Copper Transporter-CTR1 Expression and Pathological Outcomes in Platinum-treated Muscle-invasive Bladder Cancer Patients

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Abstract. *Background/Aim: Platinum (Pt)-based neoadjuvant chemotherapy (NAC) is the standard-of-care for muscle-invasive bladder cancer (MIBC). However, the survival benefit with NAC is driven by patients with pathological response at cystectomy. Non-responders are subject to adverse effects of Pt, with delay in definitive treatment. Copper transporter receptor 1 (CTR1) plays an important role in Pt uptake and the level of expression may influence Pt sensitivity. We hypothesized that tumor CTR1 expression correlated with pathological outcome. Patients and Methods: We identified matched paraffin-embedded tissues from pre-NAC transurethral bladder tumor resection (TURBT) and post-NAC radical cystectomy (RC) specimens in 47 patients with MIBC who received Pt-based NAC. Tumor and adjacent normal tissues were stained with CTR1 antibody. CTR1 expression was determined through immunohistochemistry by two pathologists blinded to the outcome (0=undetectable; 1+=barely detectable; 2+=moderate; and 3+=intense staining). Pathological response was defined as either down-staging to non-MIBC (≤pT1N0M0) or complete pathological response (pT0). Pathological outcome was compared between the CTR1 expression groups. Results: Forty-three percent of TURBT and 41% of RC specimens expressed a CTR1 score of 3+. Forty-four percent of patients had a pathological response to NAC, and 17% had pT0 disease at cystectomy. In both pre-NAC TURBT and post-NAC RC specimens, a CTR1 expression score of 3+ correlated with pathological response (p=0.0076 and p=0.023, respectively). Conclusion: This is the first study to demonstrate a correlation between CTR1 tumor expression and pathological outcome in Pt-treated MIBC. These findings suggest that CTR1 expression may be a biomarker for Pt sensitivity.*

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Platinum (Pt)-based neoadjuvant chemotherapy (NAC) improves overall survival (OS) in muscle-invasive bladder cancer (MIBC) and is considered the current standard-of-care (1, 2). Pathological response to chemotherapy, defined as either pathological complete response (pT0N0M0) or pathological down-staging to non-muscle invasive disease (NMIBC) (≤pT1N0M0), predicts improved clinical outcome (3, 4). However, response rates to chemotherapy are sub-optimal 29-55% (5, 6). Non-responders are unnecessarily exposed to the adverse effects of therapy with a delay in definitive treatment and a risk of progression.

Despite randomized studies supporting the role of Pt-based NAC, only 12% of eligible patients receive such therapy (1, 7). This has often been attributed to patient/physician preference, advanced age of patients with MIBC, and the presence of multiple medical co-morbidities in this population. At present there is no validated molecular marker or clinical characteristic that adequately predicts response to Pt-based chemotherapy. Hence, there is an urgent unmet need to discover predictive biomarkers of Pt sensitivity, which can guide therapeutic decisions.

In vitro studies have demonstrated that multiple mechanisms contribute to Pt resistance, including decreased drug accumulation, de-toxification by GSH conjugates and other anti-oxidants, increased level of DNA damage repair enzymes, changes in DNA methylation status, inactivation of apoptosis pathway, alteration of membrane protein trafficking as a result of defective organization, and disruption of the cytoskeleton (8-12). While Pt resistance is multi-factorial, reduced intracellular drug accumulation has been one of the

most consistently identified features of cisplatin-resistant cell lines (12), including bladder cancer cell lines (13, 14).

CTR1, an uptake transporter of copper, also plays a significant role as an influx Pt transporter, thereby modulating intratumoral Pt concentration in cancer cell lines (15). In patients with advanced-stage ovarian cancer who underwent optimal cytoreductive surgery (residual masses 1 cm or less) and subsequent Pt-based therapy, lower levels of tumor *CTR1* mRNA levels were associated with poor clinical outcome (16). Similarly in Pt-treated non-small cell lung cancer patients, we demonstrated a significant correlation between post-chemotherapy tumor CTR1 expression with intratumoral Pt concentration and clinical outcome (17, 18). To date, however, there exists no clinical study evaluating the relationship between pre-chemotherapy tumor CTR1 expression levels and pathologic outcome for any tumor type. We hypothesized that tumor CTR1 expression would correlate with pathological outcome in MIBC following Pt-based NAC.

Patients and Methods

Patients. The institutional review board at the Medical College of Wisconsin approved this study. A retrospective review of the surgical pathology database between the years 2007-2014 yielded 47 patients with MIBC, who received Pt-based NAC and subsequently underwent radical cystectomy (RC). Patients' characteristics are given in Table I. None of the patients had previously received chemotherapy for any malignancy before diagnosis of bladder cancer. Secondary to acute renal failure, only one cycle of the planned course of NAC was administered in 5/47 (10%) of patients, all of whom proceeded directly to RC. All patients underwent cystectomy within 8 weeks of completion of NAC. Gemcitabine with cisplatin was the NAC regimen administered in 53% of patients. Methotrexate, vinblastine, Adriamycin and cisplatin were administered in 26% of patients. Carboplatin with gemcitabine was administered in 6% of patients primarily due to pre-existing renal dysfunction. All patients with small cell differentiation (15%) received etoposide-based regimens. A pathologic response was noted in 21/47 (44%) of patients while a pathologic complete response (pT0/pCR) was noted in 8/47 (17%) of patients who received NAC. Long-term clinical outcome data were not available for 30% of patients included in the study, due to either a short follow-up or the patient receiving care elsewhere after cystectomy.

Table I. *Patients' characteristics*.

N	47	
Age, median (range), yr.	68 (42-81)	
Characteristic	N	%
Gender		
Male	25	53
Female	22	47
Ethnicity		
Caucasian	34	72
African-American	8	17
Unknown	5	11
Stage at presentation (clinical)		
T2	34	73
T3	8	17
T4/N1	5	10
Histology		
UC	38	81
UC with small cell differentiation	7	15
UC with squamous cell differentiation	2	4
Neoadjuvant Chemotherapy		
Cis - Etoposide	5	11
Carbo - Etoposide	2	4
MVAC	12	26
Gem - Cis	25	53
Gem - Carbo	3	6
Pathologic response to NAC		
p T0	8	17
p Tis	10	21
p Ta	1	2
p T1	2	4
P T2 or higher	26	56

Ta, Non-invasive papillary carcinoma; Tis, carcinoma *in situ*; T1, tumor invades subepithelial connective tissue; T2, tumor invades superficial muscularis propria; T3a, tumor invades perivesical tissue; T4, tumor invades prostatic stroma, uterus, vagina, pelvic wall, abdominal wall; N, regional lymph node metastasis; UC, urothelial cancer; Gem, gemcitabine; Cis, cisplatin; Carbo, carboplatin; MVAC, methotrexate vinblastine, doxorubicin (adriamycin) cisplatin; NAC, neoadjuvant chemotherapy; p, pathologic.

Tissue specimens. Paraffin-embedded tumor and adjacent normal urothelial tissue were obtained from pre-NAC (TURBT) and post-NAC (RC) specimens in the patients described above and tissue microarrays (TMA) were constructed. Tumor and normal urothelium (when available) were represented by triplicate tissue cores. The tissue of origin and presence or absence of malignancy in the representative sample was confirmed by the pathologists before construction of the TMAs.

Immunohistochemistry for CTR1 expression. Immunohistochemistry (IHC) was performed to determine expression of CTR1 in the TMA. The primary antibody for CTR1 (Novus Biologicals, Littleton, CO, USA) has been validated by us and several others. (19, 20) The antibody was diluted at 1:500 and incubated on tissue sections overnight at 4˚C. Negative controls included tissue from 7 MIBC patients who did not receive NAC and 5 patients who underwent cystectomy for benign conditions such as neurogenic bladder. Expression of CTR1 was scored by assessing the intensity (on a 0–3+ scale) by two pathologists who were blinded to clinical information and outcome: (0=undetectable; 1+= barely detectable staining; 2+=moderate staining; and 3+=intense dark brown staining). The percentage of positive cytoplasmic staining cells was also determined. Specimens with expression score of 1+ or greater demonstrated diffuse cytoplasmic staining (Figure 1). Thus, semi-quantitative scores based on percent of positive cells are not reported separately. Final expression scores reported are an average of triplicates for each sample.

Statistical analysis. The primary objective of the present study was to determine the relationship between CTR1 expression and

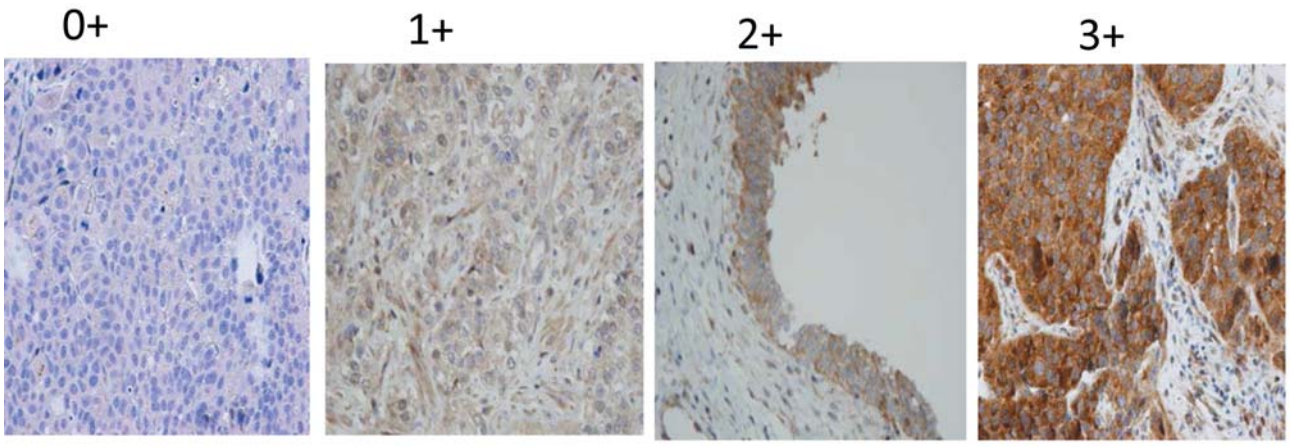

Figure 1. *Differential expression of CTR1 by IHC in bladder cancer. IHC staining intensity (0=no appreciable staining, 1+=barely detectable staining, 2+= moderate staining, 3+=dark brown staining).*

pathologic response to Pt-based NAC in patients with MIBC. Two-sided statistical tests of significance (Chi-square test and Fisher's exact test for cell frequency ≤5) were used to compare the pathological outcome between immunoexpression groups using cut-offs of 1+, 2+ and 3+. Given the sample size and distribution, the main analysis consists of a comparison between CTR1 expression groups that are dichotomized into high-expression (3+) and low-expression (≤2+). Wilcoxon sign-rank test was performed on matched samples to explore the correlation of CTR1 expression in pre-NAC and post-NAC specimens. All statistical analyses were performed using the SAS 9.3 (SAS Institute Inc., Cary, NC, USA) and a *p*-value <0.05 was used for statistical significance.

Results

CTR1 expression in bladder cancer and adjacent normal tissues. In the TMA constructed from TURBT specimens, 3 out of 47 tumor samples were not evaluable due to the absence of cancer in the region of assessment. As TURBT specimens are small and do not sample adjacent normal tissue, we did not stain for CTR1 in the normal tissue. CTR1 expression of 3+ was noted in 19 out of 44 TURBT samples. CTR1 expression of 2+ was noted in 11/44 samples, and only one patient had no CTR1 expression.

In the TMA performed from RC tumor specimens, 13 of 47 tumor samples were not evaluable. Out of these 13 patients, 8 patients had pT0 at cystectomy and 5 did not have an evaluable tumor tissue available. CTR1 expression of 3+ was noted in 14 of 34 RC tumor specimens, while CTR1 expression of 2+ was noted in 12 out of 34 RC tumor specimens, and the remaining samples had an expression score of 1+. CTR1 expression was also examined in RC adjacent normal urothelium, and 8 tissue samples were unavailable for analysis. CTR1 expression score of 3+ was noted in 24 of 39 specimens, while 8 of 39 specimens had CTR1 score of 2+, with the remaining samples scoring 1+.

Association Between CTR1 Expression and Pathological Outcome.

Pre –NAC (TURBT) specimens. Tumor CTR1 expression scores of 3+ in TURBT specimens significantly correlated with down-staging to NMIBC and pCR following NAC (p=0.0076). Table II illustrates pathological outcome based on CTR1 expression scores. In 18 out of 25 (72%) patients with CTR1 expression of ≤2, muscle invasive or more extensive disease was noted in the RC specimen following NAC. Out of the seven patients with small cell differentiation of bladder cancer, 6 had a CTR1 expression of 3+. All patients with small cell differentiation and CTR1 expression of 3+ had pathological responses with NAC.

Post-NAC (cystectomy) specimens. Tumor CTR1 expression scores of 3+ in RC specimens significantly correlated with down-staging with Pt-based chemotherapy (p=0.023). Table III illustrates outcome based on CTR1 expression scores. In 17 out of 20 (85%) of patients with CTR1 expression of ≤2, muscle invasive or more extensive disease was noted following NAC. CTR1 expression in benign urothelium from cystectomy specimens did not correlate with pathological response (p=0.4771).

Effects of Pt NAC on tumor CTR1 expression. We compared tumor CTR1 expression, when available, between matched TURBT and RC specimens (N=30). In 13 patients (43%), CTR1 tumor expression level was maintained following Pt-based chemotherapy. In 12 patients (40%), CTR1 expression was higher in post-NAC *versus* pre-NAC specimens suggesting possible up- regulation of CTR1 in response to chemotherapy and in the remaining 5 patients, CTR1 expression was lower in post-NAC *versus* pre-NAC specimens. The correlation analysis between tumor CTR1

Table II. *Association of pathologic outcomes with CTR1 expression in pre-neoadjuvant chemotherapy tumor specimens (n=44).*

Pre-chemotherapy	Pathologic outcomes			
CTR1 expression	pT0	≤pT1NO MO (Ta, Tis ,T1)	Non-muscle-invasive disease (p T0+ ≤ pT1NO M0)	Muscle-invasive or higher disease (≥pT2)
3+	4	9	13	6
≤2+	3	4	7	18

Table III. *Association of pathologic outcomes with CTR1 expression in post-neoadjuvant chemotherapy tumor specimens (n=34).*

Post-chemotherapy	Pathologic outcomes			
CTR1 expression	pT0	≤pT1NO MO (Ta, Tis ,T1)	Non-muscle-invasive disease (pT0+ ≤ pT1NO M0)	Residual muscle-invasive disease (≥pT2)
3+	0	8	8	6
≤2+	0	3	3	17

expression in matched TURBT and RC specimens was not performed due to the limited number of matched samples available for analysis. We were also unable to assess the CTR1 correlation in normal urothelium before and after NAC due to the limited availability of normal tissue in TURBT samples.

Discussion

Cisplatin also called the "penicillin of cancer," was first described in 1845 as Peyrone's salt. It was initially noted to inhibit binary fission in Escherichia coli (*E. coli*) bacteria and subsequently has remained the mainstay of treatment for various solid tumors, including MIBC (21-24). Over the last decade, advances in molecular biology and genomic (personalized) medicine have driven an exponential increase in the therapeutic options and improved outcome in various malignancies. Despite the high prevalence of actionable mutations identified in MIBC, targeted therapy has yet to benefit the majority of patients with MIBC, and no targeted therapies are currently approved for MIBC. Little has changed in the past three decades regarding management and survival for the approximately 75,000 patients diagnosed with bladder cancer annually in the US (25, 26).

Despite the proven survival benefit of Pt-based NAC for MIBC, this treatment remains under-utilized due to a multitude of factors, including the concern that non-responders are being subject to the adverse effects of NAC with a delay to definitive treatment. Since OS strongly correlates with pathologic down-staging, (27, 28) an opportunity exists to improve outcomes for MIBC by identifying patients most likely to respond to Pt-based NAC with predictive biomarkers. In pre-NAC tumor specimens of patients with complete response, *ERBB2* missense mutations and somatic ERCC2 mutations were associated with an excellent response (29, 30). However, these mutations were noted in only one-third of patients with a complete response. Emprin and survivin also were noted to predict survival in post-NAC specimens (31). However, these markers await validation in a pre-NAC setting and may be cumbersome to perform in a community setting.

In a cohort of 19 patients with MIBC treated with Pt-based NAC, we were able to measure tissue Pt concentrations in fresh-frozen archival bladder specimens and demonstrated that intratumoral Pt concentration significantly correlated with pathologic outcomes (32). Extensive pre-clinical investigation identified CTR1 as an important Pt influx transporter, and more recent studies in ovarian and lung cancer have noted that post-chemotherapy CTR1 expression correlated with clinical outcome (16, 33).

To our knowledge, this is the first study to investigate the relationship between CTR1 expression in pre- and post-NAC specimens and pathologic outcome in MIBC after Pt-based NAC. Our study demonstrated a significant correlation between CTR1 expression in both pre- and post-chemotherapy specimens and pathologic response. Our findings are consistent with reports of other tumor types, including lung and advanced ovarian cancer, where tumor CTR1 expression correlated with tumor responses and clinical outcomes (34).

In pre-clinical studies, cisplatin exposure was found to cause a rapid degradation and down-regulation of CTR1 expression (35, 36). This effect is considered functionally

significant as subsequent uptake of copper and cisplatin was noted to decrease with cisplatin resistance. However, in our samples, chemotherapy did not have a consistent effect on CTR1 expression. All patients who had down-regulation of CTR1 expression following chemotherapy had muscle-invasive disease at the time of cystectomy. Our interpretation of the effect of chemotherapy on CTR1 expression is limited by a small sample. It should be noted that for this analysis, 8 patients who had complete response following NAC and another 7 patients without matching tumor were not included. Further studies addressing the mechanism of CTR1 regulation at the cellular level are warranted.

Out of the 11 patients who were down-staged with NAC to carcinoma *in situ* (CIS), 8 had a CTR1 expression of 3+. Primary Tis (carcinoma *in situ*) accounts for approximately 10% of cases of NMIBC and is associated with a 45% incidence of progression to invasive disease (cT1 or higher) and 17% incidence of progression to MIBC in contemporary series (37). Bladder CIS is managed with local resection followed by a course of weekly intravesical therapy with BCG for 6 weeks. Alternatively, patients may receive intravesical mitomycin C if unable to tolerate BCG. Our data raise the possibility that CIS may be resistant to systemic Pt-based NAC since 73% of CIS cases had 3+ CTR1 staining even after Pt NAC and supports the recommendation for RC for patients with CIS that is refractory to BCG or recurs after treatment. Cancer outcomes after cystectomy for these patients would be needed to confirm this hypothesis.

Our findings suggest that CTR1 immunoexpression could be a potential biomarker for Pt sensitivity and can be assessed in pre-NAC TURBT specimens to inform the risk and benefit profile of NAC for an individual patient. In our cohort, there were 7 patients with small cell differentiation of urothelial cancer. Corroborating the known fact that small-cell cancers tend to be cisplatin-responsive, 6/7 of these patients had a pathological response and a CTR1 expression score of 3+. The one patient (1/7) who did not have a pathological response had a CTR1 expression of 1+.

CTR1 expression has been shown to be regulated at both transcriptional and post-translational levels by various factors including transcription factor specificity protein 1 (Sp1) as well as copper and other heavy metals such as Cd, Zn and Ag (38, 39). Modulation of copper transporter expression may be a novel therapeutic strategy to enhance the efficacy of Pt chemotherapy. Specifically, copper chelators and agents that prevent degradation of CTR1, such as bortezomib, are currently being studied in combination with Pt in a variety of solid tumors known to develop Pt resistance.

The limitations of our study include small sample size, use of archival tissue, unevaluable samples, short follow-up of survival data, and predominantly Caucasian patients. In the current study, we did not correlate progression-free survival with CTR1 expression or pathological outcome, and this was primarily due to a short follow-up or patients receiving care at another facility after RC.

A future prospective study is warranted to validate our findings. A study of the correlation between CTR1 expression *via* mRNA levels in fresh tissue and immunostaining in archival tissue should be considered. We are investigating the relationship between CTR1 expression and intra-tumoral Pt to confirm our hypothesis that CRT1 is important in promoting Pt influx in tumor cells. It is also important to assess the association between tumor CTR1 expression and Pt induced nephrotoxicity and ototoxicity. Lastly, it is possible that copper transporters CTR2, ATP7A, and ATP7B also modulate cisplatin resistance and their role should be investigated as well.

Conclusion

CTR1 expression levels in both pre- and post-chemotherapy MIBC specimens correlated with pathological response to Pt-based NAC. CTR1 expression could be a biomarker of platinum sensitivity, thereby promoting the use of Pt-based NAC in patients with the greatest likelihood of response and avoiding surgical delay and risk of chemotherapy-induced toxicity in likely non-responders. A prospective study is warranted to validate the use of CTR1 as a predictive biomarker of response to Pt chemotherapy.

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