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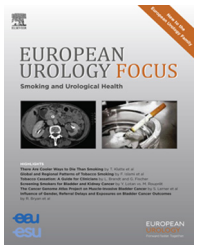

Bladder Cancer

Low T-cell Receptor Diversity, High Somatic Mutation Burden, and High Neoantigen Load as Predictors of Clinical Outcome in Muscle-invasive Bladder Cancer

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Abstract

Background: The success of cancer immunotherapies has highlighted the potent ability of local adaptive immune responses to eradicate cancer cells by targeting neoantigens generated by somatic alterations. However, how these factors interact to drive the natural history of muscle-invasive bladder cancer (MIBC) is not well understood.
Objective: To investigate the role of immune regulation in MIBC disease progression, we performed massively parallel T-cell receptor (TCR) sequencing of tumor-infiltrating T cells (TILs), in silico neoantigen prediction from exome sequences, and expression analysis of immune-related genes.
Design, setting, and participants: We analyzed 38 MIBC tissues from patients who underwent definitive surgery with a minimum clinical follow-up of 2 yr.
Outcome measurements and statistical analysis: Recurrence-free survival (RFS) was determined. TCR diversity was quantified using Simpson's diversity index. The main analyses involved the Mann-Whitney *U* test, Kaplan-Meier survival analysis, and Cox proportional hazards models.
Results and limitations: Low TCRβ chain diversity, correlating with oligoclonal TIL expansion, was significantly correlated with longer RFS, even after adjustment for pathologic tumor stage, node status, and receipt of adjuvant chemotherapy (hazard ratio 2.67, 95% confidence interval 1.08–6.60; $p = 0.03$). Patients with both a high number of neoantigens and low TCRβ diversity had longer RFS compared to those with fewer neoantigens and high TCR diversity (median RFS 275 vs 30 wk; $p = 0.03$). Higher expression of immune cytolytic genes was associated with nonrecurrence among patients with low TCR diversity or fewer neoantigens. Limitations include the sample size and the inability to distinguish $CD8^+$ and $CD4^+$ T cells using TCR sequencing.
Conclusions: These findings are the first to show that detailed tumor immune-genome analysis at definitive surgery can identify molecular patterns of antitumor immune response contributing to better clinical outcomes in MIBC.
Patient summary: We discovered that clonal expansion of certain T cells in tumor tissue, possibly targeting cancer-specific antigens, contributes to prevention of bladder cancer recurrence.

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1. Introduction

Muscle-invasive bladder cancer (MIBC), unlike the more favorable non–muscle-invasive form (NMIBC), has dismal prognosis and a high recurrence rate. Extensive genomic profiling of urothelial cancer suggests that MIBC is driven by distinct genetic differences from NMIBC [1–5]. In addition, urothelial carcinoma has a high somatic mutation burden, third in frequency only after lung cancer and melanoma [2]. Recent evidence indicates that somatic mutations are the basis for the generation of potential neoantigens recognized by antitumor T lymphocytes [6,7], which can be effectively rescued from an exhausted state by immune checkpoint blockade therapy in certain patients [8]. Tumor-infiltrating lymphocytes (TILs) carry prognostic significance in urothelial cancer, with high CD8 expression in tumors associated with prolonged disease-free and overall survival [9]. Given these results, our goal is to improve understanding of the role of immune regulation in MIBC disease progression. More specifically, we investigated how potential neoantigens derived from somatic nonsynonymous mutations influence the diversity of TILs in MIBC to impact recurrence status and the length of recurrence-free survival (RFS).

Next-generation sequencing of expressed T-cell receptor (TCR) transcripts allows for quantification of T-cell diversity [10,11] via calculation of Simpson's diversity index (DI) from sequencing reads, with low DI indicating oligoclonal T-cell expansion [12]. To comprehensively address correlations between TCR diversity and the mutational neoantigen landscape of a tumor, we performed TCR sequencing, whole-exome sequencing with neoepitope prediction, and immune-related gene expression analysis for 38 chemotherapy-naïve MIBC tumors from patients with clinical follow-up of at least 2 years. To the best of our knowledge, this in-depth level of integration of the immune and genetic landscape with clinical outcomes has not been investigated in MIBC.

2. Materials and methods

2.1. Study design

Under a protocol approved by the University of Chicago institutional review board (#15550B), tumor tissue and available adjacent normal frozen tissue were collected from chemotherapy-naïve MIBC patients at the time of definitive surgical resection of the primary tumor. Since the great majority of MIBC patients who experience recurrence (denoted Rec) will do so within 2 yr, we defined nonrecurrent (NR) patients as those who were known to be recurrence-free at least 2 yr after definitive surgery (median length of follow-up for NR patients 262 ± 59.3 wk; Supplementary Table 1). We collected all samples with at least 2 yr of clinical follow-up from the tissue bank organized by one urologist (G.S.). Of these, we included only tissues that yielded high-quality and sufficient DNA and RNA after extraction, resulting in a total of 38 cases.

2.2. TCR sequencing

cDNA libraries were prepared using previously described methods (Supplementary material) [11]. The Bowtie2 aligner was used to map sequencing reads to the human TCR loci and a previously described algorithm was applied to decompose the V-(D)-J regions of the CDR3s [11]. Simpson's DI was calculated to quantify the clonality of the TCR α and β repertoires [12] according to $\mathrm{DI} = \left[\sum_{K}^{i=1} \frac{n_i(i-1)}{N(N-1)}\right]^{-1}$, where N is the total number of sequences, n_i is the number of sequences belonging to the ith clonotype, and K is the total number of clonotypes.

2.3. Assessment of T-cell infiltration

Tumor sections were stained with hematoxylin and eosin, and T-cell infiltration into tumor and stroma was qualitatively assessed by an attending genitourinary pathologist (T.A.) who was blinded to the TCR DI scores. All tumors were given a holistic numeric score in comparison to other samples. After TCR sequencing, a subset of samples with high and low TCR diversity were also subjected to immunohistochemistry (IHC) for CD3, CD4, and CD8 to investigate the correlation between T cell number and DI score. The IHC slides were scored (T.A.) in the same holistic manner.

2.4. Gene expression

Gene expression analysis was performed on tumor cDNAs using TaqMan gene expression assays (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. *GAPDH* (assay Hs02758991_g1) was used as a housekeeping gene. Amplification of *FOXP3* and *CD4* within 40 cycles failed for two samples (A18 and A30), so these were excluded from the analysis.

2.5. Whole-exome sequencing

Libraries were prepared and analyzed as previously described (Supplementary material) [13], with peripheral blood serving as a normal control.

2.6. Bioinformatics analysis

2.6.1. Exome sequencing

Results have already been reported for 28 of the 36 whole-exome sequencing samples [13]. Eight additional samples were sequenced and analyzed in the same manner (Supplementary material).

2.6.2. Neoantigen prediction

Neoantigens were predicted for each nonsynonymous variant by defining all novel 8- to 11-mers resulting from the mutation and determining whether the predicted binding affinity to human leukocyte antigen (HLA) class I alleles was <500 nM [14,15] using NetMHCpanv2.8 software [16,17]. A total of 32 samples underwent neoantigen prediction. Four samples were excluded owing to unavailability of the HLA type. Two additional samples lacked normal controls for whole-exome sequencing.

2.7. Statistical analysis

Continuous variables were compared using the Mann-Whitney *U* test. Survival analysis was conducted using both Kaplan-Meier log-rank analysis and Cox proportional hazards models. Analysis was carried out using GraphPad Prism 6 software (GraphPad, San Diego, CA, USA), and R version 3.2.0 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. TCR sequencing conclusively distinguishes TILs from normal-tissue T-cell populations

For 20 patients, we performed TCR sequencing on both tumor and adjacent normal tissues. An attending genitourinary oncology pathologist (T.A.) verified that tumor tissue

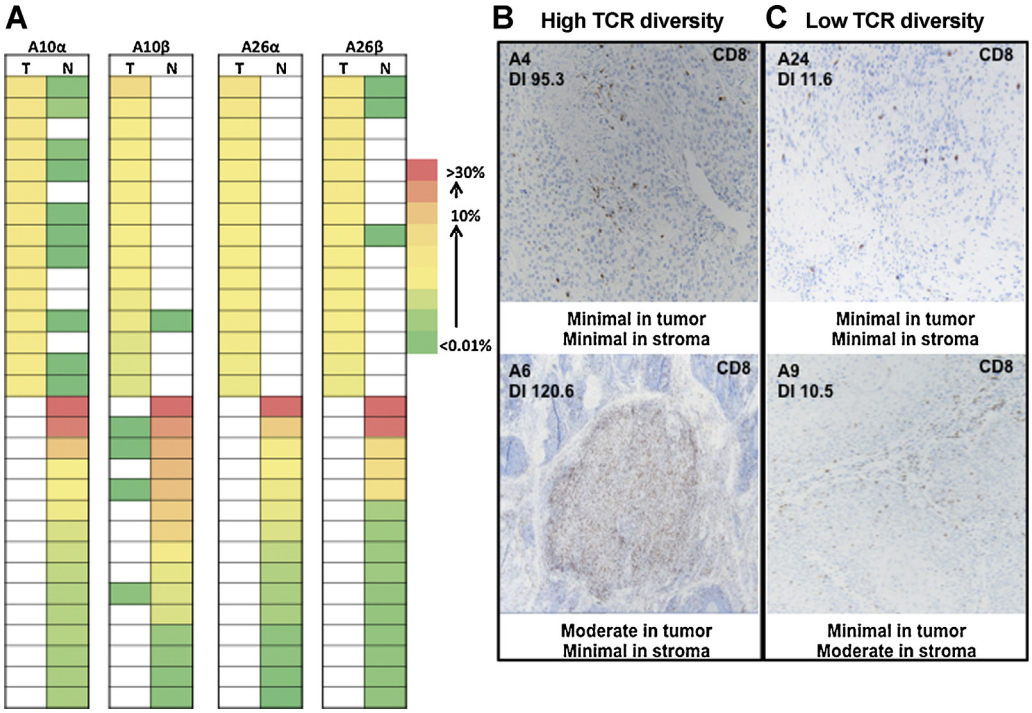

Fig. 1 – T-cell receptor (TCR) sequencing provides granular data on TCR repertoires beyond immunohistochemistry (IHC). (A) Representative heatmaps of the top 15 clonotypes identified for α and β chains for two samples, comparing tumor (T) and normal (N) distributions. White spaces indicate particular clonotypes not found in the corresponding tissue. The Supplementary material provides heatmaps for 18 additional samples. Samples were sorted according to the most abundant clonotypes in each chain and compared to corresponding tumor or normal tissue. Unique clonotypes found in tumor tissue were absent or hardly detectable (frequency <0.1%) in corresponding normal tissue for most samples. (B) Representative IHC examples for CD8 in muscle-invasive bladder cancer tumors with high TCR diversity. Tumor A4 (DI 95.3) has minimal CD8 staining in tumor and stroma, while tumor A6 (DI 120.6) has moderate staining in tumor and minimal staining in stroma. (C) CD8 staining of two tumors with low TCR diversity. Tumor A24 (DI 11.6) has minimal staining in tumor and stroma, while tumor A9 (DI 10.5) has minimal tumor staining and moderate stromal staining. TCR DI scores did not correlate with qualitative scoring of T-cell infiltration according to IHC (performed by a pathologist blinded to DI scores). DI = Simpson's diversity index.

contained a high percentage of tumor nuclei and that normal tissue excluded tumor cells. Analysis of V-(D)-J combinations with complementarity-determining region 3 (CDR3) sequences demonstrated that unique TCR clonotypes in tumor tissues were absent or hardly detectable in the adjacent normal tissues (frequency <0.01; Fig. 1A and Supplementary Fig. 1). We concluded that the tumor TCR repertoires were significantly different from those of adjacent normal tissues, and then focused our TCR analysis only on tumor tissue for the remaining 18 patients because of our interest in TIL diversity.

TCR sequencing was performed on a total of 38 tumors (Table 1, Supplementary Table 1). To assess whether the number of T cells influences DI quantification, we performed IHC for CD3, CD4, and CD8 on a subset of samples (Fig. 1B and Supplementary Fig. 2). A pathologist blinded to the sample DIs independently and qualitatively scored the degree of T-cell infiltration into tumor centers and the surrounding stroma. We observed that even patients with very low DI values (DI <10) had varying degrees of T-cell infiltration into tumor centers and surrounding stroma, ensuring that these low DI scores were not simply due to low TIL numbers. Since it was noted that high-DI samples (DI >50) also had similar variations in TIL numbers, DI is truly a representation of the clonal expansion of T cells and not simply a reflection of the total number of T cells.

3.2. *TCR diversity in primary tumors foreshadows recurrence risk*

Notably, we observed that tumors from NR patients had a lower TCRβ DI than tumors from Rec patients (mean ± standard deviation [SD] 36.1 ± 31.8 vs 129.8 ± 159; $p = 0.08$, Fig. 2A). We pursued this finding by classifying tumors into binary DI groups using the median DI as a cut-point. Kaplan-Meier survival analysis revealed that patients with low TCRβ DI had significantly longer RFS than patients with high TCRβ DI (median not reached vs 34.7 wk; log-rank test $p = 0.018$, Fig. 2B and Supplementary Fig. 3). The relationship between RFS and TCRβ DI remained significant after adjusting for pathologic tumor stage, node status, and receipt of adjuvant chemotherapy in a Cox proportional hazards model ($p = 0.033$, hazard ratio [HR] 2.67, 95% confidence interval [CI] 1.08–6.60). We also stratified patients according to tumor stage/node status and observed a positive correlation between DI and tumor stage/node status when assessed without other covariates (analysis of variance $p = 0.028$; Fig. 2C). These results indicate that primary tumors at a more advanced pathologic stage at the time of surgery already have less oligoclonal T cell expansion.

We hypothesized that in Rec patients with low TCRβ DI, immunosuppressive regulatory T (Treg) cells rather than cytolytic $CD8^+$ T cells may be expanding. We therefore

Table 1 – Patient demographic data: detailed clinical variables associated with prognosis in the 38 patients with muscle-invasive bladder cancer included in the study

Patient ID	Rec	Time to FU or Rec (wk)	Gender	Age at surgery (yr)	pT	pN	Histology	Site of recurrence	Adjuvant therapy
A3	Y	6.1	F	72	pT4a	pN0	Adenocarcinoma	Pulmonary nodules, gastric masses	N
A4	Y	23.1	M	70	pT3b	pN0	UC	Iliac masses	N
A10	Y	30.0	M	65	pT3b	pN1	UC	Bone	Y
A11	Y	36.9	F	74	pT3a	pN0	UC	Pulmonary nodules	N
A14	Y	5.4	M	59	pT4a	pN2	UC	Liver, lungs, LN, bone	Y
A15	Y	29.4	M	57	pT3a	pN1	UC with sarcomatoid features	Pelvic mass and LN	Y
A18	Y	188.1	M	77	pT2a	pN0	UC	Pelvic mass, liver	N
A20	Y	27.0	M	50	pT3b	pN0	UC	Pelvic mass, LN	Y
A24	Y	48.7	M	45	pT4a	pN1	UC	RP LN	Y
A25	Y	86.6	M	70	pT4a	pN2	UC with small-cell or NE features	Pelvic LN, brain	Y
A26	Y	68.4	F	78	pT3a	pN1	UC	Bone	N
A27	Y	62.3	M	70	pT4a	pN2	UC	Bone, pelvic LN	Y
A28	Y	39.3	M	68	pT4a	pN2	UC	Pelvic mass and LN	Y
A29	Y	9.7	F	80	pT4a	pN2	UC	Lungs, mediastinum, hila, liver, RP LN	N
A30	Y	12.3	M	49	pT4a	pN2	Adenocarcinoma	Bowel	N
A32	Y	22.4	M	76	pT3b	pN2	SCC	Pelvic mass, periportal LN	N
A34	Y	138.1	M	62	pT3a	pN2	UC	RP LN	Y
A36	Y	16.9	M	68	pT3a	pN1	UC	Unspecified sites	Y
A37	Y	25.1	M	70	pT3b	pN0	UC	Pulmonary nodules	N
A38	Y	3.6	M	59	pT4	pN0	SCC	Pelvic, lower abdominal LN	N
A1	N	360.6	M	78	pT3b	pN0	SCC	NA	N
A2	N	287.1	M	57	pT2b	pN0	UC with squamous features	NA	N
A5	N	261.7	M	67	pT2b	pN0	UC	NA	N
A6	N	262.6	F	68	pT3a	pN0	UC	NA	N
A7	N	347.7	M	74	pT4a	pN0	UC	NA	N
A8	N	239.0	F	60	pT3a	pN0	UC with squamous features	NA	N
A9	N	166.6	F	43	pT2b	pN0	UC with squamous features	NA	N
A12	N	265.4	M	59	pT2b	pN0	UC	NA	N
A13	N	234.1	M	55	pT3a	pN0	UC	NA	N
A16	N	220.4	M	84	pT3a	pN0	UC	NA	N
A17	N	71.9	M	68	pT2a	pN0	UC	NA	N
A19	N	289.7	F	74	pT2b	pN2	UC	NA	Y
A21	N	249.1	M	39	pT3a	pN0	UC	NA	Y
A22	N	330.9	M	57	pT2a	pN0	UC	NA	N
A23	N	209.6	M	56	pT3a	pN0	UC	NA	N
A31	N	212.6	M	78	pT3a	pN1	UC with squamous features	NA	N
A33	N	378.3	M	74	pT3a	pN1	UC	NA	Y
A35	N	323.7	M	52	pT3b	pN1	UC	NA	Y

REC = recurrence; FU = follow-up; M = male; F = female; pT = pathologic primary tumor stage; pN = pathologic node status as defined by the American Joint Committee on Cancer; UC = urothelial carcinoma; NE = neuroendocrine; SCC = squamous cell carcinoma; RP = retroperitoneal; LN = lymph node; Y = yes; N = no; NA = not applicable.

classified our cases into groups with high or low *CD8/FOXP3* expression using median expression in all tumors (equal to 3.9) as the cut-point. Among Rec patients, those with lower TCR DI scores typically also had low *CD8/FOXP3* expression, although the difference was not statistically significant (Supplementary Fig. 4A). More interestingly, we observed a significantly positive correlation between *CD8/FOXP3* expression and DI among Rec patients that was not seen in NR patients ($R^2 = 0.57$; $p < 0.001$, Supplementary Fig. 4B). These two observations indicate that Rec patients with low T-cell diversity may have dominant expansion of certain Treg cells and, similarly, Rec patients with high *CD8/FOXP3* ratios may lack clonal expansion of antitumor $CD8^+$ T cells, as evidenced by their comparatively higher TCRβ DI values.

3.3. *Predicted neoantigens impact on clinical outcome*

Whole-exome sequencing of 36 of the 38 MIBC tumors revealed a higher somatic non-synonymous mutation

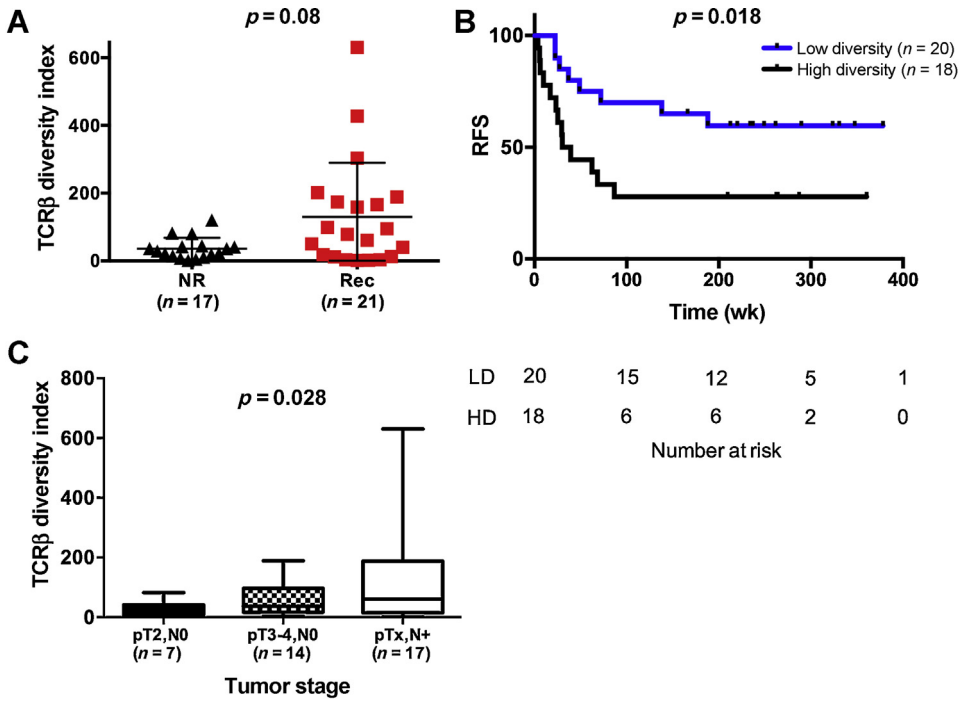

Fig. 2 – Low T-cell receptor (TCR) diversity index (DI) correlates with clinical outcome. (A) Patients without disease recurrence (NR; *n* = 17) had lower average TCR β DI compared to patients who experienced recurrence (Rec; *n* = 21) (36.1 ± 31.8 vs 129.8 ± 159, *p* = 0.08). (B) Kaplan-Meier curve of RFS, with patients divided into groups with high and low TCR DI groups (median DI 40.4; *p* value by log-rank test). (C) Stratification according to tumor size and nodal status (p value by analysis of variance).

burden in NR compared to Rec tumors, a finding that has been validated in other tumor types (mean ± SD 217 ± 152 vs 103.4 ± 94; $p = 0.007$, Supplementary Fig. 5). We performed in silico potential neoantigen or neoepitope prediction from these nonsynonymous mutations [8,18,19] for 32 of our 38 MIBC tumors (Supplementary Tables 2 and 3). The number of somatic nonsynonymous mutations had a tightly linear positive correlation with the number of predicted neoantigens ($R^2 = 0.89$; $p < 0.0001$, Supplementary Fig. 6). Tumors from NR patients had a higher average number of predicted neoantigens (177.8 vs 103.9; $p = 0.032$, Fig. 3A). To ensure we selected only those neoantigens most likely to be presented to T-cells, we applied two additional filtering criteria: (1) HLA-binding affinity of >500 nM for the corresponding wild-type peptide; and (2) affinity at least three times greater for the mutant than for the wild-type peptide (larger numbers indicate weaker affinity). The relationship between filtered neoantigen load and RFS was not statistically significant (median RFS not reached vs 52.6 wk for high vs low neoantigen group; log-rank $p = 0.11$, Fig. 3B). We next compared the relationship between filtered neoantigen load and DI, and observed that there was no relationship between neoantigen load and TCR diversity ($p = 0.9$) in the NR group, but a positive correlation in the Rec group ($p = 0.07$), with a statistically significant difference in slope between the two cohorts ($p = 0.038$; Fig. 3C). This indicates that NR tumors maintain low TCR diversity independent of the number of predicted neoantigens, while in Rec tumors TCR diversity simply increases in the presence of more neoantigens.

Finally, we compared RFS using stratification according to both neoantigen load and TCRβ diversity. Patients with a high neoantigen load and low TCRβ diversity had longer RFS compared to those with low neoantigen load and high TCR diversity (median RFS 275 vs 30 wk; $p = 0.03$, Supplementary Fig. 7).

3.4. *Immune-related gene expression signature differentiates clinical outcome*

We performed gene expression assays for *CD4*, *CD8*, *FOXP3*, indoleamine 2,3-dioxygenase 1 (*IDO1*), granzyme A (*GZMA*), and perforin-1 (*PRF1*). High expression of *CD8*, *GZMA*, and *PRF1* is associated with an immunostimulatory profile, while high expression of *CD4*, *FOXP3*, and *IDO1* is associated with immunoregulation. For each of these factors, we calculated the NR/Rec expression ratio. We found that NR tumors had higher levels of *CD8*/*CD4* expression ($p < 0.01$, Table 2). Among tumors with either low DI or low neoantigen load, NR patients had significantly higher levels of *CD8*, *CD8*/*CD4*, *GZMA*, and *IDO1* compared to Rec patients (Fig. 4A). For visual representation, we generated a heatmap of immunostimulatory gene expression according to recurrence, diversity, and neoantigen classification (Fig. 4B). Of note, there was a highly linear positive correlation between *CD8* and *IDO1* expression ($R^2 = 0.82$, $p < 0.001$); *IDO1* expression is probably upregulated in response to *CD8* upregulation in a negative feedback mechanism [20,21]. These data suggest that a degree of both intratumoral oligoclonal T-cell expansion and high expression of

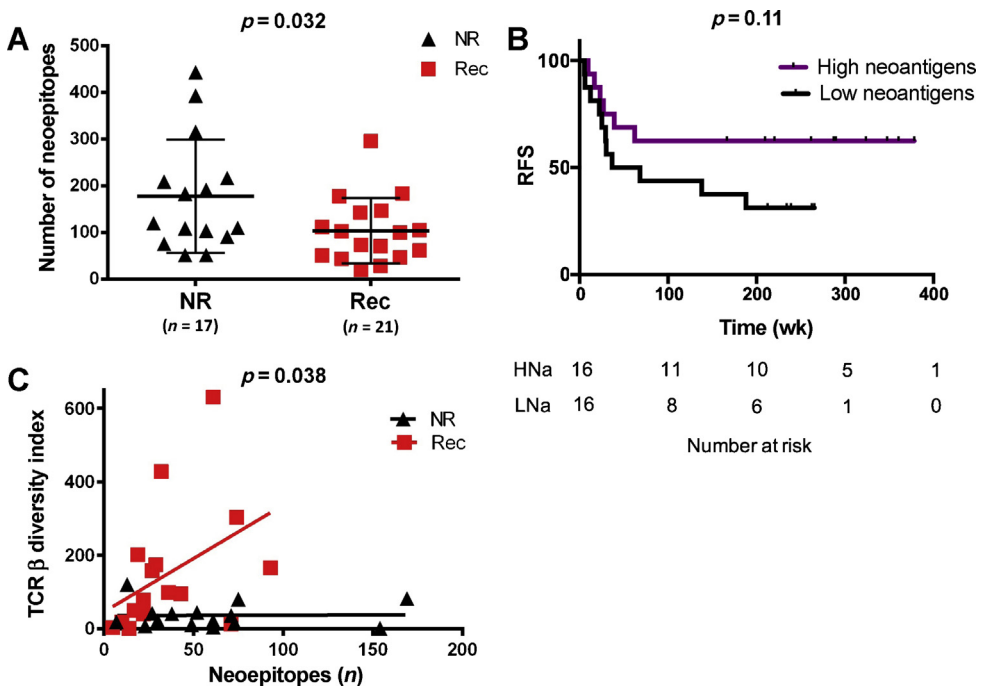

Fig. 3 – A combination of a high number of neoantigens and low T-cell receptor (TCR) diversity index (DI) mediates survival and nonrecurrence. (A) Tumors from patients without recurrence (NR) had a higher number of predicted neoantigens than tumors from patients who experience recurrence (Rec; 177.8 vs 103.9; $p = 0.032$). (B) Kaplan-Meier survival analysis reveals a nonsignificant recurrence-free survival (RFS) benefit for patients with tumors with a high number of predicted neoantigens (RFS median not reached vs 52.6 wk for high vs low neoantigens; $p = 0.11$). (C) Tumors from NR patients exhibit no association between neoantigen number and TCR DI ($p = 0.9$), while there is a positive correlation for Rec patients ($p = 0.07$). The difference in slope is significant ($p = 0.038$).

Table 2 – Expression ratios for immunocytolytic genes according to clinical outcome [a]

	All patients ($n = 36$)		Low diversity ($n = 17$)		Lower NNA ($n = 13$)		High diversity ($n = 19$)		Higher NNA ($n = 16$)	
	NR/Rec	*p* value	NR/Rec	*p* value	NR/Rec	*p* value	NR/Rec	*p* value	NR/Rec	*p* value
CD8	2.97	0.342	11.85	0.06	10.55	0.029	1.90	0.898	0.75	1
CD8/CD4	2.17	0.003	4.52	0.027	6.96	0.007	1.60	0.179	0.70	0.562
GZMA	10.22	0.071	26.12	0.027	41.99	0.019	4.90	0.416	2.10	0.635
IDO1	27.62	0.077	57.17	0.048	149.86	0.019	2.00	0.373	2.10	0.679
CD8/FOXP3	1.43	0.232	2.42	0.45	3.35	0.029	1.50	0.21	0.70	0.635

NNA = number of neoantigens; NR/Rec = expression ratio between patients without recurrence (NR) and patients with recurrence (Rec).
[a] Expression levels of the genes listed in the table differed significantly by clinical outcome according to genetic and diversity variables. In samples with low diversity, relative increases in *CD8*, *GZMA*, and *IDO1* expression were observed in tumors from Rec patients. Similar trends are seen for tumors with fewer than the median number of neoantigens (low neoantigen load). These trends were not seen for tumors with high diversity or those with high numbers of neoantigens. The Mann-Whitney *U* test was used to determine *p* values.

immunostimulatory genes may be necessary to protect against recurrence.

4. Discussion

Our study is the first to examine the relationship between predicted neoantigen load and TCR diversity with clinical outcomes in chemotherapy-naïve MIBC. No study has quantitatively characterized the TIL receptor repertoire in depth via TCR sequencing in MIBC. Our results demonstrate that DI is a measure of the expansion of T cells that captures the functionality of TILs in a manner that cannot be achieved with IHC quantification alone. TCR sequencing may therefore more objectively capture the immune environment within the entire tumor sample and be a novel method for predicting disease course in MIBC.

The finding that TCR DI remains associated with RFS even after correcting for prognostic clinical variables implies that it is the immune response to genetic (and epigenetic) alterations, rather than simply the genetic alterations themselves, that influences clinical outcome. As evidence, TCR DI was associated with RFS, but neoantigen load alone was not. Such a hypothesis is validated by a recent trial demonstrating a strong clinical response to anti-PDL1 therapy in MIBC [22]. Just as boosting the

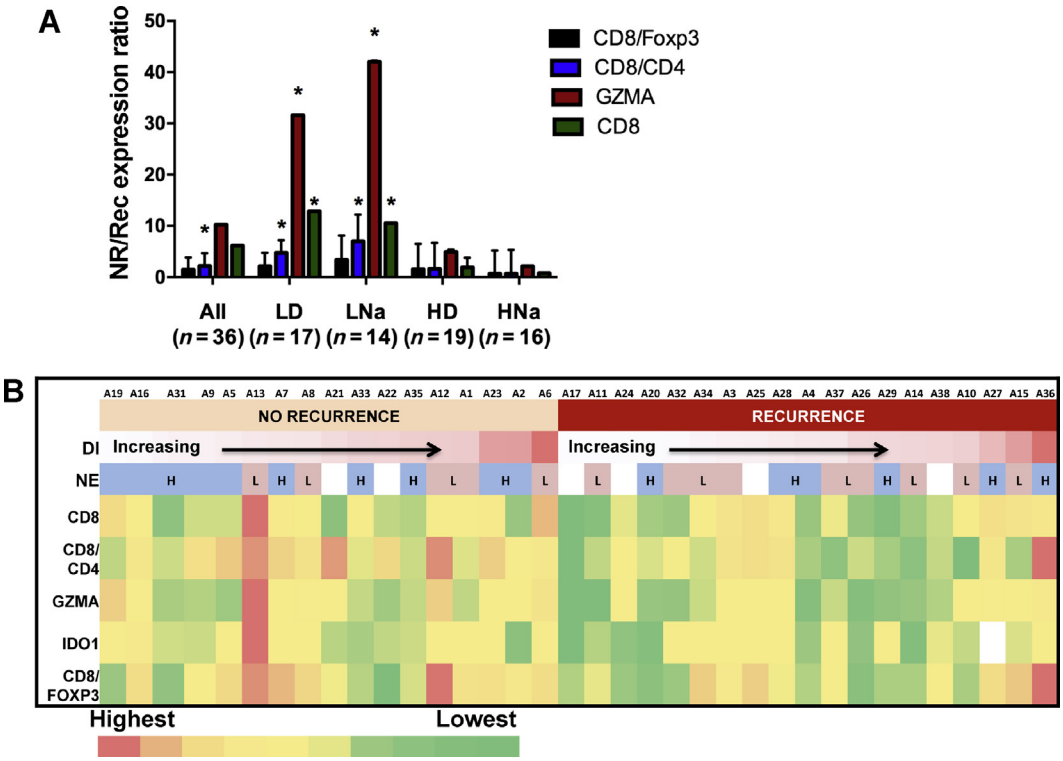

Fig. 4 – Patients without recurrence (NR) exhibit higher levels of immunocytolytic gene expression than patients who experienced recurrence (Rec) when stratified by diversity and neoantigen load. (A) For each group indicated, we calculated the relative expression of *CD8*, *CD8/CD4*, *GZMA*, and *CD8/FOXP3* for tumors from NR patients over Rec patients. T-cell populations with high *CD8/FOXP3* expression have high ratios of cytolytic CD8$^+$ T cells to Treg cells, while T-cell populations with a low *CD8/FOXP3* expression ratio correspond to a low CD8$^+$ T cell to Treg ratio. Bars marked with asterisks reached statistical significance ($p < 0.05$). The difference in immunocytolytic gene expression is greatest for tumors with a low number of predicted neoantigens ($n = 5$ NR, $n = 9$ Rec). In calculating the NR/Rec gene expression ratio, we used the standard error of the mean to represent error bars. HD = high diversity; LD = low diversity; HNa = high neoantigen load; LNa = low neoantigen load. (B) Heatmap of gene expression of immune-related factors according to recurrence status, TCR diversity, and neoantigen classification. Red indicates the highest expression and green indicates the lowest. The scale is normalized for each gene. NR tumors with low diversity tended to have higher expression of immunostimulatory factors and *IDO1*. H = high; L = low.

immune response via anti-PDL1 therapies results in clinical improvement in MIBC, our study shows that patients who intrinsically have more robust intratumoral immune responses independently have improved long-term clinical outcomes. Patients with low TCR DI at the time of cystectomy may therefore be optimal candidates for immunotherapy.

The lack of correlation between RFS and neoantigen load also suggests that while a higher mutation burden increases the likelihood of generating relevant neoantigens [8], NR tumors may simply have "won the neoepitope lottery" to generate those few true neoantigens that elicit strong oligoclonal T-cell expansion, probably via high-affinity binding to both TCR and HLA class I molecules [23]. In other words, having a greater number of neoantigens increases the probability, but without guarantee, of capturing those strongly immunogenic neoantigens capable of stimulating a very effective antitumor immune response. T-cell anergy may also contribute to the inability of Rec tumors to mount an effective immune response to presented neoantigens. Assessment of PD1 or PDL1 may therefore clarify the relationship between TCR DI and neoantigen load. In the interim, identification of potential critical neoantigens in MIBC, or those already capable of inducing strong T cell–mediated responses, could potentially serve as a basis for peptide vaccines.

The limitations of our study include its small sample size, making it critical to validate the results by analyzing future MIBC patient cohorts with appropriate clinical follow-up to improve our understanding of these relationships. Second, variant tumor histology is a potential confounder in our analysis that could not be corrected. Third, our TCR sequencing approach cannot distinguish subsets of T cells; however, flow cytometry cannot be used to perform these analyses owing to the limited availability of cancer tissues and technical difficulty in separating subsets of cells in solid tumors. Finally, next-generation sequencing does not allow definition of the TCR α and β pair that recognizes neoantigens. Single-cell sequencing, by contrast, could potentially help to identify the pair of TCR chains that respond to neoantigens.

5. Conclusions

Taken together, our results have important implications for prognostic prediction in MIBC, as well as for the future use of immunotherapies. In particular, identification of the neoepitopes capable of stimulating oligoclonal T-cell

expansion could offer new avenues for adaptive T-cell therapy and peptide vaccines for various types of human tumor by either boosting the natural antitumor system or by compensating for deficiencies in genetic alterations. Our results demonstrate that integration of the genetic and immune landscapes of bladder tumors provides valuable prognostic information on the clinical course of MIBC that can be harnessed for therapeutic measures.

Author contributions: Yusuke Nakamura 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: Park, O'Donnell, Nakamura.
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Analysis and interpretation of data: Choudhury, Park, Kiyotani, Nakamura, O'Donnell.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euf.2015.09.007.

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