Divergent Biological Response to Neoadjuvant Chemotherapy in Muscle-invasive Bladder Cancer

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Abstract

Purpose: After cisplatin-based neoadjuvant chemotherapy (NAC), 60% of patients with muscle-invasive bladder cancer (MIBC) still have residual invasive disease at radical cystectomy. The NAC-induced biological alterations in these cisplatin-resistant tumors remain largely unstudied.

Experimental Design: Radical cystectomy samples were available for gene expression analysis from 133 patients with residual invasive disease after cisplatin-based NAC, of whom 116 had matched pre-NAC samples. Unsupervised consensus clustering (CC) was performed and the consensus clusters were investigated for their biological and clinical characteristics. Hematoxylin & Eosin and IHC on tissue microarrays were used to confirm tissue sampling and gene expression analysis.

Results: Established molecular subtyping models proved to be inconsistent in their classification of the post-NAC samples. Unsupervised CC revealed four distinct consensus clusters. The CC1-Basal and CC2-Luminal subtypes expressed genes consistent with a basal and a luminal phenotype, respectively, and were similar to the corresponding established pretreatment molecular subtypes. The CC3-Immune subtype had the highest immune activity, including T-cell infiltration and checkpoint molecule expression, but lacked both basal and luminal markers. The CC4-Scar-like subtype expressed genes associated with wound healing/scarring, although the proportion of tumor cell content in this subtype did not differ from the other subtypes. Patients with CC4-Scar-like tumors had the most favorable prognosis.

Conclusions: This study expands our knowledge on MIBC not responding to cisplatin by suggesting molecular subtypes to understand the biology of these tumors. Although these molecular subtypes imply consequences for adjuvant treatments, this ultimately needs to be tested in clinical trials.

Introduction

Muscle-invasive bladder cancer (MIBC) is an aggressive malignancy associated with a 50% mortality rate at 5 years (1, 2). Optimal treatment consists of neoadjuvant cisplatin-based chemotherapy (NAC) followed by radical cystectomy. While NAC has been shown to improve the overall survival rate by 5%–7%, in the real-world setting, only approximately 40% of patients experience a major response, as defined by having no residual MIBC in the radical cystectomy specimen (3). Persistent invasive bladder cancer after NAC is considered chemoresistant (3), and patients with residual cancer at radical cystectomy are at a high risk for disease progression (4).

Gene expression–based molecular subtyping of chemotherapy-naïve MIBC has advanced our understanding of this heterogeneous disease (5–10). Response to NAC or immunotherapy, especially checkpoint blockade, has been shown to correlate with molecular subtype (8, 11–14). However, platinum-based chemotherapy and immunotherapy are highly likely to shift the biological and clinical behavior of a tumor, which would be expected to impact molecular subtyping.

Molecular alterations induced by chemotherapy are poorly characterized, but early evidence suggests that significant alterations occur at the level of both the genome and transcriptome. For example, chemotherapy has been shown to affect the clonal evolution of an individual patient’s tumor (15). An early study on molecular subtypes suggested that NAC may induce subtype switching (8), indicating that NAC induces phenotypical changes on the transcriptome level, or selects one subtype over another in a mixed, heterogeneous tumor.

In this study, we assembled a large dataset of residual invasive bladder cancer after NAC and investigated these tumors using...
Transcriptional Relevance

Despite cisplatin-based neoadjuvant chemotherapy (NAC), 60% of patients with muscle-invasive bladder cancer still have residual invasive disease at radical cystectomy. The NAC-induced biological alterations in these cisplatin-resistant tumors remain largely unstudied. We analyzed gene expression from 116 matched pairs pre- and post-NAC. Hematoxylin & Eosin and IHC were used to confirm tissue sampling and gene expression analysis. Established molecular subtyping models proved to be inconsistent in their classification of the post-NAC samples. Unsupervised consensus clustering revealed four distinct consensus clusters (CC). The CC1-Basal and CC2-Luminal subtypes expressed genes consistent with a basal-like and a luminal-like phenotype, respectively. The CC3-Immune subtype had the highest immune activity, including T-cell infiltration and checkpoint molecule expression, but lacked both basal and luminal markers. The CC4-Scar-like subtype expressed genes associated with wound healing/scarring and was associated with favorable prognosis. Further evaluation is necessary to determine whether this subtyping could impact the selection of second-line treatment.

Materials and Methods

Patients

A consecutive cohort of patients with MIBC from seven institutions was enrolled. All patients were treated with curative intent. After initial diagnosis of MIBC by transurethral bladder tumor resection (TURBT), each patient received at least three cycles of cisplatin-based NAC followed by radical cystectomy. After completion of consensus clustering (CC), it was recognized that 1 patient had been treated with carboplatin-based NAC. Although, this patient was included for CC, it was removed for all subsequent analysis. Briefly, this cohort included 295 TURBT and 144 radical cystectomy specimens. After quality control filtering (see below), 134 patients with post-NAC gene expression profiles were available for analysis (Supplementary Table S1); for subsequent analysis, 133 patients were included after removal of the case treated with carboplatin. In addition, a total of 21 nonneoplastic scar tissue samples were collected from the tumor bed of radical cystectomy specimens with a complete pathologic response (pT0N0).

The human ethics board of each institution approved this study and all patients consented to analysis of their tumor tissues (protocol numbers: Bern, Switzerland, KEK-Be 219/2015; Vancouver, British Columbia, Canada, H09-01628; Southampton, United Kingdom, 10/H0405/99; Seattle, WA, FHCRC: #7116; Amsterdam, the Netherlands, CJMPB-104; UIC Davis, Sacramento, CA, 438935-6; and Rotterdam, the Netherlands, MEC-2014-642). This research was carried out in accordance with the approved guidelines.

Tissue sampling and gene expression profiling

Whole transcriptional analysis was performed on formalin-fixed, paraffin-embedded tumor tissue with GeneChip Human Exon 1.0 ST Array (Affymetrix) in a Clinical Laboratory Improvement Amendments–certified laboratory (16). A total of 134 of 144 post-NAC samples and 21 of 21 scar samples passed quality control, respectively. Microarray data from the corresponding pre-NAC samples were taken from our previously published data set (14). For subsequent analysis, the patient treated with carboplatin was excluded and only 133 samples were used. Microarray data surpassing all quality measures were available for matched pairs of pre- and post-NAC samples from 116 patients, all of whom received cisplatin-based NAC. Microarray data were normalized and genes summarized using single-channel array normalization (17). Array files for these cases are available under GEO accession code GSE124305 (http://www.ncbi.nlm.nih.gov/geo/).

Unsupervised CC

Unsupervised CC was performed using the ConsensusClusterPlus (18) package in R (R Core Team 2014). Low-varying genes were filtered out by selecting the 2,000 genes with the highest median absolute deviation. Bootstrap clustering was performed with 10,000 iterations, using Pearson correlation as the similarity metric, and Ward algorithm for clustering. We focused on two, three, and four cluster solutions that showed a sequential separation of each group.

Gene set enrichment analysis

Gene set enrichment analysis software from the public domain (http://www.broad.mit.edu/gsea/; ref. 19) generated enrichment plots and calculated significance of gene signatures. Genes were ranked with signal-to-noise and a weighted enrichment statistic was used to calculate normalized enrichment scores, P-values, and FDR.

Immunophenotype scoring

The immunophenotyped model was originally developed by Charoentong and colleagues (2017; ref. 20). Using machine learning and the published lists of immune genes, we created a weighted average signature based on the authors’ published model. This model was then applied to our cohort to generate the immunophenotype scores.

Quantification of tumor for tissue sampling

Tumor content was assessed by two expert investigators (R. Seiler and H.Z. Oo) using hematoxylin & Eosin (H&E) sections taken from the same tissue blocks sampled for gene expression analysis. Both investigators were blinded to the CC. For each sample, the percentage of tumor area in relation to the total area of tissue on the section was estimated. The largest coherent tumor focus on the section was measured and within this focus, the percentage of cancer cells in relation to all cells was estimated. For all quantifications, no differences were observed between the estimates of both researchers (P > 0.05).

Tumor regression grading

A subset of post-NAC tumors from three centers were classified according to previously published methods into tumor regression grades (TRG; ref. 21). The TRG were determined previously for a different project and therefore, investigators were blinded to the CC. TRG was defined by histologic estimation of the size of the residual viable tumor relative to the size of the original tumor bed, which was indicated by zones of fibrosis. TRG 3 and TRG 2 were defined as having ≤50% and >50% tumor regression,
Assignment to subtypes using additional models
Assignment of the pre- and post-NAC samples to the molecular subtypes was performed as described previously (14). Briefly, we used five established subtyping models including the Genomic Subtyping Classifier (GSC; ref. 14). The four other models included the University of North Carolina (UNC, Chapel Hill, NC; refs. 7, 9), The Cancer Genome Atlas (TCGA; ref. 6), Lund University (Lund, Lund, Sweden, 2012; ref. 5), and MD Anderson (MDA, Houston, TX; ref. 8).

Construction of tissue microarrays and IHC
The tissue microarrays (TMA) were prepared for IHC analysis by taking a maximum of four samples per patient. Two were harvested from the primary tumor before NAC (TURBT) and, if still present, two from the residual tumor after NAC (radical cystectomy). The freshly cut tissue sections/TMAs were used to determine the protein expression of targets of interest.

In brief, the TMAs were incubated in buffer Tris-EDTA (cell conditioning 1, CC1) at 95°C for 60 minutes, followed by incubation with primary antibody at 37°C for 60 minutes. The following antibodies were used: PPARy (anti-rabbit, clone #24355, Cell Signaling Technology, 1:100 dilution; ref. 22), CD8 (anti-mouse, NCL-L-CD8-4B11, Leica, 1:100 dilution; ref. 22), KRT5/6 (anti-mouse, clone #D5/16B, Millipore, 1:400 dilution), GATA3 (anti-mouse, clone #50-823, Cell Marque, 1:1,600 dilution), Ki67 (anti-mouse, clone #M7240, Dako, 1:100 dilution), and CD44 (anti-rabbit, clone #EPR1013Y, Abcam, 1:100 dilution; ref. 23). The TMAs were washed and incubated with Ventana universal secondary antibody at 37°C for 32 minutes before being visualized using Ventana DAB Map Detection Kit. All IHC staining was performed using the Ventana Discovery Ultra Autostainer.

When assessing the staining intensity, the nuclear (GATA3, Ki67), cytoplasmic (KRT5/6), and membranous/cytoplasmic (PPARy, CD8, and CD44) subcellular localization was taken into consideration. The scoring for GATA3, KRT5/6, PPARy, and CD44 was determined using a 4-point scale system as well as percentage of stained cancer cells. Score 0 represents no staining or absence of any tumor cells, score 1 represents a weak stain, score 2 represents a moderate intensity stain, and score 3 is a strong stain. The overall protein expression was determined by multiplying the intensity score by the percentage. For Ki67, the percentage of stained cancer cells in relation to all cancer cells was considered. For CD8, the score by the percentage. For Ki67, the percentage of stained cancer cells in relation to all cancer cells was considered. For CD8, the score by the percentage. For Ki67, the percentage of stained cancer cells in relation to all cancer cells was considered. For CD8, the score by the percentage.
Figure 1.
Molecular subtyping of pre-NAC TURBT and post-NAC RC bladder cancer samples using the GSC. **A,** Prognosis using the GSC in the pre-NAC setting (n = 114). **B,** Prognosis using the GSC in the post-NAC setting (n = 114). **C,** Distribution of subtype cells in the pre-NAC and post-NAC setting (n = 116). **D,** Comparison of subtype cells using different models (n = 116); samples are ordered according to the GSC model in both the pre- and post-NAC settings. **E,** Concordance of models with respect to the basal/luminal axis (n = 116), where complete model agreement is the darkest blue (or red) and scored as 5 (see Materials and Methods for additional details). The MDA p53-like and Lund-Infiltrated subtypes have zero weighted votes. The gray box indicates a tie between the agreement of the models. The Fleiss' Kappa indicates the concordance across the different models and is significantly higher in pre-NAC when compared with the post-NAC samples.
Taken together, these results highlight that molecular subtyping can provide prognostic information both before and after chemotherapy, but the classification of MIBC using these models will change after exposure to NAC.

Cisplatin-resistant bladder cancer can be classified into four distinct biological subtypes

Current evidence suggests cisplatin-based therapies can drive the evolution of bladder tumors by inducing genomic alterations and/or by selecting for cisplatin-resistant clones within a molecularly heterogeneous tumor (8, 15). We therefore hypothesized that the relative shift in the distribution of subtypes in the post-NAC setting was a consequence of cisplatin-induced or cisplatin-selected changes in the biological and genomic characteristics of the cisplatin-resistant tumors (defined as \( \text{y}_{\text{PT}} \geq 2 \) or \( \text{y}_{\text{PN}} \geq 1 \)).

To test this hypothesis, we employed unsupervised CC with a subset of highly variant genes and identified a robust 4-cluster solution (Supplementary Fig. S3). Importantly, CC using different input gene sets identified essentially the same classes (Supplementary Table S2; Supplementary Fig. S3). An investigation of the biological characteristics of the individual clusters revealed that CC1 and CC2 were consistent with established basal and luminal MIBC molecular subtypes (Fig. 2). The CC1 tumors tended to have higher expression of basal-associated genes (i.e., KRT5, KRT14, and CD44; Fig. 2A and B), while the CC2 tumors had higher expression luminal-associated genes (i.e., KRT20, PPARG, and GATA3; Fig. 2A and C).

To confirm the cluster assignment on the basal and luminal axis, TMA cores were stained with basal (KRT5/6 and CD44) and luminal (GATA3 and PPARy) markers (Fig. 2D–F). Importantly, two different areas of each tumor correlated strongly between matched cores (\( R = 0.91 \), Supplementary Fig. S4). As expected, tumors in CC1 showed higher protein expression of KRT5/6 and CD44 (Fig. 2E), whereas the protein expression of GATA3 and PPARy (Fig. 2F) was low. The opposite pattern was observed for tumors in CC2, which showed high protein expression for GATA3 and PPARy (Fig. 2F) and low expression of basal markers (Fig. 2E). Given these data, we named these subtypes CC1-Basal and CC2-Luminal.

Immune signaling defines the biology of one group of cisplatin-resistant tumors

Higher expression of immune-associated genes (i.e., CTLA4, MPEC1, and CD27) was noted in both the CC1-Basal and CC3, with lower expression in CC4 and little in the CC2-Luminal (Fig. 2A; Supplementary Fig. S5). However, CC3 lacked robust expression of basal or luminal markers that defined CC1-Basal and CC2-Luminal, respectively.

To explore the immunologic activity of CC3, we applied two different immune signatures to the four clusters. First, we found that the expression patterns of genes from the Pan Cancer Immune Profiling Panel (24) were significantly higher in CC3 compared with the other clusters (Fig. 3A; \( P = 0.01 \), NES = 1.88). A second immune signature (immune 190; ref. 22) showed low scores for the CC2-Luminal subtype, with higher scores associated with the CC3 and the CC1-Basal subtype (Fig. 3B).

As T-cell infiltration can significantly impact the tumor microenvironment, we hypothesized that the highest immune scores may be related to T-cell activity. Consistent with this hypothesis, we found higher T-cell and Th cell signature scores in the immune infiltrated clusters, with the highest scores in CC3 and the lowest in CC2-Luminal (Fig. 3C and D). These findings were confirmed with IHC, where particularly high T-cell infiltration was observed in CC3 and almost none in CC2-Luminal (Fig. 3E and F).

Interestingly, high PPARy expression (Fig. 2) and lower immune activity observed in the CC2-Luminal subtype provide support suggesting that PPARy activity can drive an immune-deficient tumor environment in MIBC, even in the post-NAC setting (22). Moreover, CC1-Basal and CC3 not only had higher immune signature scores, but also showed higher expression of the immune suppressor genes PD-L1, PD-L2, and CTLA4, suggesting an immune-suppressive microenvironment (Supplementary Fig. S5).

Finally, we also determined that the enrichment of immune activity observed for CC3 was not exclusively driven by T cells, as this cluster also robustly expressed many chemokines and cytokines (Fig. 3G; \( P = 0.018 \), NES = 1.77). Collectively, due to the lack of basal or luminal marker expression and the higher level of immune cell infiltration and signaling, we named this subtype CC3-Immune.

Wound-healing and scar-like characteristics define the biology of a group of cisplatin-resistant tumors

Many of the highly expressed genes in CC4 were associated with the p53-like signature described in the MDA classifier (refs. 8, 25; Fig. 2A). We also found higher expression of genes that were consistent with wound healing/scarring (MITH1, CN1N1, and DES) or with epithelial-to-mesenchymal transition (EMT; i.e., ZEB1, ZEB2, and VIM), suggesting these patients had response to therapy (Fig. 2A; Supplementary Fig. S6).

Histologic examination of these patient tumors’ revealed significant tumor content, confirming these patients were in fact pathologic nonresponders (Fig. 4A; Supplementary Fig. S7). The CC1-Basal tumors had the largest tumor diameter (Supplementary Fig. S7A and S7B); however, the median percentage of tumor cells in the sampled tumor area was above 60% in all four clusters (Supplementary Fig. S7C). The presence of tumor cells was also confirmed with Ki67 IHC, where marked staining was observed in samples from all four clusters (Fig. 4B and C). Interestingly, a cell-cycle gene signature showed lower proliferation for CC4 tumors, suggesting these tumors may be more quiescent (Fig. 4D). The area of tumor sampled contained a similar proportion of stromal elements between all four CC (Supplementary Fig. S7), nonetheless we found additional gene signatures that supported a scar-like phenotype (Fig. 4E).

When we compared the CC4 tumors with tissue sampled from the tumor bed (nonneoplastic scar tissue in radical cystectomy sample) of patient who had a complete response to NAC (i.e., pT0N0), we found highly consistent gene expression features (Fig. 4F). Using principal component analysis (PCA), we found CC4 tended to separate with true scar tissues and away from CC1-C3 tumors on PC1, but grouped with the tumor samples on PC2 (Fig. 4G, left). On PC3 we observed a distinct separation of the CC1-Basal and CC2-Luminal tumors, with minimal separation of CC3 and CC4 (Fig. 4G, right). Collectively, the three PCs tended to separate the CC1–C3 tumors from the CC4 and scar tissues, which clustered together (Supplementary Fig. S8; Supplementary Table S3). These data indicate that the CC4 and scar tissues share similar genomic profiles.

To determine whether there was an association between the CC and prognosis, we performed a survival analysis. This revealed...
that CC4 has a favorable prognosis compared with CC1–CC3 in the Kaplan–Meier plot (Fig. 4H) but was not associated with lower tumor stages at radical cystectomy (Supplementary Fig. S9). In a univariate analysis, the relative risk in CC4-Scar–like was 2.8 times less than CC2-Luminal (HR = 0.36, P = 0.038), and 3 times less than CC3-Infiltrated (HR = 0.33, P = 0.018) for predicting overall survival.
In a multivariable analysis adjusting for age, stage, and nodal status, CC4 had significantly better survival compared with CC3 (HR = 0.36, P = 0.038) and CC2 (HR = 0.38, P = 0.071).

We have previously reported on histologically determined TRG after NAC in bladder cancer, and established criteria that define partial responders (TRG 2) within the cohort of patients with residual MIBC after NAC (21). We showed that these partial responders had improved overall survival compared with nonresponders (TRG 3). Here we hypothesized the Scar-like subtype may reflect a gene expression correlate of TRG 2. Analysis of a subset of the patient cohort confirmed that TRG 2 was enriched among the Scar-like tumors (Supplementary Table S5; P = 0.026).

**Figure 3.** Enrichment of immune cells and immune signaling pathways in CC3-Immune. A, Pan cancer immune gene enrichment signature (41). B, A generalized immune signature (Immune190; ref. 22). C, T-cell gene signature (9). D, Th cell gene signature (9). E, IHC with anti-CD8 antibodies showing enrichment of T cells. F, Summary of CD8 IHC across the entire TMA. G, A gene enrichment signature for cytokines/chemokines (42).
Figure 4.
Matched comparisons: NAC leads to diverse alterations in MIBC

Pairwise comparison of tumors before and after NAC revealed that approximately 42% (49/116) of tumors remained static with respect to subtype after chemotherapy (Fig. 5). These tumors were basal (11)/claudin-low (16) or luminal (18)/luminal-infiltrated (4) at TURBT and clustered with the CC1-Basal or CC2-Luminal subtypes, respectively, at radical cystectomy (Fig. 5).

The next most conspicuous change after NAC was a loss of luminal and basal marker expression combined with enrichment for immune infiltration (CC3-Immune), which occurred in 34% (39/116) of cases. Of the pre-NAC luminal tumors, which lacked immune infiltration, 32% (13/41) became immune infiltrated post-NAC. We did not observe the CC3-Immune subtype arising out of any one of the pre-NAC subtypes more than another, which may imply that this is a more generalized immune response associated with chemotherapy-induced cell death (26, 27).

In contrast, we found luminal tumors were more likely than basal tumors to become CC4-Scar-like after NAC (12/55 vs. 2/61, \( P = 0.006 \)). In all cases, we observed similar patterns for the other subtyping models used in this study (Supplementary Fig. S8).

Discussion

Until recently, the treatment of MIBC has remained static for years, despite high mortality rates and optimal treatment (1, 2). The era of targeted therapies has up to now bypassed bladder cancer, and cisplatin-based chemotherapy remains a critical component of MIBC therapy. Recent studies have begun to detail NAC responses at a higher resolution, where basal tumors have been shown to benefit the most with respect to overall survival after NAC (14, 28). Specific mutations in ERCC2 and other DNA repair genes also predict for excellent outcomes after NAC (29, 30). In the metastatic setting, immunotherapy has had a major impact on
Classification of Bladder Cancer not Responding to Cisplatin

Figure 6.
Scheme indicating the classes identified after NAC and their phenotype and biological characteristics.

cisplatin-resistant MIBC, with approximately 20% of patients achieving a durable objective response to checkpoint blockade (11, 13). However, alternative second-line treatment options for the other 80% of nonresponding patients are lacking (11, 13, 31, 32). Therefore, an improved understanding of cisplatin-resistant MIBC genomics could help identify patients who may benefit from existing or emerging second-line treatments.

In this study, we investigated a large cohort of matched pre- and post-NAC MIBC samples and developed a novel subtyping scheme to define the genomics of cisplatin-resistant MIBC. In the post-NAC setting, we identified four distinct subtypes that we named CC1-Basal, CC2-Luminal, CC3-Immune, and CC4-Scar-like (Fig. 6). Notably, the CC1-Basal and CC2-Luminal subtypes were highly consistent with the established chemo-naïve basal and luminal subtypes, respectively. Although cluster assignments using different parameters resulted in similar clustering solutions, the silhouette widths for CC3-Immune and CC4-Scar-like were wide, suggesting instability, similar to the previous descriptions of treatment-naïve MIBC as "p53-like" or "infiltrated" (5, 8).

After chemotherapy, it is assumed that even cisplatin-resistant tumors will undergo some degree of apoptosis, resulting in chemokine release and immune cell recruitment to the tumor site (33). In both the CC1-Basal and CC3-Immune, we observed an enrichment of immune infiltration and chemokine/cytokine activity consistent with this occurrence. However, these tumor-infiltrating immune cells are likely in a quiescent state given the lack of tumor clearing and higher expression of immunosuppressive genes including checkpoint markers in both the CC1-Basal and CC3-Immune. Patients in the CC3-Immune cluster had the worst prognosis, which indicates both the greatest need and simultaneously the greatest potential benefit from second-line treatments, such as checkpoint inhibition.

The CC4-Scar-like transcriptome profile was highly similar to nonneoplastic scar tissue. This subtype lacked basal or luminal marker expression, but robustly expressed fibrosis and ECM markers, consistent with the wound-healing response that leads to scarring (34). CC4-Scar-like tumors share similarities with the previously described p53-like subtype (8). However, this post-NAC stromal/p53-like signature was found to be distinct from pre-NAC p53-like tumors (8). Work in kidney has shown that damaged epithelial cells can be induced to a partial EMT state, which coincides with a loss of epithelial marker expression and an arrest of cell-cycle activity (35). Given the tumor content in these samples, we hypothesize the gene expression patterns driving this cluster were not only derived from infiltrating fibroblasts, but also from tumor cells that are shifting to a transcriptional program consistent with fibrosis. Dvorak and colleagues offered the compelling idea that these "scar cancers," referring to tumors that did not arise in a previously healed wound site (scar), but rather were responsible for generating the desmoplastic stroma in which they are embedded (36). This may be most consistent with our CC4-Scar-like cluster, as these samples still have significant tumor content, but a concurrent robust desmoplastic stromal (p53-like) signature. Alternatively, it is possible that the tumor cells themselves have become quiescent in response to chemotherapy, facilitating the infiltration of fibroblasts and subsequent "wound healing" signature observed in these samples. In truth, it may be something closer to the intersection of these two ideas. Biologically, this may be interpreted as a partial response to NAC and, indeed, the patients in this group seem to have improved prognosis. Finally, these findings also corroborate previously reported histologic TRGs (21).

While an obvious pattern for the shift of subtypes before and after NAC did not emerge, we still made several important observations. We characterized one subtype on the basis of a loss of luminal and basal marker expression with concomitant persistence or gain of marked immune infiltrate after chemotherapy, leaving the immune infiltration as the defining feature of this subtype. Immune infiltration is not unique to this subtype, because the pre-NAC GSC luminal-infiltrated tumors are also defined in part by immune infiltration, and both basal and claudin-low subtypes also have significant immune infiltration. In the future, a more granular analysis of pre- and post-NAC...
immune characteristics could focus on qualitative differences in the nature of immune infiltration related to therapy.

We hypothesize that the shift in subtypes after NAC is due to tumor plasticity. However, we acknowledge that some of the shift in subtypes may reflect inherent tumor heterogeneity. Furthermore, it is possible that NAC selects for one subtype over another so that a minor subtype in the TURBT sample becomes the predominant subtype in the radical cystectomy sample. Thomsen and colleagues recently reported on intratumoral heterogeneity in 4 patients with MIBC. Both basal and luminal clones were present in two cases with multifocal disease (1 patient had both MIBC and non-MIBC), but the subtyping was uniform in the other two cases with unifocal MIBC (37). Other reports have suggested that multiple MIBC clones have highly consistent gene expression profiles (38). In the current analyses, there was close correlation in IHC staining between different tumor areas. Furthermore, the shift of approximately 40% of cases to two different subtypes (CC3 and CC4) that were not observed in the pre-NAC setting would imply that the changes are due at least in large part to real changes in biology and not necessarily tumor heterogeneity.

We acknowledge several other potential confounders in our analysis. First, this study is limited by its retrospective nature and the limited sample size of our dataset. In addition, there is a potential for gene expression profiles to vary between TURBT and radical cystectomy samples (39). It is possible that some differences in gene expression are not only due to NAC but may reflect a wound healing reaction in response to TURBT. Finally, although the majority of patients in this study had a single tumor, we cannot exclude that multifocality was present in a small number of cases, and that different tumor sites were sampled at TURBT and radical cystectomy in some cases.

This study advances our understanding of the biology of cisplatin-resistant MIBC and allows us to speculate about the potential implications for postchemo treatment options. Critically, we have shown that there are two unique immune-infiltrated subtypes (CC1-Basa and CC3-Immune). It will be important to determine in future studies whether these two subtypes will respond differentially to subsequent immunotherapy. The unfavorable outcome of CC2-Luminal tumors suggests that targeted therapies may be most important for these tumors (40). On the other hand, further treatment may not be necessary in CC4-Scar-like tumors, which showed a favorable outcome after NAC and radical cystectomy, despite a lack of complete pathologic response. Prospective clinical trials will be necessary to determine the benefit of specific adjuvant or salvage treatments in each tumor class in order for this work to gain clinical utility.

Disclosure of Potential Conflicts of Interest

R. Seiler is an employee of Genomedx and is listed as a coinventor on a patent for a genomic classifier for molecular subtyping in muscle-invasive bladder cancer to be licensed to Genomedx Biosciences. N.Q. Wang owns stock in Genomedx. S.J. Crabb reports receiving commercial research grants from Clovis Oncology and Alexis Pharmaceuticals, and is a consultant/advisory board member for Merck and Roche. E. Davicioni is an employee of and holds ownership interest (including patents) in Genomedx. J.L. Boormans is a consultant/advisory board member for Roche Pharmaceuticals, MSD, and Janssen Pharmaceuticals. P.C. Black reports receiving speakers bureau honoraria from GenoTex and is listed as a co-inventor on a patent regarding a genomic bladder cancer classifier that will be shared between GenoTex, himself, and UBC. No potential conflicts of interest were disclosed by the other authors.

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