METABOLIC, ENDOCRINE, AND GENITOURINARY PATHOBIOLOGY

Amplification of 7p12 Is Associated with Pathologic Nonresponse to Neoadjuvant Chemotherapy in Muscle-Invasive Bladder Cancer

Renate Pichler,^{*} Andrea K. Lindner,^{*} Eva Comprat,[†] Peter Obrist,[‡] Georg Schfer,[§] Tilman Todenhfer,[¶] Wolfgang Horninger,^{*} Zoran Culig,^{*} and Gerold Untergasser^{||**}

From the Departments of Urology,^{*} Pathology,[§] and Internal Medicine V,^{||} Medical University Innsbruck, Innsbruck, Austria; the Department of Pathology,[†] Hpital Tenon, HUEP, Sorbonne University, Paris, France; the Pathology Laboratory Obrist and Brunhuber,[‡] Zams, Austria; the Department of Urology,[¶] Eberhard Karls University, Tbingen, Germany; and the Tyrolean Cancer Research Institute,^{**} Innsbruck, Austria

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Address correspondence to
Renate Pichler, M.D., Ph.D.,
and Zoran Culig, M.D., Section
of Experimental Urology,
Department of Urology, Medi-
cal University Innsbruck,
Anichstra 35, A-6020 Inns-
bruck, Austria. E-mail: renate.pichler@i-med.ac.at or zoran.culig@i-med.ac.at.

Pathologic downstaging (pDS) to neoadjuvant chemotherapy (NAC) is one of the most important predictors of survival in muscle-invasive bladder cancer (MIBC). The use of NAC is limited as pDS is only achieved in 30% to 40% of cases and predictive biomarkers are still lacking. We performed a comprehensive immunomolecular biomarker analysis to characterize the role of immune cells and inhibitory checkpoints, genome-wide frequencies of copy number alterations, mutational signatures in whole exome, and tumor mutational burden in predicting NAC response. Our retrospective study included 23 primary MIBC patients who underwent NAC, followed by radical cystectomy. pDS to NAC was a significant prognostic factor for better recurrence-free survival ($P < 0.001$), with a median time to recurrence of 41.2 versus 5.5 months in nonresponders. DNA damage repair alterations were noticed in 38.1% ($n = 8$), confirming a positive correlation with high tumor mutational burden ($P = 0.007$). Chromosomal 7p12 amplification, including the genes *HUS1*, *EGFR*, *ABCA13*, and *IKZF1*, predicted nonresponse in patients with a sensitivity, a negative predictive value, and a specificity of 71.4%, 87.5%, and 100%, respectively. Total count of CD3⁺ T cells/mm² tumor was a significant predictor of NAC response. In conclusion, 7p12 amplification may predict nonresponse to NAC and worse survival in MIBC. Multicenter, prospective trials with sufficient statistical power may further fortify these findings. (*Am J Pathol* 2020; 190: 442–452; <https://doi.org/10.1016/j.ajpath.2019.10.018>)

In muscle-invasive bladder cancer (MIBC) with clinical stages T2 to T4a, cNOM0, cisplatin-based neoadjuvant chemotherapy (NAC) followed by radical cystectomy (RC) is currently the gold standard in cisplatin-fit patients.¹ The absence of residual cancer (pT0) and pathologic downstaging (pDS) after NAC (ypT0 to ypT1) at RC specimens is a strong predictor of survival.^{2,3} Significantly more patients in the combination-therapy group confirmed a higher rate of complete response compared with RC alone at the time of surgery (38% versus 15%),⁴ resulting in a survival benefit, with an absolute increase of 5% to 8% in 5-year overall survival (OS)^{5,6} and an absolute improvement of disease-free survival of 9%.⁷

Nevertheless, nearly half of patients undergoing RC are cisplatin ineligible to receive NAC on the basis of renal

function status.⁸ Moreover, the utilization of NAC in clinical practice is low, only slightly increasing over time, and influenced, besides clinical factors, by nonclinical parameters related to access to care.^{9,10} Possible causes of the low clinical acceptance for NAC is the time delay of RC in NAC nonresponders, the potential chemotherapy-induced toxicity rate, and the lack of predictive, clinically applicable biomarkers and tools with a high accuracy to identify patients most likely to benefit from NAC.¹¹ Molecular subtyping

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represents the first step forward to a more efficient personalized precision medicine in MIBC, showing the highest benefit of NAC in basal tumors,¹² whereas patients with claudin-low tumors and luminal II subtypes do not benefit from NAC and may be ideal candidates for immunotherapy.^{12,13}

Several mechanisms are involved in cisplatin resistance, such as a decreased intracellular drug accumulation and/or an increased drug efflux, drug inactivation by increased levels of cellular thiols, and alterations in drug target. Another essential way in which cancer cells might become resistant to cisplatin is based on the ability to remove cisplatin-DNA adducts and to repair cisplatin-induced DNA lesions by the presence of certain DNA repair proteins.¹⁴ First trials have shown that DNA-damage repair (DDR) *APOBEC* genetic alterations drive the most common mutations in MIBC,^{15,16} resulting in a better response to cisplatin-based NAC via genomic alterations in DDR genes¹⁷ and a survival benefit (75% 5-year survival) by *APOBEC*-related mutational signature.¹⁵

We performed a comprehensive biomarker analysis in primary MIBC of transurethral resection specimens before NAC on tumor microenvironment (TME), molecular and genetic level, to characterize driver mutations, chromosomal somatic changes, genome-wide frequency of copy number alterations, mutational signatures, tumor mutational burden, and the role of infiltrating immune cells and inhibitory checkpoints in predicting response to NAC.

Materials and Methods

Patients and Cohorts

Consecutive medical records of patients with centrally reviewed primary MIBC (cT2 to cT4a, N0 to N3, M0) diagnosed by transurethral resection from a single-center institution who received neoadjuvant cisplatin-based chemotherapy (gemcitabine, 1000 mg/m², on days 1, 8, and 15; and cisplatin, 70 mg/m², on day 2, every 28 days) followed by radical cystectomy (RC) with extended bilateral pelvic lymphadenectomy were evaluated. The samples were collected consecutively. Repeated imaging was performed in all patients after completed NAC to exclude distant metastases or locally advanced tumor spread. pDS was defined as ypT0 to T1N0 stage, and pathologic nonresponse (pNR) was defined as $\geq$ ypT2N+ on RC specimens. In all patients, the same follow-up investigations and visits after RC were scheduled, according to our institutional practice, as described previously.¹⁸ Patients who were followed up elsewhere postoperatively and patients with evidence of distant metastasis on standard imaging after NAC were excluded. This retrospective observational study was approved by the local Ethics Committee of the Medical University Innsbruck (Innsbruck, Austria; study number 1006/2017).

Whole Exome Sequencing and Calculation of Tumor Mutation Burden

DNA isolation was performed after macrodissection of the tumor with the formalin-fixed, paraffin-embedded (FFPE) tissue extraction kit (Qiagen, Hilden, Germany) on the automated Qiasymphony workstation. After shearing 100 ng genomic DNA with the Covaris M220 sonicator (Covaris Ltd, Brighton, UK), whole exome sequencing libraries were generated by the use of the TruSeqDNA Exome kit (Illumina Inc., San Diego, CA) combined with target enrichment by xGen Lockdown Probes (IDT, Coralville, IA) with an automated workflow on the Hamilton NGS-Star (Hamilton Robotics, Reno, NV); whole exome sequencing libraries were quantified with the Qubit 3.0 (Thermo Fisher Scientific, Waltham, MA) and sequenced on the NextSeq500 (Illumina Inc.) with a mean on-target coverage of 150 $\times$. Generation of FASTQ files and demultiplexing were performed on the BaseSpace Server (Illumina Inc.). The Enrichment App 3.1.0 of the BaseSpace Server was used for rapid alignment and variant detection tool. In fact, reads were aligned against the reference genome using IsaacCall (https://github.com/sequencing/isaac_variant_caller, last accessed February 10, 2019), and structural variants were called using Manta (<https://github.com/Illumina/manta>, last accessed February 10, 2019). Small germline variants (single-nucleotide variants and insertions-deletions) were called using Starling (https://github.com/sequencing/isaac_variant_caller, last accessed March 12, 2019), or somatic variants were called using Pisces (<https://github.com/Illumina/Pisces>, last accessed March 12, 2019). The Variant Calling Assessment Tool VCAT 3.0.0 (Illumina Inc.) was used for the comparison of variant call sets using VCF files as input and the Platinum Genomes version 2016-1.0 (Illumina Inc.) and dbSNP build 147 (<http://www.ncbi.nlm.nih.gov/SNP>, last accessed January 13, 2019) as reference databases. Genomic VCF files were filtered for 10% mutation frequency, EXAC allele frequency <1%, and nonsynonymous or frame-shift mutations to calculate the respective tumor mutational burden (TMB) for each MIBC specimen.

Analysis of Chromosomal Aberrations in Tumor Cell DNA Using Array-Based Comparative Genomic Hybridization

The OncoScan CNV FFPE Assay Kit (Thermo Fisher Scientific) working with 220,000 markers distributed with a resolution of 10 Mb all over the genome was used to monitor copy number variations and loss of heterozygosity. In addition, it covers approximately 900 oncogenes and tumor suppressors with a higher resolution of 50 to 125 kb. Genomic DNA (100 ng) was extracted from macrodissected tumor with the FFPE tissue extraction kit on the automated Qiasymphony Workstation. Chips were hybridized in the Gene Chip Hybridization Oven 645, processed in the Gene Chip Fluidics Station 450, and then scanned in the Gene Chip Scanner 7 G (Affymetrix, Santa Clara, CA; Thermo Fisher Scientific). Scanned data were

imported in the Chromosome Analysis Suite software version 4.0 (ChAS 4.0; Thermo Fisher Scientific) and, thereafter, analyzed for DNA gain or loss (ie, changes in ≥ 25 SNP markers for defining a region with copy number variations). Regions with loss of heterozygosity were defined to be >5 Mb. Copy number gains or losses were detected for small chromosome arms, regions, and whole chromosomes; and they were analyzed in respective cohort analysis (Chromosome Analysis Suite software 3.1). Small genomic regions showing high-level amplifications (defined as $\log_2$ ratio >1), as well as regions indicating homozygous deletions (defined as $\log_2$ ratio <-1), could also be identified. [Supplemental Figure S1](#) shows representative examples of the high-resolution analysis of two MIBC samples (one patient with pDS and one patient with pNR).

Multispectral Analysis of Tumor-Infiltrating Lymphocytes and PD-L1 Expression by Digital Image Analysis

The Opal 7 color immunohistochemistry kit (Perkin Elmer, Waltham, MA) was used for simultaneous detection of multiple biomarkers in FFPE tissue, specifically CD3, CD8, programmed death-ligand 1 (PD-L1), forkhead box P3 (FoxP3), and cytokeratin (CK) 7/20, together with a nuclear counterstain (DAPI) on tumor FFPE sections. Tissue staining was performed with an automated protocol for the respective antibodies in the automated stainer Bond RX (Leica, Vienna, Austria) with the respective Leica antigen retrieval solution. Thereafter, slides were mounted and scanned in the Mantra quantitative pathology workstation (Perkin Elmer) with the Mantra Snap software version 1.0.3 for image acquisition and the inForm Advanced Image Analysis Software version 2.3.0 (Akoya Biosciences Inc., Menlo Park, CA) for trainable image analysis and automatization of multispectral imaging. The immunoscore was performed on areas with no necrosis and high lymphocyte infiltration on whole slide sections of the transurethral resection specimens. Tumor-infiltrating lymphocytes were counted in the tumor area and the respective tumor stroma and calculated in percentage of positive cells/slide. CK7/20-positive tumor cells were scanned for coexpression of PD-L1 and, thereafter, percentage PD-L1 positivity in tumor cells was calculated. Only tumor cells with membranous staining were taken into consideration. Digital pathology with quantification of tumor-infiltrating lymphocytes and PD-L1 is presented in [Figures 1 and 2](#).

Molecular Subtyping by Immunohistochemistry

Intrinsic molecular subtyping, referred to herein as luminal and basal, was performed using mouse monoclonal antibody against human GATA3 (luminal marker) and keratin 5/6 (KRT5/6; basal marker), as described previously.¹⁹ Paraffin-embedded tissue sections were deparaffinized and hydrated in xylene and graded alcohol series. Thereafter, antigen

Figure 1 Immunoscoring of programmed death-ligand 1 (PD-L1) quantification of transurethral resection specimens by multicolor-immunofluorescence and digital image analysis on the Mantra system. PD-L1-negative (**A**) and PD-L1-positive (**B**) muscle-invasive bladder cancer. PD-L1 expression is marked in green, and cytokeratin 7/20 is marked in red. Original magnification, $\times 200$.

retrieval was performed by microwave treatment in citrate buffer (10 mmol/L; pH 6.0) and endogenous peroxidase activity was blocked with 3% H_2O_2 /methanol. Sections were incubated in blocking solution containing 10% bovine calf serum (Dako Cytomation, Glostrup, Denmark) for 45 minutes and then stained for 1 hour with primary antiserum [rabbit monoclonal anti-human CK5 and CK6 Cocktail (Epitomics-Abcam, Cambridge, MA) clone CK5 (EP24) and CK6 (EP67); dilution 1:200]. Moreover, serial sections were incubated with a monoclonal mouse anti-GATA3 (Roche, Unterhaching, Germany; clone L50-823; dilution 1:100). Primary antiserum was detected after incubation with the respective biotinylated secondary antibody (biotinylated horse anti-rabbit IgG or biotinylated rabbit anti-mouse IgG; Vector Laboratories, Burlingame, CA) using the Vectastain Elite ABC Kit (Vector Laboratories) and the FAST DAB Tablet Set (Sigma, Vienna, Austria). Sections were counterstained with Meyer's haemalaun and mounted with Pertex (Histolab, Gothenburg, Sweden). KRT5/6 and GATA3 were semiquantitatively assessed and scored as follows: – indicates negative staining; +, 10% to 25% (weak expression); ++, $>25\%$ to 50% (moderate expression); and +++,

Figure 2 Immunoscoring of tumor-infiltrating lymphocyte (TIL) expression of transurethral resection specimens by multicolor-immunofluorescence and digital image analysis on the Mantra system. Representative stains and quantification showing a cold tumor (few TILs; **A**) and a hot tumor (high TILs; **B**). CD3, CD8, forkhead box P3 (FOXP3), and cytokeratin 7/20 expression is labeled with yellow, pink, blue, and green, respectively. Original magnification, $\times 200$.

>50% (strong expression) positive tumor cells. An overview of all used antibodies is summarized in [Table 1](#).

Statistical Analysis

Statistical analyses were performed using SPSS software version 22 (IBM Corp., Armonk, NY) with two-sided $P < 0.05$ considered as statistically significant. The sensitivity, specificity, positive predictive value, and negative predictive value for *7p12* amplification in predicting NAC response were calculated. Correlations between parameters were assessed with Spearman's ρ correlation coefficient (r^s). Patient immune and molecular characteristics were compared between the two groups (pDS versus pNR) by the *U*-test. OS and recurrence-free survival (RFS) were defined as the time period from the date of primary tumor diagnosis to death of any cause and the detection of local recurrence and distant lymphatic or hematogenous metastases, respectively. The median value of each marker was used as the cutoff point to dichotomize patients into two groups for Kaplan-Meier survival analysis and comparison by the log-rank test. Graphic diagrams were produced with GraphPad

PrismTM6 version 8 (GraphPad Software Inc., La Jolla, CA). All values were presented as means $\pm$ SEM.

Results

Demographic and Clinicopathologic Characteristics and Molecular Subtyping

At diagnosis of primary MIBC, the mean ($\pm$ SD, range) age was 66.5 (± 6.8 , 48 to 76) years, including five females (21.7%) and 18 males (78.3%). Seven patients (30.4%) were defined as pNR, and 16 patients were classified as pDS. [Supplemental Table S1](#) summarizes patient characteristics and response to NAC by clinical tumor-node stage. Transurethral resection before NAC confirmed at least pT2a urothelial carcinoma of the bladder in all patients.

Median follow-up was 18 months (range, 6 to 89 months). There was a significant RFS benefit associated with pDS to cisplatin-based NAC ($P < 0.001$). Concerning pNR, six of seven patients (85.7%) developed tumor recurrence, whereas only two patients with pDS (12.5%) showed recurrent disease (both patients had ypT1 disease). Median time to recurrence was 5.5 months in the pNR group and 41.2 months in the pDS (ypT0 to ypT1) population. Focusing on patients with pDS after NAC, eight patients (50%) showed ypT0 and another eight patients showed ypT1 disease. The median RFS was 64.3 months in the ypT0 group compared with 18 months in patients with ypT1 tumor.

Of 21 MIBCs, 14 (66.7%) were classified as luminal on the basis of positive GATA3 expression and negative KRT5/6 expression: six of seven patients (85.7%) were pNR, and 8 of 14 (57.1%) were pDS patients. However, a clear overlap with coexpression of GATA3 and KRT5/6 was observed in 6 of 21 cases (28.6%; pNR: $n = 1$; pDS: $n = 5$); of them, no KRT5/6-positive case had strong coexpression of GATA3 (staining in >50% of tumor cells). An example of GATA3/KRT5/6 coexpression is illustrated in [Supplemental Figure S2](#). Double-negative tumors for GATA3 and KRT5/6 accounted for 1 of the 21 cases (4.7%) ([Supplemental Figure S3](#)).

TMB Shows No Significant Differences concerning Pathologic Response to NAC and Survival Outcomes

The landscape of the 16 most coding somatic mutations related to nucleotide excision repair genes, chromatin-modifying genes, DNA replication, DNA repair, and driver genes is reported in [Figure 3](#), with *TP53* (45%), *ARID1A/B* (40%), and *KMT2B/C/D/E* (35%) as the three most frequently mutated genes. Of 21 patients, 8 (38.1%) confirmed alterations in DDR genes (*ATM*, *ATRX*, *BRCA1*, *CHEK2*, *ERCC2/4/5*, *FANCA*, *FANCD2*, *FANCG*, *MLH1*, *PALB2*, *PMS1*, and *FANCC*). DDR alterations were significantly associated with a higher TMB (means $\pm$ SD: 15.2 ± 7.7 versus 9.1 ± 1.4 mutations/Mb; $P = 0.007$)

Table 1 Overview of All Antibodies, Providers, Clone Number Concentrations, and Antigen Retrieval Applied for Molecular Subtyping, Multispectral Analysis of Tumor-Infiltrating Lymphocytes, and PD-L1 Expression by Digital Image Analysis

Antibody	Opal, nm	Company	Clone	Dilution	pH
CD3	650	Dako Agilent	Code: A0542	1:250	9
CD8	570	Dako Agilent	C8/144B	1:200	9
CK7	690	Dako Agilent	OV-TL 12/30	1:150	9
CK20	690	Dako Agilent	KS 20.8	1:150	9
FoxP3	620	Abcam	236A/E7	1:150	6
PD-L1 (CD274)	520	Dako Agilent	22C3	1:150	9
DAPI	450	Perkin Elmer		1:20	6
GATA3		Roche Diagnostics	L50–823	1:100	6
KRT5/6		Epitomics	CK5 (EP24) and CK6 (EP67)	1:200	6

CK, cytokeratin; FoxP, forkhead box P; KRT, keratin; PD-L1, programmed death-ligand 1.

(Supplemental Figure S4). The three patients with the highest TMB load had the highest frequency of DDR alterations [Patient 9: TMB 30.14 with seven DDR alterations (*ATM*, *ATRX*, *ERCC2/4*, *FANCD2*, *PMS1*, and *FANCC*); Patient 1: TMB 23.96 with three DDR alterations (*ATRX*, *ERCC5*, and *FANCA*); and Patient 2: TMB 13.51 with two DDR alterations (*BRCA1* and *PALB2*)]. No significant differences in DDR alterations concerning response to NAC (pNR: 25% versus 27.3%) and RFS ($P = 0.271$ by log-rank test) were noticed. However, a trend toward better RFS was observed for patients with DDR alterations compared with DDR-unaltered tumors (Supplemental Figure S4).

Among 21 MIBC patients undergoing NAC, TMB was higher in the pDS group compared with nonresponders, but without statistical significance (median: 10.33 versus 9.41 mutations/Mb; 334.5 versus 317 mutations/exome; $P = 0.201$), with a broad range (6.67 to 30.14 mutations/Mb; 240 to 1052 mutations/exome) (Figure 4A). Evaluating by median TMB, there was no statistically significant difference between patients with high TMB (≥ 10 mutations/Mb) and low TMB (< 10 mutations/Mb) concerning RFS ($P = 0.071$) and OS ($P = 0.122$), respectively (Figure 4B).

High CD3⁺ T-Cell Infiltration Is Associated with Improved Response to NAC

Digital pathology with quantification of tumor-infiltrating lymphocytes and PD-L1 is presented in Figures 1 and 2. Significant differences in mean expression levels of total counts (cells/mm² tumor) based on NAC response were confirmed only for CD3⁺ T cells (pDS versus pNR: 733.3 versus 208.6; $P < 0.001$). In contrast, the levels of CD8⁺ T cells ($P = 0.090$), FOXP3⁺ regulatory T cells (T_{regs} ; $P = 0.103$), CD3/FoxP3 ratio ($P = 0.659$), CD8/FoxP3 ratio ($P = 0.495$), and CD3/CD8 ratio ($P = 0.129$) did not differ significantly in regard to response to NAC, although there was a trend toward higher CD8⁺ T-cell infiltration in patients with NAC response (Figure 5, A–C).

Differences in the localization pattern (stromal and tumoral) of immune cell percentage (CD3, CD8, and FoxP3) are shown in detail in Supplemental Figure S5. Moreover,

mean (range) pretreatment tumor PD-L1 expression was not statistically different in patients with pDS to NAC (42.3%; 0% to 93%) compared with nonresponders (39.4%; 0% to 84%) (Figure 5D). Stratifying tumoral PD-L1 expression in three groups (0%, 1% to 50%, and $> 50\%$), pDS to NAC was seen in 31.2% ($n = 5$), 18.8% ($n = 3$), and 50% ($n = 8$), respectively; and in nonresponders in 20% ($n = 1$), 40% ($n = 2$), and 40% ($n = 2$), respectively. There was no significant association between tumoral PD-L1 expression and TMB ($r^s = -0.136$; 95% CI, -0.566 to 0.350 ; $P = 0.577$). The median TMB was 13.5, 9.5, and 8.7 mutations/Mb in the PD-L1 expression group of 0%, 1% to 50%, and $> 50\%$, respectively (Supplemental Figure S6).

Amplification of 7p12 Is Associated with Nonresponse to NAC

Virtual karyotype analysis confirmed a loss of 17p13, encoding for *TP53*, in five of seven nonresponders (71.4%) and only in 2 of 14 pDS patients with loss of heterozygosity of *TP53* (14.3%) and in eight pDS patients (57.1%). Interestingly, in five of seven samples of nonresponders (71.4%), an amplification of the chromosomal region 7p12 was observed. Focusing on the sequence of interest, 7p12.3-p11.2 (55 315 232- 47 323 074; a 7 990 000-bp fragment), this chromosomal region encompasses several genes that are implicated in the acquisition of resistance to cisplatin, such as *HUS1*, *ABCA13*, epidermal growth factor receptor (*EGFR*), *FIGNL1*, and *IKZF1*. All five MIBC samples of patients with *HUS1/ABCA13/IKZF1/EGFR* amplification showed a strong expression of GATA3 and were negative for basal cytokeratins 5 and 6 on immunohistochemistry analysis (Supplemental Figure S3). The combined up to 8 $\times$ amplification of *HUS1/ABCA13/IKZF1/EGFR* was confirmed in all five of seven nonresponders, whereas none of the 14 pDS patients (0%) had an amplification in one or more of these genes ($P < 0.001$) and 2 of 14 pDS patients showed loss of heterozygosity of *HUS1/ABCA13/IKZF1/EGFR* (Supplemental Figure S7).

The decision rule for nonresponse to NAC in case of *HUS1/ABCA13/IKZF1/EGFR* amplification results in a

Figure 3 Whole exome sequencing on formalin-fixed, paraffin-embedded DNA of transurethral resection bladder cancer specimens to identify non-synonymous mutations/exome. The landscape of the 16 most common coding driver mutations related to the RTK/RAS/phosphatidylinositol 3-kinase, T53/cell cycle, DNA repair, and oxidative damage pathways in our cohort. Samples were subdivided into nonresponders (left section of the graph) and pathologic downstaging (right section of the graph) and sorted by the total number of all mutations in descending order.

sensitivity, a specificity, a positive predictive value, and a negative predictive value of 71.4%, 100%, 100%, and 87.5%, respectively.

Concerning survival outcomes, median time to recurrence after RC was three times shorter in the *HUS1/ABCA13/IKZF1/EGFR* amplification group compared with *HUS1/ABCA13/IKZF1/EGFR* wild-type patients (6.8 versus 18.1 months; $P < 0.001$). The *HUS1/ABCA13/IKZF1/EGFR* amplification was also predictive of poor RFS (hazard ratio, 4.0; 95% CI, 0.16–100.9; $P < 0.001$) and a trend toward poor OS (hazard ratio, 2.57; 95% CI, 0.12–55.2; $P = 0.394$) (Figure 6).

Discussion

As cisplatin induces cell cytotoxicity via interference with transcription and/or DNA replication mechanisms,¹⁴ it is

well known that mutations in DDR genes, especially in *ERCC2*, *ERBB2*, and *ATM/RB1/FANCC*, are associated with increased sensitivity to cisplatin-based chemotherapy in bladder cancer.^{17,20,21} In line with our data, the presence of alterations in DDR-related genes has been shown to correlate with a high overall TMB.^{16,21,22} Although high TMB has been associated with better response to immune checkpoint inhibitors,²³ no statistically significant differences were noticed between TMB and response to NAC or survival outcomes. However, a trend toward better survival was observed for patients with high TMB (≥ 10 mutations/Mb). Moreover, no statistically significant increase in overall mutational or neoantigen load was observed with cisplatin-based NAC when comparing prechemotherapy and post-chemotherapy specimens.²⁴ Thus, it seems that it cannot be proposed that DNA-damaging chemotherapy, such as cisplatin, necessarily leads to increased mutational load in post-treatment tumors. In addition, no significant

Figure 4 Determination of the tumor mutational burden (TMB) by whole exome sequencing. **A:** Association between TMB (mutations/exome) and pathologic response to neoadjuvant chemotherapy. *P* values determined by *U*-test. **B:** Kaplan-Meier survival curves with recurrence-free survival (RFS) and overall survival (OS) in days according to TMB stratified by predefined cutoff points (<10 mutations/Mb indicates low TMB; ≥ 10 mutations/Mb, high TMB). *P* = 0.07 (log-rank test). Data are expressed as means $\pm$ SEM (**A**).

correlation was noticed between TMB and tumoral PD-L1 expression, suggesting that PD-L1 and TMB have been shown to be independent, not correlated, predictive biomarkers.²⁵

In our cohort, the most frequently altered driver genes were *TP53* (45%), *ARID1A/B* (40%), *KMT2B/C/D/E* (35%), and *CDKN1A* (25%), as already described previously,^{15,24} but without significant differences concerning response to NAC. As the overall intratumoral heterogeneity itself, defined as the proportion of mutations that were subclonal in a tumor sample, influences survival outcomes,²⁴ it is necessary to improve and refine a specific predictive mutational signature that can be applied into clinical practice to detect patients who most benefit from NAC.¹⁷

Therefore, somatic copy number alterations were assessed by Affymetrix arrays. Herein, we represent for the first time that a specific amplification on the chromosomal region *7p12* was associated with nonresponse to NAC in MIBC and, consecutively, significantly worse survival outcomes regarding RFS. When analyzing the sequence of interest in detail, *7p12.2-p11.2* prioritized genes that had not previously been reported as regulators of platinum-based chemotherapy response in bladder cancer included *HUS1*, *ABCA13*, *IKZF1*, *EGFR*, and *FIGL1*. Approximately two-thirds of nonresponders to NAC confirmed an amplification of up to eight times of one of the four genes *HUS1*, *ABCA13*, *IKZF1*, or *EGFR*, whereas none of the patients with pDS had amplified *HUS1/ABCA13/IKZF1/EGFR*

Figure 5 Multispectral analysis of tumor-infiltrating lymphocytes and programmed death-ligand 1 (PD-L1) expression of transurethral resection bladder cancer specimens by digital image analysis. Total expression levels (cells/mm² tumor) of immune cell subsets [CD3, CD8, and forkhead box P3 (FoxP3)] and tumoral PD-L1 expression (**A–C**) based on response to neoadjuvant chemotherapy (NAC; **D**). Data are expressed as means $\pm$ SEM (**A–D**). ****P* < 0.001 (*U*-test).

Figure 6 Recurrence-free survival (RFS) and overall survival (OS) by *HUS1/ABCA13/IKZF1/EGFR* amplification status. Amplification in *HUS1/ABCA13/IKZF1/EGFR* predicts statistically significant poorer RFS (A), also with a trend for OS (B), but without statistical significance [$P = 0.394$ (log-rank test)]. **** $P < 0.0001$ (log-rank test).

genes. These findings resulted in a sensitivity and a specificity of 71.4% and 100%, respectively, for nonresponse to NAC on the basis of *HUS1/ABCA13/IKZF1/EGFR* amplification.

The gene encoding for *EGFR/ErbB1* is located on 7p12.3-p12.1.7. Mutations leading to *EGFR* overexpression/amplification play an important role in bladder cancer pathogenesis, with rapid tumor proliferation, aggressive tumor behavior, and poor prognosis.^{26,27} Focusing on The Cancer Genome Atlas cohort, analysis of focal somatic copy number alterations by SNP-array and low pass whole genome also identified *EGFR* as an amplified gene in bladder cancer.^{15,28} Besides breast and gastric cancer, *HER2/ErbB2* overexpression and/or amplification is frequently found across other tumor types of epithelial origin, with a *HER2* positivity rate of 12.4% for bladder cancer.²⁹ As an example, *HER2* amplification has been reported to identify a subset of high-grade non-muscle-invasive bladder cancers with a markedly aggressive behavior, closely related to clinical and pathologic stage, higher risk of recurrence, and progression.³⁰ As protein overexpression (57%) is much more common than *HER2*

gene amplification (7%) in MIBC, and it is well known from breast cancer that only tumors with gene amplification benefit from anti-*HER2* trastuzumab therapy, the role of anti-*HER2* targeted therapy in bladder cancer remains controversial.³¹ Moreover, a further interesting candidate gene, *HUS1*, a member of the multifaceted DDR network to maintain genomic integrity, is involved in the cell cycle arrest and DNA repair in response to DNA damage and regulates response to genotoxic chemotherapies *in vivo*.^{32,33} The gene for *ABCA13*, an ATP binding cassette transporter, is also located on the detected amplified region. ATP binding cassette transporters play a crucial role in the development of resistance by the efflux of anticancer agents outside of cancer cells.³⁴ *ABCA13* overexpression is associated with reduced sensitivity to the alkylating temozolomide-based chemotherapy and decreased RFS in glioblastoma patients.³⁵ Another gene located in the amplified region is Fidgetin-like 1 (*FIGNL1*), being essential in the homologous recombination pathway, being overexpressed in small-cell lung cancer patients and enhancing cell resistance to cisplatin and etoposide.³⁶ Finally, *IKZF1* is known to encode a transcription factor that belongs to the family of Ikaros zinc-finger DNA-binding proteins associated with chromatin remodeling and regulating cell-to-cell interactions. Ikaros plays a crucial function in the hematopoietic system, and *IKZF1* deletions have been associated with the development of acute lymphoblastic leukemia of B-cell progenitors, progression of chronic myeloid leukemia to lymphoid blast crisis, and poor clinical outcome.³⁷

Concerning the TME, it seems that cisplatin-based neo-adjuvant chemotherapy reinforces the antitumor response by activating CD8⁺ effector T cells and decreasing T_{regs} in a dose-dependent manner in MIBC and epithelial ovarian cancer.^{38,39} Thus, an increased ratio of CD8⁺/T_{reg} tumor-infiltrating lymphocyte density in prechemotherapy tissues was strongly associated with response to NAC in MIBC,⁴⁰ suggesting that the immune system plays a critical role in the response to chemotherapy. Moreover, high levels of stromal tumor-infiltrating lymphocytes within the TME correlated significantly with favorable survival rates^{38,41} and an inflammatory tumor phenotype.⁴¹ In line with these findings, a high tumoral and stromal CD3⁺ T-cell density (total count/mm² tumor and percentage) was significantly associated with response to NAC in our study population. A possible explanation for the direct interaction of the cytotoxic effect of cisplatin-based NAC on tumor cells and simultaneous immune effect is that cisplatin enhances the antitumor function of CD8⁺ T cells by decreasing the strong immunosuppressive activity of granulocytic myeloid-derived suppressor cells on TME *in vitro*.⁴² Thus, drugs that can simultaneously target the TME and tumor cells may represent a novel approach to overcome therapeutic resistance. Although the degree of PD-L1 expression correlated positively with the overall density of tumor-infiltrating lymphocytes,⁴⁰ supporting an adaptive immune resistance

mechanism, we and other investigators^{40,43} confirmed no significant differences between PD-L1 expression at transurethral resection of bladder specimens and responses to NAC.

Stromal components within the TME, such as fibroblasts or myofibroblasts, are key players in the tumor immune escape mechanism and may regulate resistance to platinum-based chemotherapy.⁴⁴ Thus, high expression of stromal gene signature was associated with worse disease-specific survival in patients with NAC and RC.⁴⁵ Accumulation of tumor-infiltrating T_{regs} in the TME is also considered to be a negative prognostic factor by inhibiting antitumor response in bladder cancer.⁴⁶ In line with other studies,⁴⁰ we confirmed no significant differences in mean expression levels of total counts (cells/mm² tumor) based on NAC response for FOXP3⁺ regulatory T cells. Interestingly, frequency of T_{regs} (CD4⁺ FoxP3⁺) was significantly higher in NAC naïve patients in comparison to those patients after receiving NAC, suggesting a dynamic steady decrease of T_{reg} frequency during cisplatin-based NAC by a dose- and cycle-dependent manner.³⁹ This fact means that TME-mediated therapeutic resistance may be induced by various soluble factors secreted by tumor or stroma cells. Moreover, immune cells can vary in their activation status within the TME.⁴⁷ The positive stimulatory effect of special NAC protocols on immune response with direct cytotoxic antitumor effect on tumor cells may be an option to increase the beneficial immune effect of chemotherapy in nonresponders.³⁹ Concerning molecular subgroups using two immunohistochemical markers (basal KRT5/6 and luminal GATA3),¹⁹ a high rate of coexpression of GATA3 and KRT5/6 was identified in 6 of 21 cases (28.6%). It is difficult to define the basal subtype in this study group by the sole immunohistochemistry analysis without genomic expression profiling as KRT5/6 staining was not necessarily associated with negative GATA3 staining. Our findings are in line with the recent results presented by Wang et al,⁴⁸ confirming also a GATA3 and KRT5/6 coexpression in 48.35% of MIBC samples, although GATA3 and KRT5/6 demonstrated a significant negative correlation. Moreover, on the basis of the coexpression of immunohistochemical luminal and basal markers, 28% of basal tumors were coclustered with luminal tumors.¹⁹

These preliminary findings must be interpreted with caution because of several major limitations. First, this was a retrospective study with limitation of statistical power and, thus, results must be reevaluated in further multicenter, prospective trials with sufficient statistical power before drawing any final conclusion. Nevertheless, our study population was homogeneous as all included patients underwent the identical cisplatin-based NAC protocol and number of chemotherapy cycles with follow-up visits at the same institution with equal time intervals. Second, the median follow-up was only 18 months, resulting in limitation of statistical power concerning OS analysis. Third, given the limited cohort size, the significant differences on digital

karyotype analysis between the two groups of NAC response should be considered as hypothesis generating for now and further analyses are needed: To corroborate our preliminary results, the validation of a correlation of the expression level of the genes implicated in the 7p12 amplification would be necessary. Unfortunately, the gene expression of these genes was not analyzed as such analysis would need good-quality RNA from tissue blocks and RNA sequencing or real-time PCR analysis. Perhaps, many tissue blocks displayed already strong degradation of RNA, as determined by a quality check on the Agilent Bioanalyzer (Agilent, Santa Clara, CA). In addition, fluorescence *in situ* hybridization analysis that uses a fluorescently labeled, locus-specific indicator DNA probe to assess cells for the identified 7p12 amplification may reconfirm our preliminary results.

In conclusion, DDR gene alterations were significantly associated with a high TMB. Nevertheless, no significant correlation was confirmed between DDR alterations or TMB and response to NAC, although there was a trend toward better survival rates after NAC. More important, chromosomal amplification of the region 7p12.3-p11.2, including the genes *HUS1*, *EGFR*, *ABCA13*, and *IKZF1*, predicted nonresponse to cisplatin-based NAC and worse RFS and OS outcomes after NAC in MIBC compared with patients with wild type. Thus, the detection of an amplified *HUS1/EGFR/ABCA13/IKZF1* region resulted in a sensitivity, a specificity, a positive predictive value, and a negative predictive value of 71.4%, 100%, 100%, and 87.5% for nonresponse to NAC. Given the limited patient number, these significant differences on virtual karyotype analysis must be considered hypothesis generating at the moment and must be validated in further larger independent prospective data sets with further analysis (fluorescence *in situ* hybridization and validation of gene expression levels implicated in the 7p12 amplification).

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.10.018>.

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