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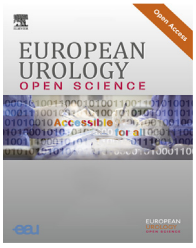

European Association of Urology

Bladder Cancer

Alterations in DNA Damage Repair Genes Before and After Neoadjuvant Cisplatin-based Chemotherapy in Muscle-invasive Bladder Cancer

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Abstract

Background and objective: The role of genetic variants in response to systemic therapy in muscle-invasive bladder cancer (MIBC) is still elusive. We assessed variations in genes involved in DNA damage repair (DDR) before and after cisplatin-based neoadjuvant chemotherapy (NAC) and correlation of alteration patterns with DNA damage and response to therapy.

Methods: Matched tissue from 46 patients with MIBC was investigated via Ion Torrent–based next-generation sequencing using a self-designed panel of 30 DDR genes. Phosphorylation of γ-histone 2A.X (H2AX) was analyzed via immunohistochemistry to evaluate DNA damage. Genetic variants were analyzed along with clinical data and quantitative phospho-H2AX data using the Kaplan-Meier method, Cox regression analysis, and factor analysis of mixed data.

Key findings and limitations: Twenty-five patients (54%) had a response (<pT2 pN0 cM0) to NAC. Responders had more somatic DDR gene variants in preNAC (53 vs 11; $p < 0.001$) and postNAC (51 vs 9; $p = 0.038$) tumor tissue in comparison to nonresponders, as well as significantly greater phosphorylation of H2AX after NAC. *ERCC2* was significantly co-mutated with *REV3L* among responders. Owing to the small cohort, no specific mutation was significantly positively associated with therapy response. However, accumulation of *CDK12*, *NBN*, *MSH3*, *MLH1*, *ATR*, *BRCA1*, *BRCA2*, *REVL3L*, and *SLX4* variants was observed for responders.

Conclusions and clinical implications: Patients with MIBC who responded to cisplatin-based NAC had more somatic DDR gene variants than nonresponders. Moreover, responders exhibited significantly greater DNA damage after NAC.

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Patient summary: Patients with muscle-invasive bladder cancer who have mutations in genes that are involved in repair of DNA damage are more likely to respond to cisplatin-based chemotherapy. Testing to identify these gene mutations could help in selecting the patients who are most likely to benefit from this treatment.

1. Introduction

Cisplatin-based neoadjuvant chemotherapy (NAC) followed by radical cystectomy (RC) is the standard treatment for platinum-fit patients with muscle-invasive bladder cancer (MIBC) [1]. However, up to 50% of patients experience disease progression and eventual lethality [2–4]. No biological tool suitable for routine care that can predict treatment responses, avoid unnecessary overtreatment, identify the patients most likely to benefit from NAC, and help in planning tailored therapy has yet been identified [1,5–7].

Previous studies have shown that patients with alterations in DNA damage repair (DDR) genes are more likely to have tumors that are sensitive to cisplatin, presumably because of a reduction in or even prevention of DDR, which results in higher tumor cell death [8,9]. Prediction of the likelihood of a response could potentially allow for a reduction in the therapy dose and/or the number of cycles, might help in modifying NAC schemes, and could guide alternative strategies such as combination therapy, thereby avoiding overtreatment and unnecessary side effects. In addition, early identification of responders could help in identifying potential candidates for bladder preservation.

The aim of our study was to establish a customized panel for next-generation sequencing (NGS) to investigate the 30 most frequently mutated DDR genes in MIBC according to The Cancer Genome Atlas (TCGA) database. We assessed the correlation of patterns DDR gene variation with clinical outcomes and immunohistochemistry (IHC) results for phosphorylated γ-histone 2A.X (H2AX), a marker of DNA damage, to investigate their impact on therapy response. DNA alterations were analyzed in matched tumor tissues before and after NAC, as well as in nontumorous urothelial tissue if it was available. We hypothesized that the NGS panel could be a useful tool for predicting treatment response. Causal mechanisms regarding the impact of mutations on therapy response have not been investigated owing to a lack of suitable models.

2. Patients and methods

2.1. Patients and samples

This retrospective single-center study involved a cohort of 83 patients with nonmetastatic MIBC treated with cisplatin-based NAC between 2014 and 2020. Only chemotherapy-naïve patients with histologically confirmed MIBC (≥T2) on transurethral resection of bladder tumor (TURBT) were included. We excluded 37 patients because of an incomplete sample set or an ineligible therapy regimen, which left 46 patients for final analysis. A flowchart of the patient selection process is shown in Supplementary Figure 1. PreNAC tissue was obtained via TURBT and postNAC tumor tissue was retrieved during RC. For 27 patients, tumor-free urothelium obtained at RC was also analyzed. Responses were defined according to a combination of pathological downstaging to non-MIBC (<pT2 N0 M0) and a radiological response (Response Evaluation Criteria in Solid Tumors v1.1), defined as a reduction in tumor size of >20% in comparison to the size before NAC [10]. Principal component analysis of the clinical data allowed clustering of patients into an overall response group generated from radiological and pathological responses.

All analyses were performed on formalin-fixed, paraffin-embedded tissue. For patients with a complete response (n = 8), tissue from the previous tumor site was examined in postNAC analysis. The study was approved by the institutional ethics committee (reference 2093/2019).

2.2. Target gene selection and panel design

Sequencing data for 130 MIBC tumors from the TCGA database (http://www.cbioportal.org) were reviewed. The 30 most frequently mutated DDR genes in this cohort were selected as target genes for a customized NGS panel (Fig. 1B).

2.3. Next-generation sequencing

Library preparation was conducted using genomic DNA and the customized Ion AmpliSeq DNA repair gene panel (IAD138776_167; Supplementary Table 3). NGS was performed with Ion Torrent technology (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions [11].

2.4. NGS data processing

Data were filtered and analyzed using Ion Reporter (Torrent Variant Caller 5.10.1.19) according to the manufacturer's instructions (Supplementary Section 2.4). Variants that were detectable in all tissues from an individual patient or/and had a variant allele frequency >40% were considered germline variants [12,13]. All other mutations were considered somatic mutations. Unless stated otherwise, all variants detected were used for further analysis.

2.5. Immunohistochemistry

H2AX phosphorylation (Ser139) was visualized via IHC. Regions of interest were marked by a dedicated uropathologist on slides stained with hematoxylin and eosin. Three different images from three different areas per slide were recorded at 20× magnification for each patient and each time point.

B

DDR gene mutations in BC-TCGA database

DDR gene NGS panel		
Gene	Mutation prevalence %	Significant co-occurrence
ATM	12	POLQ, FANCD2
ERCC2	12	RAD51C, NBS1
BRCA2	8	
MDC1	7	FANCC, MSH3
PRKDC	7	RAD51C
FANCD2	6	SLX4, ATM
REV3L	6	FANCA, FANCM
BRIP1	5	MSH6
CDK12	5	ERCC1, POLQ, FANCM
ERCC6	5	
POLQ	5	ATR, RAD51, MSH3, CDK12
SLX4	5	
BAP1	4	FANCA
FANCA	4	BAP1, REV3L, ERCC1
FANCM	4	ERCC1
KNTC1	4	
MSH3	4	
NBN	4	ERCC2
TOPBP1	4	
ATR	3	
BRCA1	3	
MLH1	3	
PARP1	3	
RAD51C	3	
XRCC1	2.3	
FANCC	1.5	
FANCF	1.5	
MRE11A	1.5	
MSH2	1.5	
MSH6	1.5	

C

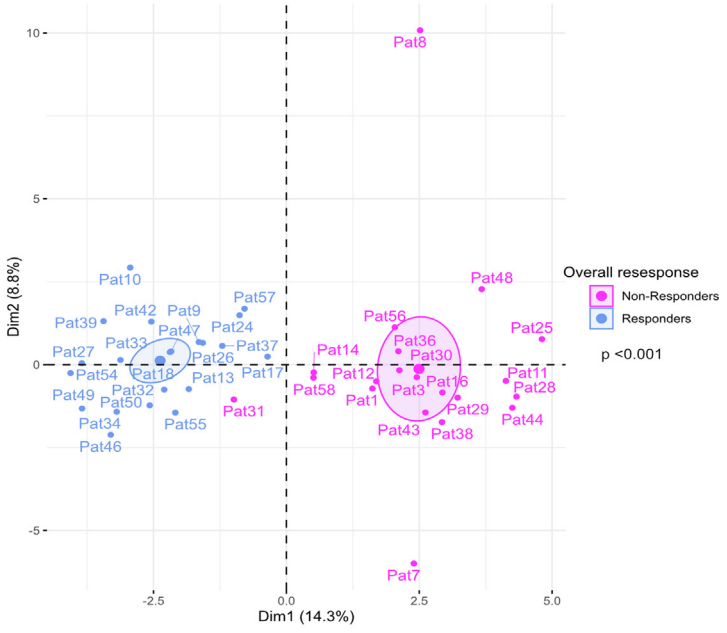


Fig. 1 – (A) Experimental overview. (B) Mutation frequency and mutational co-occurrence ($p < 0.05$) of the 30 most frequently altered DDR genes in MIBC in the TCGA database used for the customized NGS panel. (C) Factor analysis of mixed data map showing clustering of clinical parameters with total response (<pT2a). Ellipses represent a confidence interval of 95%. BC = bladder cancer; DDR = DNA damage repair; IHC = immunohistochemistry; NGS = next-generation sequencing; MIBC = muscle-invasive bladder cancer; Pat = patient; TCGA = The Cancer Genome Atlas; TURB = transurethral resection of the bladder; NAC = neoadjuvant chemotherapy.

2.6. *Statistical evaluation*

Inverted grayscale images derived from RGB IHC images were analyzed using NuSet, a neural network trained to detect nuclei in Python [14]. Semiquantitative H2AX phosphorylation data were subjected to factor analysis of mixed data (FAMD) along with the corresponding genetic variations and clinical data. *maftools* v2.14.0 was used to generate Kaplan-Meier survival curves [15]. Contingency data were analyzed using principal component analysis, univariable Cox regression, Fisher's exact test, and the Mann-Whitney U test. Overall survival (OS) was calculated from the start of NAC to death from disease (coded as an event) or from other causes (coded as censored) within a follow-up period of 36 mo. There was no loss to follow-up. For survival endpoints, we report hazard ratios (HRs) and 95%

confidence intervals (CIs). Statistical significance was set at $p < 0.05$ and all tests were two-sided. All statistical analyses were performed in R v4.2.2 and GraphPad Prism v8. Details are report in the Supplementary material.

3. Results

3.1. Patient demographics and treatment

We included 46 patients treated with NAC for MIBC in our retrospective study (Supplementary Fig. 1). Demographic data are listed in Table 1. Several clinical parameters were significantly associated with OS (Supplementary Table 2). In terms of NAC, 41 patients (89%) received gemcitabine/-cisplatin and five (11%) received methotrexate, vinblastine, doxorubicin, and cisplatin. All patients underwent RC within a maximum of 8 mo (mean 5 mo) from MIBC diagnosis (Fig. 1A). The median number of therapy cycles was four (range 2–6 cycles). Both NAC regimens were equivalent in terms of treatment success ($p = 0.4$; Supplementary Fig. 2A).

Overall, 25 patients were classified as responders and 21 as nonresponders to cisplatin-based NAC (Fig. 1C). FAMD maps revealed that radiologically confirmed progressive disease ($n = 13$), partial response ($n = 14$), and complete response ($n = 8$) correlated with the corresponding pathological response; stable disease ($n = 11$) deviated from the corresponding pathological response, with patients clustered on both sides (Supplementary Fig. 2C). Patients who received two NAC cycles ($n = 9$) or six NAC cycles ($n = 4$) clustered on the nonresponder side, while patients who received four cycles predominantly clustered on the responder side (Supplementary Fig. 2B).

Table 1 – Clinicopathological characteristics of 46 patients with MIBC

Variable	Response[a]		Died of disease
	Yes	No	
Patients, *n* (%)	25 (54)	21 (46)	22 (48)
Median age, yr (range)	58 (47–79)	62 (28–83)	63 (28–83)
De novo MIBC, *n* (%)	22 (58)	16 (42)	13 (34)
Sex, *n* (%)			
Male	21 (57)	16 (43%)	19 (80%)
Female	4 (44)	5 (56%)	3 (33%)
Clinical stage at baseline, *n* (%)			
cT2	12 (60)	8 (40)	11 (55)
cT3	13 (52)	12 (48)	11 (44)
cT4	0	1 (100)	0
cN0	17 (59)	12 (41)	12 (43)
cN1	6 (60)	4 (2)	6 (60)
cN2	2 (29)	5 (71)	3 (43)
cM0	25 (54)	21 (46)	22 (50)
Lymphovascular invasion, *n* (%)	7 (50)	7 (50)	8 (57)
Carcinoma in situ, *n* (%)	8 (50)	8 (50)	10 (62)
Therapy, *n* (%)			
Gemcitabine + cisplatin	22 (54)	19 (46)	21 (51)
MVAC	3 (60)	2 (40)	1 (20)
2 cycles	2 (22)	7 (78)	9 (100)
3 cycles	3 (50)	3 (50%)	3 (50)
4 cycles	18 (72)	7 (28)	5 (20)
5 cycles	1 (50)	1 (50)	0
6 cycles	1 (25)	3 (75)	1 (25)
Response to NAC (<pT2), *n* (%)			
Yes	25 (100)	0	4 (8)
No	0	21 (100)	14 (30)

MIBC = muscle-invasive bladder, NAC = neoadjuvant chemotherapy; MVAC = methotrexate, vinblastine, doxorubicin, and cisplatin.
[a] Percentages refer to rows for response versus no response.

3.2. Accumulation of somatic DDR gene alterations in the responder group

A total of 316 valid gene alterations were detected in the cohort of 46 patients. The numbers of total variants and somatic variants per patient, gene, locus, and time point are listed in Figure 2A. Overall, the responder group was characterized by higher numbers of alterations in preNAC tissue (116 vs 92 variants; $p = 0.3$) and postNAC tissue (124 vs 88 variants; $p = 0.072$) versus the nonresponder group, but the differences were not significant (Fig. 2B). However, there were significant differences between the responder and nonresponder groups in the number of somatic mutations in preNAC tissue (53 vs 11; $p < 0.001$) and postNAC tissue (51 vs 9; $p = 0.038$; Fig. 2B). Before NAC, more than 80 different variants were detected in each group. Overall, the groups shared only 14 variants in preNAC tissue and 16 variants in postNAC tissue. There were 108 alterations that were detected in preNAC tissue, postNAC tissue, and tumor-free bladder tissue, and therefore most likely represent germline variants.

There was no difference in the total number of alterations per patient, but the responder group had significantly more somatic mutations in preNAC tissue ($p < 0.001$) and in postNAC tissue ($p = 0.038$; Supplementary Fig. 3A,B). Three responders only had genetic variants in preNAC tissue.

3.3. Distinct pattern of DDR gene alterations in the responder group

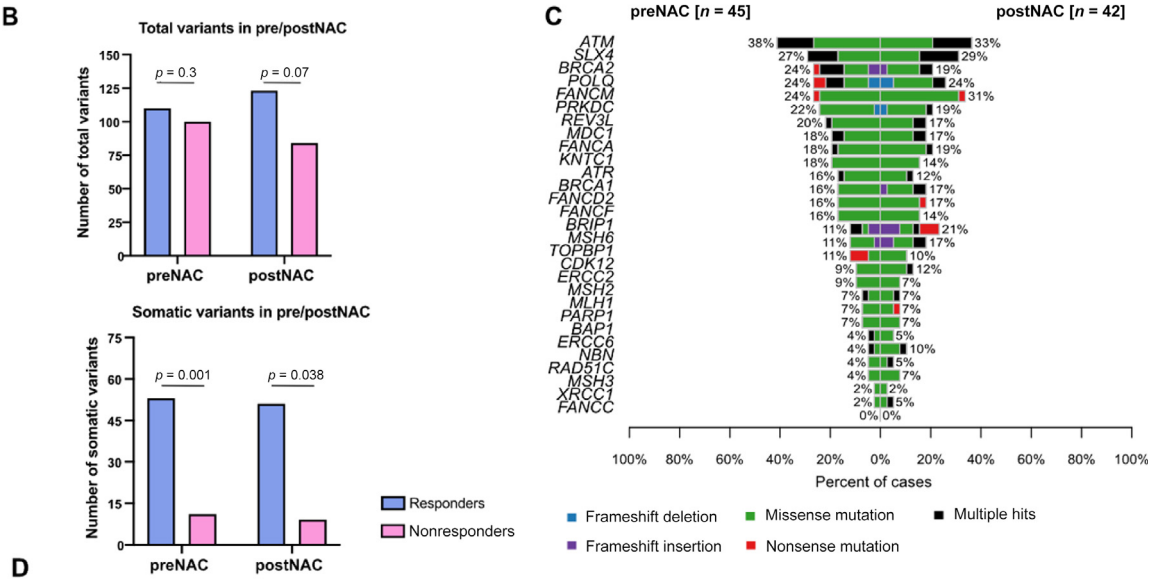
Different types of genetic alteration were observed for DDR genes, with missense mutations being the most common at both time points (Fig. 2C and Supplementary Fig. 5A,B). The alteration map combined with clinical data did not reveal any correlation between variants and NAC type, number of cycles, or pT stage (Fig. 3A,B). However, the alteration frequency for each gene differed between the responder and nonresponder groups (Fig. 2D and Supplementary Fig. 3C).

In our cohort, *ATM, SLX4*, *BRCA2* (preNAC), and *FANCM* (postNAC) had the most variants in both groups (Fig. 2D, Supplementary Fig. 3C, and Supplementary Fig. 5). Overall, variants in *CDK12*, *MSH3,* and *NBN* were only detectable in the responder group at both time points, whereas *FANCF* alterations were exclusively observed in postNAC tissue in the nonresponder group (Fig. 2D). In addition, variants of *BRCA1* (5 vs 1), *ATR* (6 vs 2), *REV3L* (7 vs3), *ATM* (15 vs 10), *BRCA2* (10 vs 5), and *SLX4* (11 vs 7) were more frequent in the responder group (at least 50% more). Comparison of the two time points revealed that *NBN* (2 vs 5) and *MSH6* (2 vs 7) had the greatest increases in the number of somatic variants in the responder group (Fig. 2D). The highest loss of somatic mutations was observed for *BRCA2* (7 vs 3), all detectable only in the responder group (Supplementary Fig. 3C). Overall, the highest enrichment in alteration load was observed for *ERCC6* (4% vs 10%) and *BRIP1* (11% vs 21%; Fig. 2C and Supplementary Fig. 3C).

A

		Number of total variants														
		Patients			Genes				Loci							
		Total	Mutated	%	Total	%	Specific	%	Total	%	Different	%	Specific	%	Only	%
preNAC	R	25	24	52	30	100	4	13	116	56	89	43	67	32	41	35
	NR	21	21	46	25	83	1	3	92	44	81	39	60	29	10	11
	Total	46	45		30				208				127		51	
postNAC	R	25	22	48	28	93	5	17	124	58	106	50	87	41	12	10
	NR	21	21	46	24	80	1	3	88	42	70	33	45	21	3	3
	Total	46	43		30				212				132		15	

		Number of somatic variants														
		Patients			Genes				Loci							
		Total	Mutated	%	Total	%	Specific	%	Total	%	Different	%	Specific	%	Only	%
preNAC	R	25	15	33	23	77	12	40	53	83	43	67	40	63	0	0
	NR	21	8	17	11	37	1	3	11	17	9	14	8	13	0	0
	Total	46	23		30				64				127			
postNAC	R	25	8	17	25	83	19	63	51	85	51	85	51	85	0	0
	NR	21	8	17	6	20	1	3	9	15	9	15	9	15	0	0
	Total	46	16		30				60				60			



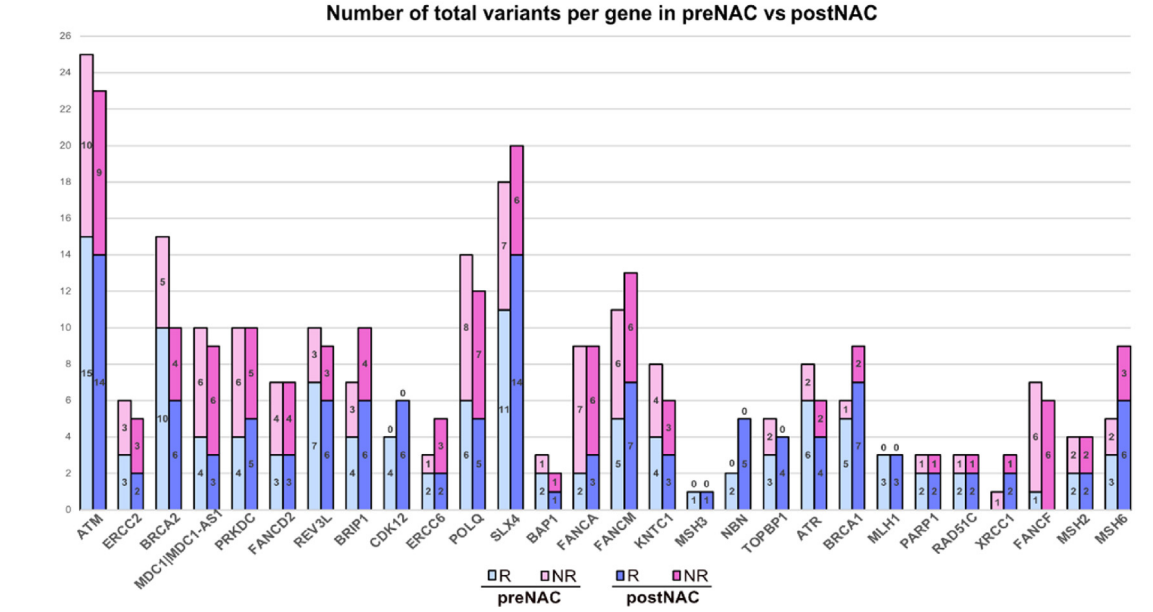


Fig. 2 – Distribution of variants in the course of therapy. (A) Summary table of total variants and somatic variants per gene and locus by patient and time point. "Specific" = exclusive to the responder/nonresponder group; "Different" = number of different variants; "Only" = exclusive to this time point. (B) Numbers of total and somatic variants preNAC and postNAC in the two groups. (C) Type of variants per gene and percentage among all variants preNAC and postNAC. (D) Numbers of all variants per gene preNAC and postNAC in the two groups, with genes listed according to The Cancer Genome Atlas mutation frequency. R = responder; NR = nonresponder; NAC = neoadjuvant chemotherapy.

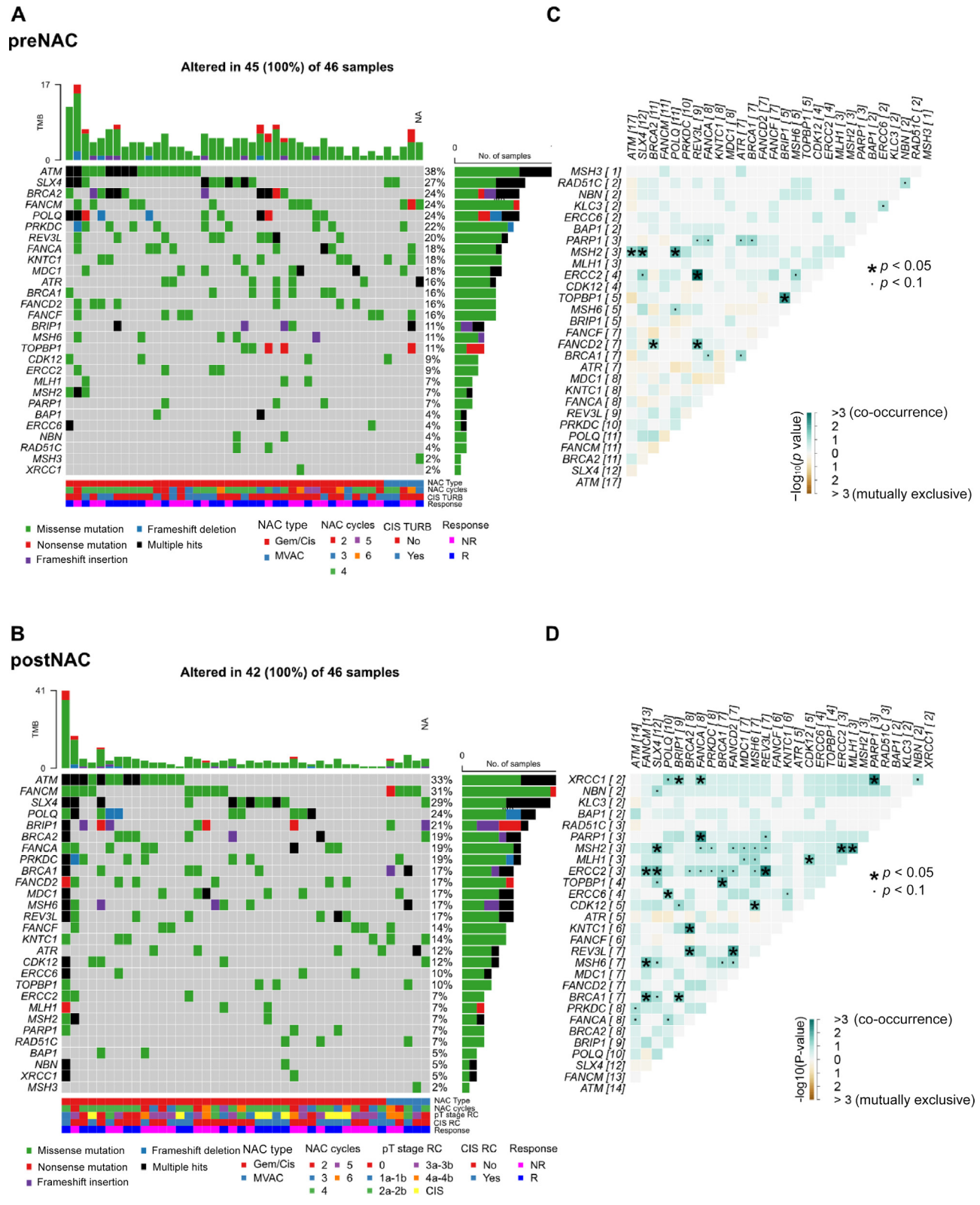


Fig. 3 – Gene alteration map and co-occurrence plot. The gene alteration landscape was correlated to (A) preNAC and (B) postNAC clinical data. Each column represents a tumor and each row represents a gene. Genes are sorted in descending order of mutation frequency. (C,D) Mutational co-occurrence plotted for all variations for both time points. Statistical significance is indicated by an asterisk, and the levels of co-occurrence (green) and mutual exclusivity (yellow) are highlighted in ascending intensity (log_{10}). TMB = tumor mutational burden; NA = not altered; CIS = carcinoma in situ; TURB = transurethral resection of the bladder; RC = radical cystectomy; Gem/Cis = gemcitabine/cisplatin; MVAC = methotrexate, vinblastine, doxorubicin, and cisplatin.

We observed significant co-mutation of some genes (Fig. 3C,D). Interestingly, all three responders who had *ERCC2* mutations also had *REV3L* alterations, which reached statistical significance ($p < 0.001$).

3.4. Accumulation of loss-of-function variants in the responder group

We assessed the effect of each variant on protein function by combining different prediction models, as previously described [16,17]. The impact patterns of all variants in Rs and NRs were plotted for every gene and patient in tumors before and after NAC (Supplementary Fig. 4). The number of benign variants (not impairing), likely pathogenic variants (likely impairing), and variants of uncertain impact was higher for the responder group than for the nonresponder group (Fig. 4A). In addition, the difference in the number of variants between preNAC and postNAC tissue was significantly higher in the responder than in the nonresponder group (81 vs19 variants; $p < 0.001$; Fig. 4B).

3.5. Association of FANCF and PRKDC variants with lower probability of survival

No variants were positively associated with therapy response or survival (Supplementary Table 1). However, *FANCF* and *PRKDC* alterations were significantly associated with higher risk of nonresponse and therefore lower probability of survival (Supplementary Fig. 6A,B). Supplementary Figure 4 shows that alterations in these two genes were enriched in the nonresponder group at both time points. Kaplan-Mayer curves and altered loci are shown in Supplementary Figure 6C. The hazard ratios for OS were 4.4 for *FANCF* ($p = 0.003$) and 2.8 for *PRKDC* ($p = 0.049$).

3.6. Greater DNA damage after cisplatin-based NAC in the responder group

Phosphorylation of H2AX was assessed by quantifying the number of positive nuclei and their staining intensity on IHC (Fig. 5A). The number of positive nuclei was significantly downregulated in postNAC tumors in the nonresponder group, while upregulation was observed in the responder group, indicating more DNA damage ($p = 0.043$; Fig. 5B,C). Changes in phosphorylation between preNAC and postNAC tissue are highlighted in the heatmap in Figure 5C. There was no correlation between any specific gene mutation and the percentage or staining intensity of phospho-H2AX-positive nuclei.

4. Discussion

Identification of biomarkers for predicting the response to NAC in MIBC is a major unmet need, and one of the biggest challenges in bladder cancer research. The mutational landscape of bladder cancer has been described as highly heterogeneous, with various disrupted genes and pathways. In contrast to other tumor entities, establishment of a prognostic or predictive marker for clinical use in bladder cancer might be more complicated. DDR genes might represent a promising target in this setting. Alterations in DDR genes are associated with a greater mutational burden and better clinical outcomes in several platinum-treated cancers, including metastatic urothelial carcinoma, in which DDR gene mutations are highly prevalent [18,19]. To the best of our knowledge, this is the first study to compare the mutational load of DDR genes before and after NAC, investigate correlation of all the variants detected with response to therapy and histological DNA damage, and examine which of these variants are enriched, depleted, or acquired de novo during therapy. Our study generated several interesting findings.

In the responder group we found a significantly higher number of overall alterations that were exclusive to the preNAC setting (41 vs10) and of somatic variations (40 vs 8) in comparison to the nonresponder group. In addition, the responder group had a distinct genetic alteration signature and greater phosphorylation of H2AX after cisplatin-based NAC. Accumulation of DDR gene mutations renders tumors more susceptible to DNA damage, and somatic variants in responders might also have an effect on the response to therapy. This is supported by the fact that 38% of the gene variants in the responder group changed after NAC, while only 9% of the mutations in the nonresponder group differed between preNAC and postNAC sequencing.

ATM (38%), *SLX4* (27%), and *BRCA2* (24%) were the genes most frequently altered, and we did not detect a single *FANCC* variant. In previous studies, genetic alterations in *ATM*, *BRCA1/2*, *FANCC*, *ERCC2*, and *RAD51* were detected in approximately 15–25% of urothelial carcinomas and were associated with response to cisplatin-based NAC [9,19–21].

Despite having a similar cohort in terms of number (30–60 patients), sex distribution, age, race, and the number of responders, the gene mutations and their frequency in our cohort differ slightly from those in previous studies by Liu et al [23] ($n = 30$; whole-exome sequencing [WES]), Plimack et al [9] ($n = 34$; targeted exome sequencing [TES]), Liu ($n = 48$, WES), Desai et al [8] ($n = 48$; Illumina NGS), van Allen et al [22] ($n = 50$; WES), and Teo et al [19] ($n = 60$; TES). Reasons might be the similarly small study cohorts, preselection of patients, and the sequencing technique used, as WES and Illumina-based NGS have higher sensitivity for detection of gene variants than Ion Torrent–based NGS.

Another promising DDR gene for therapy response in MIBC is *ERCC2,* which has the highest missense mutation frequency in MIBC of up to 20% [9,6,8,24]. In two similarly sized cohorts, *ERCC2* mutations were associated with a higher overall mutational burden and accumulation among responders [6,8,9,22,24]. In our cohort, *ERCC2* was altered in three responders (6.5%) and in three nonresponders. All variants were considered as likely pathogenic because of predicted disruption of protein function. Interestingly, we found a significant co-mutation with *REV3L* among responders, but not nonresponders. None of these responders experienced a complete response to therapy. The reason for our discordant data could be the preselection of patients by van Allen et al [22], with sequencing only performed for patients with an extreme response to cisplatin-based combination NAC. The authors did not investigate whether nonresponders also harbored *ERCC2* variants. In their follow-up study, Liu et al [24] found that nonresponders also had *ERCC2* alterations in the same (peri-) helicase regions as

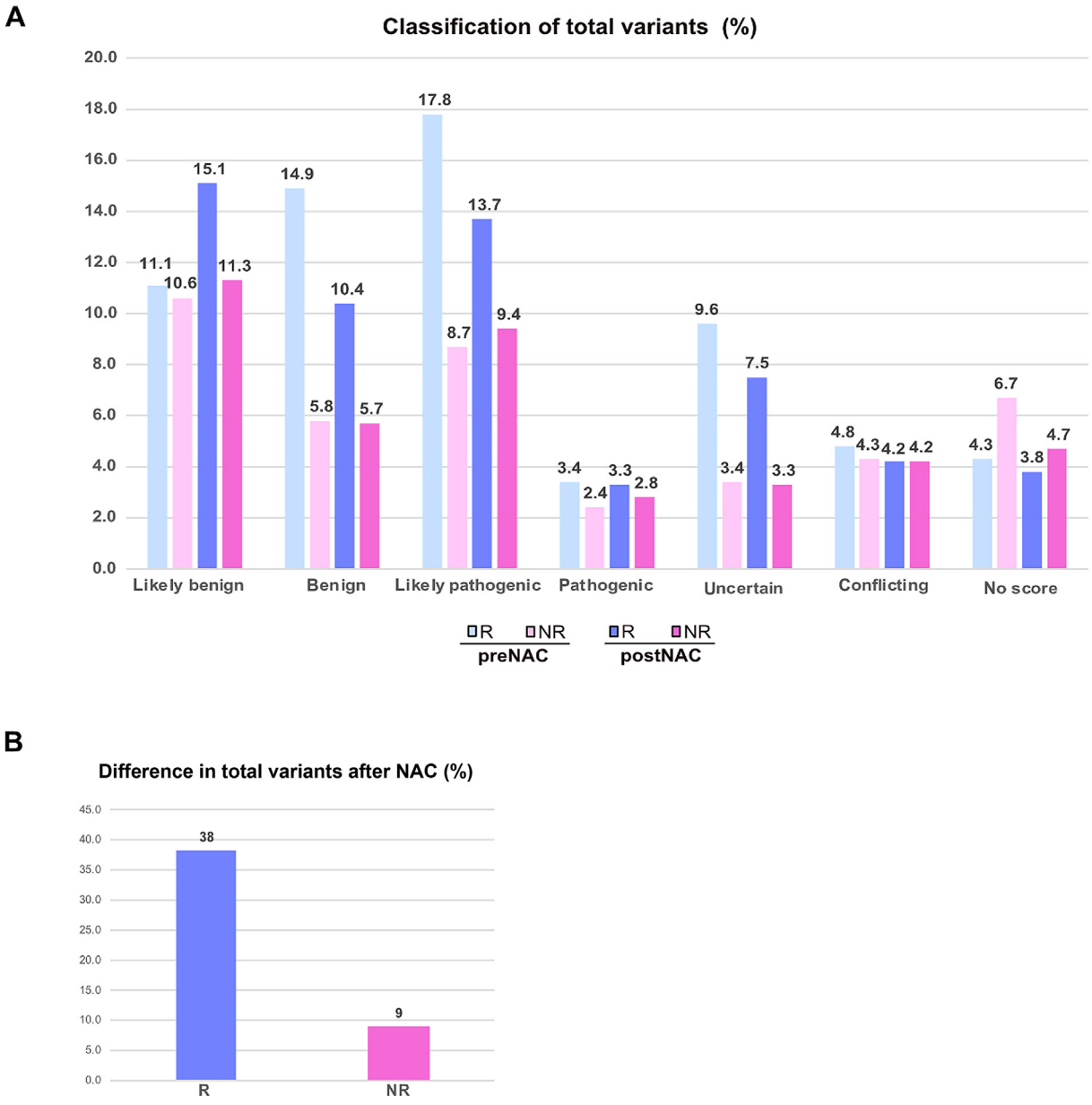


Fig. 4 – Classification and distribution of variants. (A) Classification of all variants according to their predicted impact on gene function via a combination of SIFT (https://sift.bii.a-star.edu.sg/), PolyPhen-2 scores, and the ClinVar database in a seven-tier system. The distribution of the variant classes is shown by response group and sequencing time point. (B) Difference in total variants between preNAC and postNAC tissue in the responder and nonresponder groups. Both bar charts show the proportion of the respective variants as a percentage of all variants. NAC = neoadjuvant chemotherapy; NR = nonresponder; R = responder.

observed for responders. Interestingly, two responders in our cohort had no detectable *ERCC2* variants in postNAC tumors, and there was no loss among nonresponders. This might indicate that these mutations (p.Glu120Lys and p. Glu570Gln) render tumors more sensitive to cisplatin. However, none of the mutations we identified have been described in MIBC to date [6,8,9,22].

In a retrospective study, Liu et al [23] sequenced samples from 30 patients before and after NAC via WES. Despite the similar cohort, we cannot compare their results to our data, as the authors selected for nonresponders, and the genes most frequently mutated in their cohort were not included in our NGS panel (*TP53* 68%, *KMT2D* 28%, *CDKN2A* 23%, *ARID1A* 22%, *PIK3CA* 22%, and *RB1* 20%). In agreement with Liu et al, we found a similar global level of copy number alterations in matched tumor samples. Only a few patients had a significant difference in mutation pattern between preNAC and postNAC tissue. Similar to Liu et al [23], we did not find an increase in overall mutational load after cisplatin-based chemotherapy, but instead observed a decrease in variants after NAC. One notable exception was patient 47, who had three likely pathogenic gene alterations (*ATM* c.2119T>C and c.6067G>C, *BRCA2* c.8503T>C) before cisplatin treatment, but 41 mutations in postNAC tumor tissue. The patient is a responder and was still alive at 36-mo follow-up.

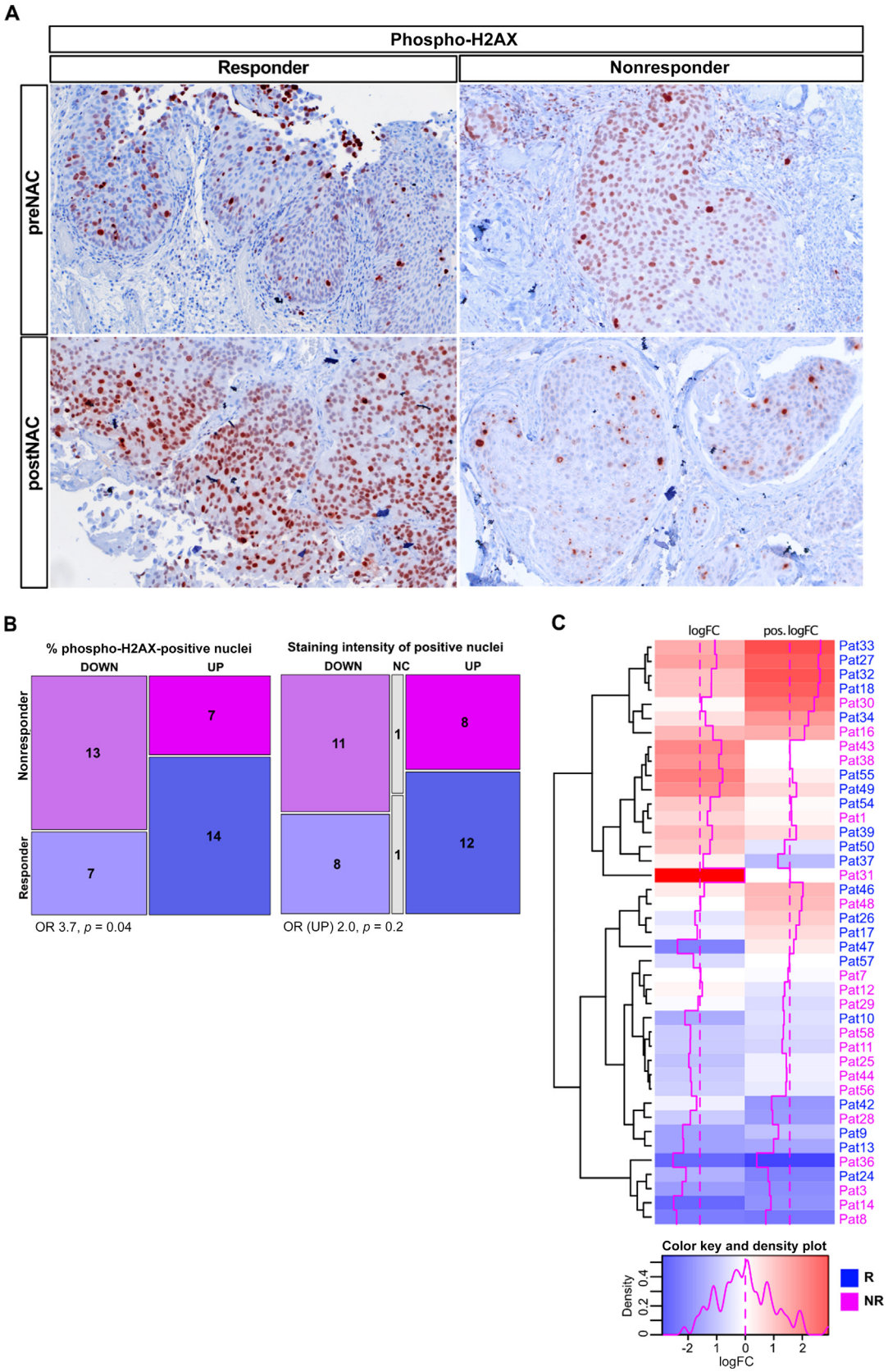


Fig. 5 – Phosphorylation analysis of H2AX. (A) Examples of phospho-H2AX histology in matched tissue before and after NAC in one responder and one nonresponder. (B) Mosaic plot of changes in the regulation of H2AX phosphorylation in tumors after NAC in comparison to the corresponding preNAC tissue, calculated as the percentage of positive nuclei relative to all nuclei and the intensity of phosphorylation. (C) Heatmap of log fold changes in the intensity of phosphorylation and in positive nuclei after NAC. DOWN = downregulation; FC = fold change; H2AX = γ-histone 2A.X; NAC = neoadjuvant chemotherapy; NR = nonresponder; OR = odds ratio; pos. = positive; R = responder; UP = upregulation.

Overall, we did not identify any genetic variants that were positively associated with response to therapy or survival. Of 63 pathogenic or likely pathogenic variants predicted to affect protein function, only 36 were detectable both before and after NAC, 27 new pathogenic or likely pathogenic variants were found in postNAC tumor tissue. However, it is not clear if the mutations arose during advanced carcinogenesis, were caused by the mutagenic effect of cisplatin, or were just missed in TURB samples because of the heterogeneity of tumor tissue. The high proportion of lost pathogenic or likely pathogenic variants in comparison to the total number of variants could indicate that cells with these defects in DNA repair are more vulnerable to cisplatin. Although the impact of germline variants and variants of uncertain significance is still elusive, there is increasing evidence that these alterations affect the prevalence of tumor development and the response to therapy in ovary, colon, and renal cell carcinoma [25–27].

Liu et al [23] described a cisplatin-specific mutation signature in postNAC tissue. In our cohort we were able to sequence tumor-free tissue obtained at RC from 27 patients (Supplementary Table 4). Interestingly, the mutation patterns were quite similar to those in the corresponding tumor tissue. Thus, we did not detect a cisplatin-induced mutation signature among the 30 genes analyzed in our NGS panel. The similar mutation patterns among the matched samples is an indication of the field effect and highlights that apparently healthy bladder tissue is not suitable as a control.

Comparison of different mutation studies in MIBC suggests that the method used for sequencing may have an impact and that WES, NGS, and targeted exon sequencing can result in discordant sequencing data. Possible reasons could be the sensitivity of the technique for detection of mutations, the coverage of primers, and the homogeneity of the gene sequences in a panel, as well as the big-data analytical packages used to process the raw data. NGS can facilitate personalized and customized therapy, such as in lung cancer (*EGFR*) and colorectal (*KRAS*) cancer [28]. However, establishment of a suitable NGS panel to guide therapy decisions in MIBC faces several obstacles, such as the lack of key mutations and the heterogeneity of tumors, among others. Another limitation of our study is its retrospective nature, so analysis of blood for more reliable determination of germline mutations was not possible.

There is still an urgent need for alternative therapies and markers of response to the limited therapies available for MIBC. However, we need to consider that there may not be a single marker or key mutation as in other tumor types, and that response to therapy in MIBC is multifactorial. Nevertheless, combined results from even small cohort studies are an important tool for understanding the biology of MIBC and can contribute to risk stratification of patients.

5. Conclusions

In our MIBC cohort, we detected more somatic variants in DDR genes in preNAC and postNAC tissue for patients who responded to cisplatin-based NAC than for patients without a response. Responders also had differential distribution of certain gene alterations, a greater difference in variants between the two sampling points, and greater DNA damage after NAC. It is therefore possible that in addition to germline mutations, accumulation of somatic DDR gene mutations increases sensitivity to cisplatin-based chemotherapy in MIBC. Future prospective studies are needed to validate the most promising results.

Author contributions: Ursula Lemberger had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Hassler.
Acquisition of data: Lemberger, Ernhofer.
Analysis and interpretation of data: Lemberger, Krieger.
Drafting of the manuscript: Lemberger, Krieger.
Critical revision of the manuscript for important intellectual content: Englinger, Oszwald, Laukhtina, Compérat, Shariat.
Statistical analysis: Krieger, Lemberger.
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Other (patient enrolment): Bruchbacher, Haitl, Oszwald, Compérat.

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Appendix A. Supplementary data

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