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Expression levels of DNA damage repair proteins are associated with overall survival in platinum treated advanced urothelial carcinoma

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Abstract

Background—Combination platinum chemotherapy is standard first line therapy for metastatic urothelial carcinoma (mUC) patients. Defining platinum response biomarkers for mUC patients, could establish personalize medicine and provide insights into mUC biology. While DNA repair mechanisms are hypothesized to mediate platinum response, we sought to analyze if increased expression of DNA damage genes correlates with a worse overall survival (OS) in mUC patients.

Methods—We retrospectively identified a clinically annotated cohort of mUC patients, treated with first-line platinum combination chemotherapy. A tissue microarray (TMA) was constructed from formalin fixed paraffin embedded (FFPE) tissue from the pre-treatment primary tumor.

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Immunohistochemical (IHC) analysis of the following DNA repair proteins; ERCC1, RAD51, BRCA1/2, PAR, and PARP-1, was performed. Nuclear and cytoplasmic expression was analyzed using multispectral image. Nuclear staining was used for the survival analysis. Cox regression evaluated the associations of the percentage of positive nuclear staining and OS in multivariable analysis, controlling for known prognostic variables.

Results—In a cohort of 104 mUC patients, higher nuclear staining percentage of ERCC1 [HR=2.7 (1.5, 4.9), $p=0.0007$], RAD51 [HR=5.6 (1.7, 18.3), $p=0.005$], and PAR [with HR=2.2 (1.1, 4.4) $p=0.026$] were associated with worse OS. BRCA1, BRCA2, and PARP-1 were not associated with OS ($p=0.76$, $p=0.38$, $p=0.09$). Higher combined ERCC1 and RAD51 nuclear staining were strongly associated with worse OS ($p=0.005$).

Conclusions—High nuclear staining percentage of ERCC1, RAD51, and PAR, as assessed by IHC, are correlated with worse OS for mUC patients treated with first line platinum combination chemotherapy, supporting the evidence of DNA repair pathways' role in the prognosis of mUC. We have also produced new evidence that RAD51 and PAR may play a role in platinum response. Further prospective studies are required to determine the prognostic or predictive nature of these biomarkers in mUC.

Keywords

Urothelial Carcinoma; Immunohistochemistry; DNA damage; platinum chemotherapy

Introduction

In 2014, about 74,700 new cases of urothelial carcinoma (UC) were diagnosed in the United States, with 30% presenting with muscle-invasive or metastatic disease[1, 2]. Up to 50% of patients with muscle invasive disease will develop metastatic disease[3], the majority of whom are incurable. The median overall survival (OS) of patients with metastatic UC (mUC), who receive platinum-based therapy, is 14 months[4], and mortality rates have not substantially improved in over two decades[5]. In the first line setting, platinum drugs exhibit a response rate (RR) of approximately 50%, and a median progression free survival (PFS) of eight months[6], with select patients obtaining sustained responses[7]. Performance status and visceral metastases are well known clinical prognostic factors in mUC[8]. While significant progress in understanding the genomics of UC and identification of potential therapeutic targets has occurred, development of predictive and prognostic biomarkers for platinum chemotherapy is desperately needed.

Several studies have shown a correlation between genomic alterations in DNA damage pathways and response to platinum therapy in mUC, though none have been prospectively validated[[9, 10].

Platinum agents crosslink interstrand or intrastrand DNA, damaging DNA and leading to cell arrest [11]. Humans have six major DNA repair pathways: nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), non-homologous end-joining (NHEJ), base excision repair (BER), and translation synthesis (TLS)[12]. DNA damage induced by platinum agents is repaired by the NER and HR pathways[13]. It is

hypothesized that response to platinum is increased when these DNA repair pathways are impaired, thus unable to effectively repair DNA damage caused by platinum[13].

We studied six proteins in the HR, NER, and BER pathways. BRCA1, BRCA2, and RAD51 are components of the HR pathway. BRCA1 is a tumor suppressor that helps maintain genomic stability. It identifies DNA damage and signals the BRCA1-associated genome surveillance complex (BASC), which loads BRCA2/RAD51 onto double stranded breaks (DSBs)[14]. Genomic alterations of BRCA1, BRCA2, and RAD51 are associated with poor prognosis in multiple cancers [15–17]. Conversely, alterations in these genes also correlate with increased response rates (RR) to platinum chemotherapy as they may enhance chemotherapy-induced DNA damage [18–21]. The contradictory role of these genes may be attributed to the evolution of the genomic landscape of tumors or to the selective pressure secondary to genomic stressors, including cytotoxic chemotherapy.

PAR and PARP-1 work in the BER repair pathway. PARP-1 attaches to damaged single-stranded DNA (ssDNA), and catalyzes the formation of PAR chains onto itself and surrounding nuclear proteins, which serve as a signal for other DNA repair enzymes to localize to the DNA damage site[22].

ERCC1 functions within the NER pathway by forming a heterodimer with ERCC4 and excising bulky damaged DNA lesion at the 5' [23]. Increased expression of ERCC1 correlates with poor response to cisplatin-based chemotherapy in multiple tumors, including UC [24–27]. The aim of our study was to assess the value of variant expression of ERCC1, RAD51, BRCA1/2, PAR, and PARP-1, with OS through multispectral image analysis.

Methods

We identified a cohort of clinically annotated mUC patients treated with first line platinum chemotherapy. After Institutional Review Board approval, FFPE tissue from transurethral resection or radical cystectomy was obtained from the Department of Pathology from Hospital del Mar (Barcelona, Spain).

Tumor and normal tissue areas were identified. TMA were created by 0.6mm triplicate core biopsies from each tumor, along with sample normal bladder tissue. TMAs were analyzed for ERCC1, RAD51, BRCA1/2, PAR, and PARP-1 expression using brightfield image analysis coupled with CRi image spectroscopy. 5µm FFPE TMA's were deparaffinized in xylene, followed by a graded alcohol rehydration. After antigen retrieval, primary antibody incubation with ERCC1 antisera (1:100, 1 hr, sc-1085; Santa Cruz Biotechnology), RAD51 antisera (1:25 overnight, ab-213; AbCam), BRCA1 antisera (1:100, 1 hr, OP92T; Calbio), BRCA2 antisera (1:400, 1 hr, ab-23887; AbCam), PAR (1:400, 1 hr, MAB-3192; Millipore), and PARP-1 (1:5000, 1 hr, MCA-1522G; Serotec) was performed using a BioGenex i6000 automated staining platform (BioGenex Laboratories Inc., Fremont CA). We detected each antibody using Dako EnVision+ System Polymer-HRP secondary anti-rabbit or anti-mouse antibodies (Dako, Carpinteria, CA). Visualization of each target was accomplished using the EnVision+ 3-amino- 9-ethylcarbazole (AEC) chromogen. The TMAs were subsequently

counterstained with hematoxylin, and dehydrated in a graded series of alcohol. Examples of positive and negative staining are shown in Figure 1.

Tumor was identified by Vectra multispectral imaging system (Caliper Life Sciences, Hopkinton, MA). Using Nuance software, a spectral library was made from single stain controls (i.e. DAB only and Hematoxylin only) for the purposes of unmixing the signals for accurate expression measurements. TMA images were segmented using an in Form algorithm into two tissue categories (urothelium and stroma) and two cell categories (nucleus and cytoplasm) with confirmation by the study pathologist (RTL). Any change in tissue type (tumor, benign) on review of the images was edited in the TMA map list, as well as in the data. The numerical measurements for each nucleus or cytoplasm measured is the mean value of the stain for all the pixels found in the respective nucleus or cytoplasm. Cytoplasm was defined as a two pixel radii around each nucleus found. The optical density (OD) for each cytoplasmic and nuclear compartments tumor cell was identified. The average nuclear OD was then calculated for each patient.

Only nuclear staining level was correlated with OS, as these proteins perform their main function as DNA damage repair in the nucleus. Percent of nuclear staining was dichotomized at the median or previously identified cut-points.

Baseline clinico-pathological characteristics, prognostic factors, and clinical follow up were collected. RR was not included in the analysis because it was not prospectively collected in some patients or not RECIST assessed. OS was defined from the start date of first line chemotherapy for mUC to death or censored on the last date known alive. Median OS was summarized using the Kaplan Meier method. Cox regression evaluated the association of nuclear staining percentage with OS in multivariable analysis, controlling for prognostic variables, Eastern Clinical Oncology Group performance status (ECOG PS) and visceral metastases. All statistical computations were performed using SAS v.9.2 (SAS Institute Inc., Cary, NC, USA) and a two-sided p value <0.05 was considered statistically significant.

Results

We identified 104 mUC patients treated with first line cisplatin combination chemotherapy. Stage at initial diagnosis, metastatic sites, and clinical characteristics at the start of first line chemotherapy are summarized in Table 1. Median follow up was 24 months (range: 1–63 months). ECOG performance status of 1 or 2 and visceral metastases were significant for worse overall survival (HR: 2.3(CI: 1.2–4.1), HR: 2.1(CI: 1.2–3.6), respectively). The level of nuclear DNA repair protein expression among patients was highly variable. The median and range of nuclear staining percentages are as follows: BRCA1 63%(39–91%), BRCA2 27%(7–51%), ERCC1 79%(53–89%), PAR 4.5%(1.1–26%), PARP-1 15%(2.3–57.8%), and RAD51 88%(68–95%). The median and range of cytoplasm staining percentages are as follows: BRCA1 68%(32–93%), BRCA2 60%(26–92%), ERCC1 97%(90–99%), PAR 0.95%(0.4–2.9%), PARP-1 4%(1.3–12.6%), and RAD51 97%(90–99%). Out of 104 patients, clinical and staining data was available in 101 for BRCA1, 104 for BRCA2, 102 for PARP-1, 100 for ERCC1, 103 for PAR, and 101 for RAD51.

Initially, we tested the individual protein expression with OS to determine which proteins strongly correlate with OS. BRCA1, BRCA2 and PARP-1 nuclear staining percentage was categorized into quartiles. There was no difference in OS between the expression quartiles ($p=0.76$, $p=0.38$, $p=0.09$, respectfully).

ERCC1 nuclear staining was dichotomized at the median (79%), as previously reported [28]. Patients with less than 79% of nuclei staining had a significantly longer OS on multivariate analysis [HR=2.7 (1.5, 4.9), $p=0.0007$] (Figure 2).

PAR positive nuclear staining was defined as greater than 1%. Greater than 1% nuclear PAR staining was significantly correlated with a shorter OS on multivariate analysis [HR=2.2 (1.1, 4.4), $p=0.026$] (Figure 3).

RAD51 nuclear staining was dichotomized at 50%, as previously described[29]. Less than 50% nuclear staining was significantly associated with a longer OS on multivariate analysis [HR=5.6 (1.7, 18.3), $p=0.005$] (Figure 4).

RAD51 and BRCA2 both function within the HR pathway, thus we explored if their combined expression was more strongly associated with OS, than their individual expression. BRCA2 was dichotomized at the median and RAD51 was dichotomized at 50%. While there was a trend towards a longer OS in patients with lower RAD51 nuclear staining, it was not statistically significant ($p=0.1$).

We then performed an exploratory analysis on the combined expression of ERCC1 and RAD51, as they individually showed the strongest correlation with OS. ERCC1 and RAD51 was dicotomized as stated above. Combined low nuclear staining of both RAD51 and ERCC1 was significantly associated with a longer OS ($p=0.005$) on multivariate analysis, while increased positive nuclei of both ERCC1 and RAD51 was associated with a shorter OS (Figure 5).

Discussion

Platinum based chemotherapy is standard first-line therapy for mUC. These agents have a RR around 50–60%, with select patients obtaining durable responses [30]. Currently, most treatment recommendations are based on staging and clinical prognostic factors, including ECOG PS and renal function [24, 31–34]. Validated prognostic markers are needed to differentiate patients who may benefit from more aggressive therapy, while predictive biomarkers would likely increase RR, while directing other patients to novel therapies. Recently, multiple publications have shown correlation with gene expression patterns or mutations in DNA repair genes with pathological response[9, 10, 35, 36]. In this study, expression levels of DNA repair protein are associated with OS in mUC patients treated with platinum, indicating that DNA damage repair protein expression may be prognostic and play a role in tumor evolution, adding to existing evidence of platinum prognostic factors.

The accumulation of DNA alterations can initiate apoptosis or stimulate cell proliferation. Platinum-based drugs create bulky DNA adducts generating potentially lethal DNA damage. Removal of these adducts occur through the NER and HR DNA repair pathways[37]. It is

hypothesized that dysfunction of these pathways, through mutations or reduced protein expression, results in an increased RR to platinum chemotherapy in mUC[11].

Reduced nuclear staining of ERCC1, RAD51 and PAR was associated with a longer median OS. These results suggest that reduced efficacy of DNA repair pathways following platinum exposure, may be responsible for increased tumor lethality, as we hypothesized. While other studies have demonstrated decreased staining of ERCC1 and its correlation with response and OS in mUC, to the best of our knowledge, this is the first report correlating expression of RAD51 and PAR in mUC with OS. The strongest OS correlation observed was the combined expression of RAD51 and ERCC1, suggesting that the combined expression of select DNA damage repair proteins may be a more robust prognostic factor than individual proteins.

ERCC1 works in the NER pathway by removing distorted DNA helices created by platinum in a rate limiting fashion [46–48]. In platinum resistant ovarian cancer cell lines, reduction of ERCC1 expression restores platinum sensitivity [49]. Multiple studies have shown a correlation between ERCC1 expression and response to platinum therapy[24, 26, 50]. In UC, decreased ERCC1 expression correlated with platinum response in the metastatic and neoadjuvant setting. In this study, low ERCC1 expression was associated with longer OS, in both univariate and multivariate analysis, confirming previous findings[24]. However, it is still unknown whether ERCC1 is a predictive or prognostic factor. The association of ERCC1 expression with RR, especially in the neoadjuvant setting, indicates that ERCC1 maybe a predictive biomarker, while the correlation with OS suggests that ERCC1 expression may be prognostic, differentiating tumor phenotypes.

We also examined PAR and PARP-1 expression from the BER pathway, to determine their expression correlates with OS [51, 52]. PARP-1 acts in response to SSB by attaching PAR onto DNA along with histones, DNA-dependent protein kinases, and other nuclear molecules to signal DNA damage. PAR opens the chromatin following DNA damage[53], and subsequently signals for additional DNA-repairing enzymes[54–56]. The rate of PAR synthesis has been shown to be directly proportional to the number of damaged DNA sites[57]. In our study, lower nuclear PAR expression, defined as less than 1% of nuclei, was correlated with a longer OS. If we assume that all tumor cells exposed to platinum chemotherapy have the same amount of DNA damage, lower nuclear PAR expression may indicate decreased response to damaged DNA. Thus, our results indicate that lower PAR expression indicates a failure in the DNA damage response, making platinum chemotherapy more efficacious.

RAD51, BRCA1, and BRCA2 repair DSB through the HR pathway. RAD51 is loaded on the ends of DSBs by BRCA1/2, thus stalling replication forks. This is visualized as nuclear expression of RAD51[38, 39]. Increased expression of RAD51 has been associated with increased resistance to platinum therapy in multiple tumors[40, 41], but not in UC. In this study, increased RAD51 was associated with shorter OS, adding evidence to previous findings. Further molecular and clinical studies are required to understand RAD51 role in response to platinum chemotherapy.

BRCA1 and BRCA2 also are key genes in the HR pathway. However, in contrast to RAD51, neither correlated with OS in this study. Decreased expression of both BRCA1 and BRCA2 has been associated with increased cisplatin sensitivity in multiple tumors types [18, 21, 42]. In UC, BRCA1 expression was associated with response to cisplatin based neoadjuvant therapy in a small retrospective study[43]. Although the expression of BRCA1/2 did not correlate with OS in this study, we did observe large variation in the expression levels of BRCA1/2. Low BRCA1/2 expression suggests that PARP-1 inhibitors may be effective in selected mUC patients, as PARP inhibitors are more active in patients with BRCA1/2 alterations[44, 45]. The difference between the correlation of RAD51 and BRCA1/2 with OS indicates that more information is need about the HR pathway's interaction with platinum damaged DNA.

In this study, the most significant association was OS with low expression of ERCC1 and RAD51 and longer OS. In NSCLC cell lines, increased RAD51 expression was associated with reduced platinum efficacy, especially when co-expressed with ERCC1, however its expression did not correlate with resistance to paclitaxel, etoposide, vinorelbine, gemcitabine, 5-FU, or irinotecan [32]. These DNA repair genes may play a prognostic role in other cytotoxic chemotherapy.

The strong correlation of both low ERCC1 and RAD51 expression with prolonged survival may be due to 'synthetic lethality'. Synthetic lethality occurs when one alteration to a gene promotes cell viability, while alteration of two genes leads to cell death. In DNA repair pathways, it is hypothesized that if one pathway is impaired, the cell can still repair damage using other pathways, however if two pathways are impaired, the cell will not be able to overcome the damaging effects of platinum[58–61]. Underperformance of the HR and NER pathways, observed by low expression of RAD51 and ERCC1, had the strongest correlation with platinum response, demonstrating the synthetic lethality hypothesis. It may also be possible that combined expression or mutation of multiple DNA repair markers, including RAD51 and ERCC1 may have stronger prediction power than individual markers. This hypothesis also supports the potential clinical efficacy of PARP-1 inhibitors combined with chemotherapy in mUC. Additional studies are needed to determine how these different alterations interact with each other. An ongoing randomized phase II trial comparing neoadjuvant therapies, gemcitabine/cisplatin v. dose-dense methotrexate, vinblastine, doxorubicin and cisplatin, will evaluate the prognostic and predictive markers between the two therapies, and will provide much need prospective validation of many platinum biomarkers (NCT02177695).

There are some limitations to our study. We had a limited patient population from a single institution and was retrospective, thus selection bias may have played a role. RR was not studied to avoid inconsistencies. Expression was assessed on a TMA, therefore we may have missed evidence of heterogeneity of these markers. Expression was assessed on a TMA constructed from primary tumor tissue and not from the metastatic tissue. Primary tumors undergo expression and mutations evolutionary change as they metastasize. If we analyzed metastatic tumors immediately before therapy, we may have observed different results. Furthermore, our expression analysis used exploratory cut off points that need external validation.

Our study confirms the relevance of DNA repair proteins in mUC patients receiving cisplatin combination chemotherapy, as well as presenting evidence that RAD51 and PAR play a prognostic role in mUC. Decreased expression of PAR, RAD51, and ERCC1 nuclear staining correlated with longer OS in our study of mUC patients treated with first line cisplatin combination chemotherapy. Combined expression of RAD51 and ERCC1 shows promise as a biomarker in mUC. Further studies need to be performed to prospectively validate the role of RAD51, ERCC1, and PAR as predictive in mUC. While expression of BRCA1, BRCA2 and PARP-1 were not significantly associated with OS in this study, additional study of these proteins is warranted given the complex interplay of DNA repair pathways in response to cytotoxic damage.

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Statement of Translation

Deficient DNA damage repair proteins, including ERCC1, RAD51, BRCA1, BRCA2, PAR, and PARP-1, are associated with increased response to cisplatin chemotherapy in multiple different tumors. In metastatic urothelial carcinoma (mUC), ERCC1 expression has also been correlated with overall survival (OS). It is currently unknown how RAD51, BRCA1, BRCA2, PAR, and PARP-1 proteins correlate with overall survival in mUC. In this article, we demonstrate that ERCC1, RAD51, and PAR are strong prognostic biomarkers. We confirmed the association of ERCC1 expression and OS, and established the association between RAD51 and PAR and OS. Our study further establishes the role of DNA repair proteins in mUC, and discusses the need for prospective validation of DNA repair protein expression as prognostic biomarkers.

Clinical Practice Points

- We confirmed previous findings that decreased ERCC1 expression correlates with increased OS metastatic urothelial carcinoma.
- We present new finding that decreased RAD51 and PAR expression correlates with increased OS in metastatic urothelial carcinoma.
- Individual or combined IHC expression of DNA damage response may be used in the future to help determine the prognosis of metastatic urothelial carcinoma patients.

Figure 1. Multispectral analysis of ERCC1 IHC

A. ERCC1 IHC was carried out on a UC TMA. TMA cores were scanned using the CRi Vectra™.

B. The acquired images were analyzed using the CRi InForm software package where InForm identifies tumor cells; creates a false-color image with the identity of individual tumor nuclei (green) and associated cytoplasm (multicolor); and performs image analysis.

Figure 2.
OS by percentage of cells with positive nuclear ERCC1 staining

Figure 3.
OS by percentage of cells with positive nuclear PAR staining

Figure 4.
OS by percentage of cells with positive nuclear RAD51 staining

Figure 5.
OS by percentage of cells with positive nuclear RAD51 and ERCC1 staining

Table 1

Patient Characteristics

	N	% or median (q1, q3)
ECOG PS		
0	40	36%
1–2	64	64%
Visceral disease		
Yes	42	40%
No	62	60%
Pathological stage		
Stage 0 (Ta)	10	10%
Stage I (T1)	6	6%
Stage II (T2)	53	51%
Stage III (T3, T4)	29	28%
Stage IV (L, M)	5	5%
Missing	1	1%
Hemoglobin	102	12.7 (11.4, 13.8)
Platinum Therapy		
Cisplatin		
Carboplatin		