mutation frequency afforded adequate power (q = 0.007; Benjamini–Hochberg; Fig. 2B). Moreover, the enrichment for ERCC2 mutations in the responder group was significant when adjusted for differences in the overall mutation rate between responders and nonresponders (P = 0.04; binomial test). Toward this end, the median background mutation rate for ERCC2-mutant tumors (15.5 mutations/Mb) was significantly elevated compared with ERCC2 wild-type (WT) tumors (5.1 mutations/Mb; P = 0.01; Mann–Whitney test; Supplementary Fig. S1B), consistent with a possible DNA-repair defect and prior reports (22).

The somatic ERCC2 mutation frequency in the responder cohort was also compared with two unselected bladder
cancer populations: 130 cases from The Cancer Genome Atlas (TCGA; ref. 21) and 99 cases from a Chinese patient cohort (ref. 22; Fig. 2C). When compared with these unselected populations, ERCC2 mutations were significantly enriched in the cisplatin responder cohort (36% of cases; \( P < 0.001 \); binomial test; Fig. 2C). ERCC2 mutation status does not seem to be prognostic, as it had no impact on overall survival in the TCGA cohort (\( P = 0.77 \); log-rank; Supplementary Fig. S3). To determine the relative abundance of somatic ERCC2 mutations in other tumor types, TCGA data from 19 tumor types (\( n = 4,429 \)) were queried (25). Somatic ERCC2 mutations were observed at low frequencies (<4%) in 11 other tumor types (Fig. 3A and B). All identified somatic ERCC2 mutations occurred at highly conserved amino acid positions within or immediately adjacent to the helicase domains (Fig. 3A and C and Supplementary Fig. S4). Similarly, germline ERCC2 mutations in
patients with XP (complementation group D) and XP with combined Cockayne syndrome (XP/CS), two disorders characterized by NER function, clustered within helicase domains (Fig. 3C). Conversely, mutations causing trichothiodystrophy, a disease resulting from the alteration of ERCC2’s role in transcription, were distributed throughout the protein (26).

**ERCC2 Mutations Confer Increased Cisplatin Sensitivity In Vitro**

These observations raised the possibility that the identified mutations disrupt ERCC2 function and interfere with NER. To test this hypothesis, the first five of the identified ERCC2 mutants were stably expressed in an immortalized ERCC2-deficient cell line derived from an XP patient, and the cisplatin sensitivity profile of each cell line was measured (Supplementary Methods; Fig. 4A). Expression of WT ERCC2 rescued cisplatin sensitivity of the ERCC2-deficient cell line, whereas none of the ERCC2 mutants rescued cisplatin sensitivity (Fig. 4B). The IC\(_{50}\) for the WT ERCC2 cell line was significantly higher than that for the ERCC2-deficient parent cell line (\(P < 0.0001\); ANOVA). The difference between the ERCC2-deficient cell line and each of the mutant cell lines was not statistically significant.

**Figure 4.** ERCC2 mutants fail to rescue cisplatin sensitivity of ERCC2-deficient cells. A, immunoblot of ERCC2 expression in cell lines created by transfection of the ERCC2-deficient parent cell line (GM08207; Coriell Institute), with pLX304 (Addgene) encoding GFP (negative control), WT ERCC2, or a mutant ERCC2. The negative control ERCC2-deficient cell line (lane 1) expresses endogenous levels of inactive ERCC2 from the parent cell genome, whereas WT (lane 2) and mutant (lanes 3–7) ERCC2 cell lines show increased levels of ERCC2 expressed from the transfected gene. β-Actin is shown as a loading control. B, cisplatin sensitivity profiles of cell lines expressing WT or mutant ERCC2. Expression of WT ERCC2 in an ERCC2-deficient background rescues cisplatin sensitivity, whereas expression of the ERCC2 mutants fails to rescue cisplatin sensitivity. An IC\(_{50}\) was calculated from the survival data for each cell line, and these values are shown in C. The difference in IC\(_{50}\) between the parent [ERCC2-deficient] cell line and the cell line expressing WT ERCC2 was statistically significant, as was the difference between the WT ERCC2 cell line and each of the mutant ERCC2 cell lines (\(P < 0.0001\); ANOVA). The difference between the ERCC2-deficient cell line and each of the mutant cell lines was not statistically significant.
ERCC2 and cisplatin sensitivity in urothelial carcinoma

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Cell line was significantly higher than that for the ERCC2-deficient and mutant cell lines (P < 0.0001; ANOVA).

The NER pathway repairs DNA lesions other than cisplatin adducts, so we also tested the effect of the identified ERCC2 mutations on NER-mediated repair of UV-induced DNA damage. WT and mutant ERCC2-complemented cell lines were exposed to increasing doses of UV irradiation, and clonogenic survival was measured (Fig. 5A–C). Whereas the ERCC2-WT–complemented cell line rescued UV sensitivity, the UV sensitivities of the ERCC2-mutant–complemented cell lines were not significantly different from that of the ERCC2-deficient parent cell line. Taken together, these experiments

Figure 5. ERCC2 mutants fail to rescue UV sensitivity of ERCC2-deficient cells. A, a representative colony formation assay for the ERCC2-deficient cell line (top) as well as the ERCC2-deficient line transfected with WT ERCC2 (middle), or one of the ERCC2 mutants (D609G, bottom) following increased doses of UV irradiation. B, clonogenic survival data for negative control, WT ERCC2, and mutant ERCC2. WT ERCC2 rescues UV sensitivity of the ERCC2-deficient cell line, whereas the mutant ERCC2s fail to rescue UV sensitivity. C, UV IC50 values for cell lines. The difference between the ERCC2-deficient cell line and the WT ERCC2 cell line was significant (P < 0.0001; ANOVA), whereas the difference between the ERCC2-deficient cell line and each of the ERCC2-mutant cell lines was not statistically significant (NS).
suggest that the observed ERCC2 mutations result in loss of normal NER capacity.

Because the overall mutation rate was higher in ERCC2-mutated tumors, we hypothesized that ERCC2 mutations may be broadly contributing to genomic instability. Thus, we measured rates of chromosomal aberrations in WT and mutant ERCC2–complemented cell lines before and after cisplatin treatment. In the absence of cisplatin, background rates of chromosomal aberrations were slightly lower in the ERCC2-WT–complemented cell line than in the ERCC2-deficient or ERCC2-mutant–complemented cell lines, but this difference was not statistically significant (Fig. 6A–D). However, following cisplatin exposure, significantly fewer chromosomal aberrations were observed in the ERCC2-WT–complemented cell line than in the ERCC2-deficient parent cell line (P = 0.03; ANOVA), whereas expression of the ERCC2 mutants resulted in no rescue of chromosomal stability (P > 0.5; Fig. 6D). These data suggest that the responder-associated ERCC2 mutations may contribute to overall genomic instability in these tumors.

Other DNA Repair Gene Alterations

Finally, we sought to determine whether other DNA repair genes might undergo recurrent mutations in cisplatin-sensitive tumors. No significantly recurrent mutations were observed in other NER or homologous repair genes in responders compared with nonresponders. However, in two responder tumors that did not have ERCC2 mutations, somatic nonsense (truncating) mutations were detected in the homologous recombination DNA repair genes BRCA1 and BRCA2 (Supplementary Table S2). There were no nonsynonymous BRCA1 or BRCA2 mutations in the nonresponders (Supplementary Table S2). These results are consistent with the known sensitivity of BRCA1/2-mutant tumors (e.g., BRCA1/2-mutant breast or ovarian cancers) to platinum-containing regimens (27). Whereas somatic ERCC2 mutations were the only DNA repair gene mutations enriched in the responders, singleton missense mutations of uncertain significance were observed in DNA damage response genes in both responders and nonresponders, and are of unknown functional relevance.

DISCUSSION

In bladder cancer, the clinical benefit of neoadjuvant chemotherapy is most apparent when pathologic downstaging to pT0 or pTis is achieved at surgical resection following...
ERCC2 and Cisplatin Sensitivity in Urothelial Carcinoma

Chemotherapy. However, the lack of a predictive biomarker for clinical benefit from neoadjuvant cisplatin-based chemotherapy has limited the use of this approach in the clinical community due to toxicity concerns. Using an extreme phenotype analysis, we have identified an association between somatic ERCC2 mutations and pathologic complete response to neoadjuvant cisplatin-based chemotherapy in muscle-invasive urothelial carcinoma. Although ERCC2 mutations occur in approximately 12% of unselected cases, 36% of cisplatin-based chemotherapy responders in our cohort harbored somatic ERCC2 nonsynonymous mutations. Moreover, all ERCC2-mutant tumors responded to neoadjuvant chemotherapy.

The NER pathway is a highly conserved DNA repair system that identifies and repairs bulky DNA adducts arising from genotoxic agents such as cisplatin. The NER helicase ERCC2 unwinds duplex DNA near the damage site through the coordinated action of two conserved helicase domains. All ERCC2 mutations identified in this study occurred at conserved positions within or adjacent to these helicase domains, and the identified mutants all failed to complement cisplatin or UV sensitivity of an ERCC2-deficient cell line. Together, these data suggest that the mutations result in loss of normal ERCC2 function, leading to increased tumor cell sensitivity to DNA-damaging agents such as cisplatin.

Interestingly, in seven (78%) of the ERCC2-mutant cases, the ERCC2 mutation allelic fraction was <0.5 (Supplementary Table S2), suggesting that WT ERCC2 remains present at one allele. Therefore, the cisplatin sensitivity phenotype may result from a haploinsufficient or dominant-negative effect of a heterozygous ERCC2 mutation, rather than as a result of biallelic inactivating mutations (as in the traditional “two-hit” tumor suppressor model). The driving force for heterozygous mutation of ERCC2 is unknown; however, the prevalence of ERCC2 mutations in this study and other cohorts (such as the TCGA) suggests that loss of normal ERCC2 function may provide a selective advantage for tumors. Partial loss of DNA repair fidelity could aid tumor growth by decreasing repair-associated delays in cell-cycle progression. In addition, decreased NER capacity may result in higher rates of error-prone repair or large-scale genomic changes that further drive tumor growth.

Despite providing a potential growth advantage, mutation of one ERCC2 allele may render tumor cells susceptible to a DNA-damaging agent such as cisplatin if inadequate levels of WT ERCC2 are present to support NER (i.e., haploinsufficiency). Alternatively, the mutated version of ERCC2 may bind, but not efficiently repair, damaged DNA, thereby preventing repair by an alternative DNA-repair pathway and leading to a dominant-negative phenotype, as has been described for mutants of the yeast ERCC2 homolog Rad3 (28). Further studies are necessary to explore the effects of ERCC2 loss on tumor growth, and the mechanism by which the identified ERCC2 mutations confer changes in tumor NER capacity.

One possible explanation for the findings observed in this study is that somatic ERCC2 mutation is associated with good prognosis or smaller tumors. However, this is not supported by the survival data from the TCGA bladder cancer cohort, which excluded patients who received neoadjuvant chemotherapy. No difference in overall survival was observed on the basis of somatic ERCC2 mutation status (Supplementary Fig. S3). In the extreme response cohort reported here, patients were generally noted to have obvious disease left behind after initial transurethral resection based on operative reports and imaging, making the findings unlikely to be related to complete transurethral resection.

Although these data should not yet be used to justify avoiding cisplatin-based treatment in ERCC2 WT patients, our findings raise the possibility that somatic ERCC2 mutation status may provide a genetic means to select patients most likely to benefit from cisplatin-based chemotherapy, while directing other patients toward alternative therapeutic approaches. Of note, our study focused specifically on somatic mutations that are exclusively in the tumor, and not germline single-nucleotide polymorphisms in ERCC2 or other genes. Thus, this approach is distinct from genomewide association studies that have examined germline ERCC1 or ERCC2 polymorphisms and their mixed impact on cisplatin sensitivity (29). Broadly, these findings will require independent clinical validation in prospective trials to establish the clinical predictive power of somatic ERCC2 mutation status for cisplatin response.

It is possible that some nonresponding urothelial tumors will harbor somatic ERCC2 mutations in larger cohorts; if observed, examination of the post-chemotherapy-resistant tumor would be critical to understanding whether tumor heterogeneity played a role in resistance. The patients analyzed in this extreme phenotype analysis were treated with combination cisplatin-based chemotherapy regimens containing non-cisplatin agents; however, cisplatin was the only agent common to all regimens. Because half of patients with bladder cancer are not candidates for cisplatin-based chemotherapy due to preexisting comorbidities, less toxic carboplatin-based neoadjuvant therapies may warrant study for non-cisplatin-eligible patients with ERCC2-mutant tumors.

To date, exceptional response genomic studies have informed genomic mechanisms of response to targeted therapies, such as response to everolimus in multiple disease contexts (30, 31). However, published studies have been limited to individual case reports due to the rarity of such events with targeted therapies. This study represents a new approach for studying extraordinary responses to commonly used cytotoxic chemotherapies using a case–control design, which may be applied to other therapeutic settings in which a significant minority of patients achieve exceptional response (e.g., neoadjuvant chemotherapies in other clinical settings). Toward that end, a majority of responder cases in our cohort had no recurrent genomic determinant of cisplatin response. It is possible that alterations in DNA repair genes not readily detectable with WES (e.g., epigenetic, expression-based) may mediate cisplatin sensitivity in these cases.

In conclusion, this work provides new insights into the relationship between somatic genetic alterations and clinical response to cisplatin-based chemotherapy in urothelial carcinoma. If further validated, these results may inform therapeutic decision-making, novel therapeutic development, and clinical trial designs for urothelial carcinoma and possibly other ERCC2-mutated tumors. Finally, these results show that somatic genomic alterations may reveal the mechanistic
METHODS

Patients and Samples

Patients with muscle-invasive or locally advanced urothelial carcinoma, extreme responses to chemotherapy (defined as no residual invasive carcinoma at cystectomy or presence of persistent muscle-invasive or extravesical disease at cystectomy), and available pre-chemotherapy tumor tissue who were enrolled on Institutional Review Board (IRB)-approved tissue acquisition and DNA-sequencing protocols were identified (Dana-Farber protocols 02-021 and 11-334; Memorial Sloan Kettering Cancer Center protocols 89-076 and 09-025). All patients provided written informed consent for genomic testing used for this study. Specimens were evaluated by genitourinary pathologists (J.A. Barletta/S. Signoretti—DFCI cohort, and H. Al-Ahmadi—MSKCC cohort) to identify tumor-bearing areas for DNA extraction. The minimum percentage of neoplastic cellularity for regions of tumor tissue was 60%. Study specimens of frozen or formalin-fixed, paraffin-embedded (FFPE) tissue sections were identified at the Dana-Farber Cancer Institute (DFCI) and Memorial Sloan Kettering Cancer Center (MSKCC). Germline DNA was extracted from either peripheral blood mononuclear cells or histologically normal nonurothelial tissue. Information about the source of germline DNA is available in Supplementary Table S1.

WES and Statistical Analysis

DNA extraction and exome sequencing Slides were cut from FFPE or frozen tissue blocks and examined by a board-certified pathologist to select high-density cancer foci and ensure high purity of cancer DNA. Biopsy cores were taken from the corresponding tissue block for DNA extraction. DNA was extracted using Qiagen’s QIAamp DNA FFPE Tissue Kit Quantitation Reagent (Invitrogen). DNA was stored at −20°C. Whole exome-capture libraries were constructed from 100 ng of DNA from tumor and normal tissue after sample shearing, end repair, and phosphorylation and ligation to barcoded sequencing adaptors. Ligated DNA was size-selected for lengths between 200 and 350 bp and subjected to exonic hybrid capture using SureSelect v2 Exome bait (Agilent Technologies). The DNA was fragmented and end repaired prior to barcoding and sequencing. The DNA library was quantified with the Qubit dsDNA high sensitivity assay (Invitrogen). The libraries were size selected using a size selection kit (Kapa Biosystems) and quantified using the Qubit dsDNA high sensitivity assay. The libraries were pooled equimolar, and paired end reads were generated using the NovaSeq 6000 (Illumina). The reads were aligned to the hg19 human genome build was produced using the Genome Analysis Tool Kit (36). Alterations from all nominated significant genes from MutSigCV were considered for further analyses. Missense, nonsense, and splice-site mutations, along with short insertion/deletions, were then assigned a damaging score (range, 0–1) following previously reported methods (40). Missense mutations were scored using the Polyphen2 score (41) for the amino acid substitution. Missense mutations without available Polyphen2 scores (due to mapping errors or dinucleotide status) were listed as “unavailable” in Supplementary Table S5 and excluded. Nonsense mutations, splice-site mutations, and short insertions/deletions were assigned a damaging score of 1. Damaging scores are listed in Supplementary Table S5. Alterations with a damaging score of 0.5 were then tabulated for occurrence in responders and nonresponders. An altered gene would be considered only in one patient. The Fisher exact test was performed to compare between cohorts to derive a P value for each gene. Because a minimum of six alterations were required to observe a P value of ≤0.01, only genes with 26 alterations in the cohort (thereby representing >10% of the cohort and of highest potential clinical significance) were considered for multiple hypothesis testing. These results are summarized in Supplementary Table S6 and Fig. 2A. The ERCC2 mutation frequency in the responders was compared with the unselected TCGA (21) and Guo and colleagues (22) cohorts with a binomial test. Results from this analysis are made available in Fig. 2C. Comparison of ERCC2 mutation distribution between responders and nonresponders adjusted for elevated mutation rate in the responders was performed with the binomial test conditional on observing nine mutations and using the estimated ratio of mutation rates between responders and nonresponders as the expected frequency of ERCC2 mutations in responders under the null hypothesis [e.g., for ERCC2, binomial test (x = 9; n = 9, P = Mutation_Rate_Responders/Mutation_Rate_Nonresponders, alternative = “greater”)]. All statistical calculations were performed using the R statistical package.

Mutation Validation

Orthogonal validation of selected mutations and short insertion/deletions (those presented in this article, including ERCC2) was performed using the Fluidigm Access Array. Of 50 cases, 35 had sufficient DNA to generate sufficient read depth for analysis. A total of 85 candidate targets were submitted to Fluidigm for single-plex PCR primer assay design. This resulted in the design of 65 assays covering all 85 targets. Assay amplicons ranged from 163 to 199 bp in size, with an average of 183 bp. All available samples were run on the Access Array system (Fluidigm) using three 48.48 Access Array IFC chips following the manufacturer’s recommendations using the
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“4-Primer Amplicon Tagging protocol” for Access Array (Fluidigm; P/N 100-3370). Rev. C1), with the exception that Access Array IFC chips were loaded and harvested using a Bravo Automated Liquid Handling Platform (Agilent Technologies), following the manufacturer’s recommendations. Resulting amplicons containing sample-specific barcodes and Illumina adapter sequencers were pooled and sequenced on a MiSeq sequencer (Illumina) with two runs of 150 base paired-end reads (V2 sequencing chemistry), using custom Fluidigm sequencing primers following the manufacturer’s recommendations (Fluidigm). All sites were manually reviewed in IGV to determine the presence or absence of nonreference reads. Details about validation results for ERCC2 and additional variants are given in Supplementary Table S2. Variants in which there was inadequate sample for validation or insufficient sequencing reads in the validation data to interpret manually in IGV were listed as “unavailable.”

**Cloning and Cell Lines**

A site-directed PCR mutagenesis/8/9 method (42) was used to generate WT and mutant ERCC2 open reading frames (ORF). For each mutant, PCR products were generated such that fragments overlap at the region of the desired mutation. The fragments were then introduced into the pDONR vector through the BP reaction. The BP reaction mixture was transformed into E. coli cells and recombined to generate a pENTR vector. The pENTR vector was then used to perform the LR (attL-attR) reaction to create an expression plasmid.

The expression plasmids harboring WT ERCC2, GFP (negative control), or mutant ERCC2 constructs were expanded in E. coli TOP10 cells (Invitrogen) and purified using an anion exchange kit (Qiagen). Lentiviruses were propagated in 293T cells by co-transfection of the expression plasmid with plasmids encoding viral packaging and envelope proteins. Unless otherwise noted, all human cell lines were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Sigma) and 1% l-glutamine and grown at 37°C and 5% CO2. The 293T cell supernatants containing virus were collected after 48 hours, filtered twice (0.45-μm syringe filter; Millipore), then added to 293T cell cultures. The 293T cell culture supernatants containing virus were collected after 48 hours, harvested, and concentrated by ultrafiltration (Millipore). The 293T cells were allowed to grow for an additional 48 hours. Cells were exposed to colcemid for 2 hours, harvested using 0.75 mol/L KCl, and fixed in 3:1 methanol/acetic acid. Slides were stained with Wright’s stain, and 25 to 50 metaphases were analyzed for chromosomal aberrations (chromosome or chromatid breaks, rings, translocations, deletions, fragments/double minute chromosomes, tri- or quadraradials, di- or tricentrics, and premature chromatid separation).

**UV Clonogenic Survival Assays**

Cells were seeded in 6-well plates (Nunc) at a density of 1,500 cells per well. The following day, the cells were washed once with PBS and then exposed to increasing UV doses using a UV-B irradiator (Stratagene). Medium was replaced, and the cells were allowed to grow for 9 days. On day 10, cells were fixed using 1.5 acetic acid/methanol solution for 20 minutes at room temperature. Cells were then stained for 45 minutes using 1% crystal violet in methanol solution. Plates were rinsed vigorously with water, allowed to dry, and colonies were then manually counted. The number of colonies present at each UV dose was plotted as a ratio of the number of colonies present in mock-irradiated wells. Each data point represents the average of three independent measurements, and the error bars represent the standard deviation.

**Chromosomal Breakage Analysis**

Approximately 1 × 106 cells were seeded per 10-cm dish. After 24 hours, 400 mmol/L of cisplatin was added, and cells were allowed to grow for an additional 48 hours. Cells were exposed to colcemid for 2 hours, harvested using 0.75 mol/L KCl, and fixed in 3:1 methanol/acetic acid. Slides were stained with Wright’s stain, and 25 to 50 metaphases were analyzed for chromosomal aberrations (chromosome or chromatid breaks, rings, translocations, deletions, fragments/double minute chromosomes, tri- or quadraradials, di- or tricentrics, and premature chromatid separation).

**Immunoblots**

Frozen cell pellets were thawed and resuspended in RIPA buffer [50 mmol/L TRIS (pH 7.3), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.5% Na-deoxycholate, and 0.1% SDS] supplemented with complete protease inhibitor (Roche), NaVO4, and NaF. The cell suspensions were centrifuged, and total protein concentration of the supernatant was determined by colorimetry (Bio-Rad). Samples were boiled with loading buffer (Bio-Rad) and electrophoresed in a 3% to 8% gradient TRIS-acetate gel (Life Technologies). Resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) at 90 V for 2 hours at 4°C. The membrane was blocked for 1 hour in blocking solution (5% milk in TRIS-buffered saline-T) and incubated with primary antibody in blocking solution at 4°C overnight (mouse ERCC2, Abcam; rabbit β-actin, Cell Signaling Technology). The following day, the membrane was rinsed and incubated for 1 hour with peroxidase-conjugated secondary antibody in blocking solution (anti-mouse and anti-rabbit; Cell Signaling Technology) and rinsed. Enhanced chemiluminescent substrate solution (PerkinElmer) was added and signal was detected by film exposure (GE Healthcare).

**Disclosure of Potential Conflicts of Interest**

E.M. Van Allen has ownership interest in a patent (pending). N. Wagle has ownership interest in and is a consultant/advisory board member for Foundation Medicine. D.F. Bajorin is a consultant/advisory board member for Novartis, Foundation Medicine, and Boehringer Ingelheim. J.E. Rosenberg has ownership interest in a patent (pending). No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**


No potential conflicts of interest were disclosed by the other authors.

**Cancer Discovery**

Published OnlineFirst August 5, 2014; DOI: 10.1158/2159-8290.CD-14-0623


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.M. Van Allen, P. Kim, J. Sfakianos, I. Garcia-Grossman, R. Bambury, S. Bahl, D. Farlow, A. Qu, T.K. Choueiri, J.E. Rosenberg


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Acknowledgments

The authors thank the patients for contributing tissue for research. In addition, the authors thank Dr. Kenna Shaw, Dr. Jean C. Zenklusen, and The Cancer Genome Atlas for allowing pertinent ERCC2 results to be incorporated into their analyses. The authors also thank Dr. Joaquin Bellmunt for thoughtful feedback and clinical review of cases, the Broad Genomics Platform for sequencing and validation activities, and Lisa Moreau for help with chromosomal studies.

Grant Support

This study was supported by the Dana-Farber Leadership Council (to E.M. Van Allen), the American Cancer Society (to E.M. Van Allen), the Conquer Cancer Foundation (to E.M. Van Allen and K.W. Mouw), the NIH/National Human Genome Research Institute (U54HG003067, to L.A. Garraway and S. Gabriel), Cycle for Survival (to G. Iyer, I. Ostrovnaya, and H. Al-Ahmadi), and the NIH/National Human Genome Research Institute (U54HG003067, to E.M. Garraway, E.M. Van Allen, G. Iyer, L.A. Garraway, I. Ostrovnaya, and H. Al-Ahmadi).

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Received June 13, 2014; revised July 24, 2014; accepted July 28, 2014; published OnlineFirst August 5, 2014

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Cancer Discovery 2014;4:1140-1153. Published OnlineFirst August 5, 2014.

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