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Identification of a novel inflamed tumor microenvironment signature as a predictive biomarker of bacillus Calmette-Guerin immunotherapy in non-muscle invasive bladder cancer

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Translational Relevance Statement: We performed RNA-based profiling by NanoString nCounter on non-muscle invasive bladder cancer (NMIBC) clinical specimens and found that a novel expression signature of an inflamed tumor microenvironment (TME), but not molecular subtyping, was associated with improved recurrence-free survival after bacillus Calmette-Guerin (BCG) immunotherapy. We further demonstrate that immune checkpoint gene expression was not associated with higher recurrence rates after BCG. These findings were externally validated in a large RNAseq dataset of NMIBC suggesting our immune signature could be a robust predictive biomarker for BCG response and that an immunologically “cold” TME is a mechanism of resistance to BCG. Our results also raise concerns about treatment strategies combining BCG and immune checkpoint blockade in NMIBC and instead support approaches focused on modulating the TME. Our integrated transcriptomic and panel sequencing found FGFR3 overexpression and mutations to be associated with an “cold” TME, further supporting investigations into FGFR inhibitors for NMIBC.

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ABSTRACT

Purpose: Improved risk stratification and predictive biomarkers of treatment response are needed for non-muscle-invasive bladder cancer (NMIBC). Here we assessed the clinical utility of targeted RNA and DNA molecular profiling in NMIBC.

Experimental Design: Gene expression in NMIBC samples was profiled by NanoString nCounter, a RNA quantification platform, from two independent cohorts (n = 28, n = 50); targeted panel sequencing was performed in a subgroup (n = 50). Gene signatures were externally validated using two RNAseq datasets of NMIBC tumors (n = 438, n=73). Established molecular subtype classifiers and novel gene expression signatures were assessed for associations with clinicopathologic characteristics, somatic tumor mutations, and treatment outcomes.

Results: Molecular subtypes distinguished between low-grade Ta tumors with FGFR3 mutations and overexpression (UROMOL-class 1) and tumors with more aggressive clinicopathologic characteristics (UROMOL-classes 2 and 3), which were significantly enriched with TERT promoter mutations. However, UROMOL subclasses were not associated with recurrence after bacillus Calmette-Guerin (BCG) immunotherapy in two independent cohorts. In contrast, a novel expression signature of an inflamed tumor microenvironment (TME) was associated with improved recurrence-free survival after BCG. Expression of immune checkpoint genes (PD-L1/PD-1/CTLA-4) was associated with an inflamed TME, but not with higher recurrence rates after BCG. FGFR3 mutations and overexpression were both associated with low immune signatures.

Conclusions: Assessment of the immune TME, rather than molecular subtypes, is a promising predictive biomarker of BCG response. Modulating the TME in an immunologically “cold” tumor

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warrants further investigation. Integrated transcriptomic and exome sequencing should improve treatment selection in NMIBC.

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INTRODUCTION

Current risk stratification for non-muscle-invasive bladder cancer (NMIBC) is inadequate and relies on clinicopathologic features, with limited ability to accurately predict which tumors are most likely to recur and/or progress to muscle-invasive disease(1). Hence, patients with NMIBC undergo intensive surveillance with frequent, invasive cystoscopies, rendering bladder cancer among the most expensive cancers to manage(2). Thus, there is a critical need for prognostic and predictive biomarkers in NMIBC. Most urgently needed are molecular biomarkers that are predictive for response to immunotherapies. Intravesical bacillus Calmette-Guérin (BCG) immunotherapy has been a standard of care in NMIBC for over 40 years, but there exists no biomarker to prioritize patients for optimal treatment in this era of recurrent BCG shortages. The anti-PD-1 immune checkpoint inhibitor pembrolizumab was recently FDA-approved for BCG-unresponsive NMIBC based on a single-arm phase II trial demonstrating an initial response rate of 40% at 3 months. However, only 20% of patients remained disease-free at 12 months(3) and PD-L1 expression by immunohistochemical (IHC) staining was not associated with response, leaving little guidance on who is likely to benefit(3).

A potential approach to risk-stratify NMIBC patients and predict treatment responses is transcriptomic profiling. This strategy has led to the identification and validation of luminal and basal-like molecular subtypes of bladder cancer, similar to the luminal and basal-like breast cancer subtypes(4,5). For muscle-invasive bladder cancer (MIBC), multiple retrospective studies indicate that molecular subtyping is prognostic and predictive of response to chemotherapy and immunotherapy, and a consensus molecular classification has recently been established(4-6). In contrast, while early unsupervised microarray analyses of gene expression had focused on NMIBC, there have been only a few recent gene expression efforts in NMIBC using more contemporary expression profiling platforms(7-9). The largest of these was UROMOL, a prospective multicenter European study that established three major molecular

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subtypes of NMIBC(7). However, the 460 NMIBC specimens analyzed represent only 34% of the initial 1,372 fresh-frozen specimens obtained, after applying strict criteria on RNA quality and carcinoma cell percentage. Inclusion of only the highest-quality tumor tissue increases confidence in the resulting biologic insights but can limit the clinical translation of these findings. Most NMIBC specimens available for clinical testing are archival, formalin-fixed, paraffin-embedded (FFPE) tissues, from which RNA can be difficult to extract and is generally of poor quality(10). Thus, a clinically feasible method of multiplex gene expression profiling in NMIBC must overcome the limited amount of available tissue, low cellularity, and poor RNA quality inherent in the majority of these specimens.

RNA counting methods, such as nCounter (NanoString Technologies, Inc), that do not require enzymatic reactions allow for FFPE samples to be used in expression profiling(11), but the feasibility and value of these approaches have not been robustly assessed in NMIBC. Thus, we sought to evaluate gene expression signatures determined by RNA counting to identify associations with clinicopathologic characteristics, assess prognostic and predictive significance, and evaluate the added value of gene expression data over established DNA mutation sequencing methods in NMIBC.

MATERIALS AND METHODS

Patient samples

Clinical NMIBC specimens were collected from two sites, University of North Carolina at Chapel Hill (UNC) and Memorial Sloan Kettering Cancer Center (MSK). All specimens from UNC and MSK were archival FFPE NMIBC tissue samples procured by transurethral resection of bladder tumor (TURBT) from treatment naïve, newly diagnosed patients and were reviewed by a genitourinary pathologist (SW, HAA) to confirm grade, stage, and urothelial histology.

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Specimens in the UNC cohort were collected through the institutional review board-approved UNC Health Registry/Cancer Survivor Cohort Study, which prospectively ascertained persons with newly diagnosed with cancer seen in the UNC Hospital system. Enrolled patients gave informed consent for use of biospecimens, clinical data, and questionnaire data for approved research. Specimens in the MSK cohort were collected through an institutional review board-approved sequencing effort, in which tumor specimens and matched germline DNA were profiled by targeted panel sequencing using a panel of 341 (later updated to 410) cancer-associated genes within a CLIA-certified laboratory as previously described by Pietzak et al and available on cbioportal.org (http://www.cbioportal.org/study/summary?id=blca_nmibc_2017)(12). We included all tumors with available FFPE for RNA extraction and gene expression analysis from Pietzak et al. Treatment and management was at the discretion of the treating urologic oncologist. Patients treated with BCG immunotherapy received 6 weekly full doses of TICE BCG with only 8% receiving additional maintenance BCG. Patients were then followed every 3 months with cystoscopy and urine cytology for the first year, then every 3–6 months. All HGT1 tumors had re-staging TURBTs with confirmation of uninvolved detrusor muscle at initial diagnosis. Recurrence was defined as histologically proven cancer on biopsy or TURBT.

Expression analysis

RNA extraction and gene expression methods have been previously published(13). Briefly, RNA was isolated from a 1-mm FFPE core or two 10-µm unstained FFPE slides using the Qiagen RNeasy FFPE Kit and protocol (cat. # 73504). RNA was quantitated using a ThermoScientific NanoDrop 2000 Spectrophotometer (cat. # ND-2000) and Agilent 4200 TapeStation. After excluding samples with low concentration or low percentages of RNA molecules >300 nucleotides long, the remaining 90% of samples were processed by the UNC Translational

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Genomics Laboratory using the NanoString nCounter platform. Samples were run on a custom codeset that included gene sets for the Hedegaard classifier(7), for p53 pathway defects(14), and for 52 immune-related genes(15). Samples were randomized to batch and two Stratagene Universal Human Reference RNA samples were included to assess batch variability. Batch variability was low, with correlations between reference standards exceeding 0.98.

After counting RNA, QC procedures eliminated samples with low tumor gene expression. Four steps were used to identify such samples. (1) Expression values below the limit of detection, defined as the average of a sample's negative probe mean, was set to zero. (2) Correlations between 6 housekeeping genes (*ACTB, GUSB, KU70/XRCC6, NAGA, PGK1, RPS10*) were evaluated across samples. Pairwise correlations were all above 80%, with an overall mean correlation of 87.2%, and therefore all 6 genes were retained in the dataset. (3) The relationship between housekeeping gene missingness and endogenous gene missingness was assessed, where missingness was defined as having an expression below the limit of detection. Missingness in endogenous genes was correlated with housekeeping gene missingness. Samples with greater than half of the endogenous genes marked as missing were removed from further analysis. Principal component analysis (PCA) was then used to identify outliers. (4) Finally, we used the R package Remove Unwanted Variation (RUV) to normalize the data(16). Dimensions of unwanted variation (k) from 1 to 5 were assessed and k=1 was selected based on sample PCA plots. Upon normalization, nearest-neighbors averaging imputation was applied to all samples via the impute R package(16). Samples from MSK and UNC were assessed for quality concurrently. When duplicate assays both passed QC, these were averaged to produce a single expression vector for each patient.

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Gene expression signatures

Genes for the BASE47 gene signature were as originally described by Damrauer et al(4). Gene expression data were clustered and Prediction Analysis of Microarrays (PAM) was used to select a parsimonious set of genes that represented the three UROMOL subclasses described by Hedegaard et al.(7) For UROMOL subclasses, a total of 117 genes were included in the classifier, though the same clusters were recapitulated with 110 genes. This smaller gene set was used to cluster the samples. Classification by the p53 pathway defect expression signature originally described in breast cancer(14) and recently shown to be associated with *TP53* status in MIBC(17) was according to published methods(14,17). Finally, we developed a panel of immune genes based on work previously described by Bindea et al.(15). As NMIBC specimens either had high expression of all genes in the panel or low expression of all genes, the immune score for a tumor was defined as the average of the counts of genes comprising the immune signature panel. Tumors were grouped into tertiles by ranking tumors by gene scores and identifying the 33rd and 66th percentile thresholds adapted from Miller et. al.(18). For recurrence analyses, the top two tertiles were combined into a “high” immune signature score and compared to the bottom tertile, considered a “low” immune score. Proliferation was determined from the median expression of proliferation-related genes in Parker et. al.(19). Java TreeView was used to visualize the data according to *TP53* status and immune score(11).

Comparison of RNA-based classes with DNA mutations

Targeted panel sequencing of MSK samples was performed using the MSK-IMPACT assay(12,20,21). Coding and promoter mutations and indels, excluding silent mutations, were considered. Mutation calls were not assessed for samples that failed MSK-IMPACT QC. Specific genes were curated from Pietzak et al. based on genes mutated at >10% in NMIBC(12). Missense mutations and amplifications in known oncogenes were deemed significant if the variant was recurrent or if existing literature reported it as a functionally

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validated activating alteration. Alterations in tumor suppressor and DNA damage repair genes were deemed deleterious if truncating mutations (nonsense, frameshift indels), recurrent missense mutations, or homozygous deletions. Recurrent missense mutations were defined as those reported in either the Catalogue of Somatic Mutations in Cancer (COSMIC) or in the cBioPortal for Cancer Genomics more than 10 times. Missense mutations reported by the MSK-IMPACT bioinformatics pipeline, but not meeting our definition for recurrent alterations, were reported as “novel missense mutations”/”missense mutations of unknown significance” and not included in statistical analyses of clinicopathologic and recurrence associations.

Validation Cohorts

UROMOL Cohort: Publicly available RNAseq data from the UROMOL Project (Prediction of bladder cancer disease course using risk scores that combine molecular and clinical risk factors) were downloaded from the supplemental material section from Hedegaard et al(7). and from www.medrxiv.org (https://www.medrxiv.org/content/10.1101/2020.06.19.20054809v1) to determine BCG treatment history and recurrence outcomes. Only 19% of the UROMOL cohort received BCG and maintenance BCG was infrequently given(7).

Northwestern Cohort: Publicly available RNAseq data from GSE154261 was downloaded from Robertson et al(9). This cohort compromised of 73 primary T1 tumors treated at Northwestern University with all patients receiving induction BCG, 64% receiving some maintenance BCG, and 84% having a restaging TURBT before BCG(9).

Statistical analyses

Clustering of gene expression data was performed in R using the heatmap.plus R package(11). Pearson distance measures and complete linkage were used to determine clusters. Java

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TreeView was used to visualize the clustered data and generate heatmaps(11). The compareGroups R package was used to generate descriptive tables and to determine the statistical significance of associations among variables(11). Continuous variables were assessed using t-tests or ANOVA, where appropriate, and categorical variables were assessed using chi-squared tests or Fisher's exact tests. Cox regression modeling was used to determine the association between gene signatures and recurrence after BCG. The Kaplan-Meier method and log-rank test were used for estimations of recurrence-free survival (RFS).

RESULTS

Patient and tumor characteristics

RNA was extracted from tumor samples of 41 UNC patients and 68 MSK patients, of which 28 samples (68%) and 50 samples (74%), respectively, met QC criteria. In general, samples with low (<30%) tumor cellularity more often failed QC (**Supplemental Table 1, Supplemental Figure 1)**. Patient demographics and clinicopathologic characteristics for profiled samples are described in **Table 1**. Recurrence outcomes were available only for the MSK cohort. Clinicopathologic characteristics were not associated with recurrence risk in this cohort (**Supplemental Table 2**), consistent with our prior study(12). One patient was treated with immediate cystectomy, so their tumor was analyzed but they were excluded from recurrence analyses. Median follow-up among the MSK cohort was 39 months (IQR 13–66.2), with recurrence occurring in 23 patients. Median follow-up among the 38 patients (76%) receiving BCG immunotherapy was 38.2 months (IQR 10.7–66.6), with recurrence occurring in 18 patients.

Molecular subtyping by RNA counting

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We first sought to determine molecular subtype using the BASE47 classifier developed to separate MIBC into luminal and basal-like subtypes(4). We found limited discriminatory capacity as only 4 NMIBC tumors were classified as basal-like, whereas the remainder were luminal. We therefore focused our subsequent analyses on a gene expression classifier derived from the large UROMOL cohort of NMIBC samples(7). Our combined NMIBC sample cohort was well-distributed across the 3 previously described UROMOL subclasses (**Figure 1**) and clinical characteristics differed across the groups (**Table 2**). UROMOL class 1, previously described as luminal-like/well-differentiated, was enriched in low-grade Ta tumors that had greater tumor cellularity due to their predominantly papillary architecture. As expected, these tumors had significantly higher FGFR3 gene expression levels in both the UNC and MSK cohorts (**Supplemental Figure 2**)(7). Consistent with UROMOL class 2 tumors being the most aggressive subtype, they had the highest proliferation expression scores and expression of the proto-oncogene forkhead box M1 (FOXM1) in both cohorts (**Supplemental Figure 3**). UROMOL class 3, previously described as being an early basal-like subtype, was enriched in high-grade T1 tumors.

There were very few progression events within the MSK cohort, preventing formal analysis, but all 3 patients with progression to secondary MIBC had UROMOL class 2 tumors, consistent with the original study(7). There was no correlation between UROMOL classes and RFS within the entire MSK cohort or the subset of 38 patients treated with BCG (**Figure 2A–B**). To rule out the possibility that this lack of association was the result of small sample size, we analyzed publicly available data from the UROMOL study and found that class 2 was associated with worse RFS in the UROMOL study (n=438), but subtype was not associated with recurrence rates in the subset of patients treated with BCG (n=83) (**Figure 2C–D).** These results suggest that the UROMOL classifier is prognostic of outcome but not predictive of BCG response.

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Immune gene expression and signatures

As UROMOL classes were not predictive of recurrence following BCG treatment among either the MSK or UROMOL cohorts, we sought to evaluate other RNA-based signatures as potential predictive biomarkers of clinical benefit from BCG immunotherapy. Previous biomarker studies have identified higher pretreatment T cell infiltration and an inflamed tumor microenvironment (TME) as associated with improved response to systemic immunotherapies in various cancer types, including bladder cancer(15,22,23). We evaluated the expression of known immune-related genes to determine the degree of immune cell infiltration and inflammation within the TME(15). NMIBC tumors could be broadly classified as having either low expression or higher expression across all immune genes studied, with no groups emerging with more complex patterns (i.e., innate immune response only) (**Figure 3A**). Therefore, the median expression of all immune genes was used to calculate an immune score. Tumors were grouped into tertiles by ranking tumors by gene scores and identifying the 33rd and 66th percentile thresholds adapted from Miller et al.(18). These scores varied among UROMOL classes, with basal-like UROMOL class 3 tumors having the highest expression of immune-related genes, whereas class 1 tumors had the lowest (**Supplemental Figure 4**). Tumors with high immune scores (top two tertiles) were associated with improved RFS in the MSK cohort (hazard ratio [HR]=0.33, 95% confidence interval [CI] 0.14–0.78, p=0.01) as well as among the subset treated with BCG (HR=0.23, 95% CI 0.09–0.59, p=0.002) (**Figure 3B–C**, **Supplemental Table 2)**. We also sought to externally validate the immune score using the UROMOL cohort (**Supplemental Figure 5**) and found that higher immune scores were associated with improved RFS among the 83-patient UROMOL subgroup treated with BCG (HR=0.5, 95% CI 0.24–1.00, p=0.05), but not among the entire 438-patient UROMOL cohort (HR=0.96, 95% CI 0.70–1.33, p=0.84) (**Figure 3D–E).** No statistically significant difference was seen for high immune score with improved RFS in the Northwestern HGT1 cohort (HR=0.7, 95% CI 0.52-3.6 p=0.4) (**Supplemental Figure 6**).

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We next sought to determine which individual immune-related genes were associated with favorable outcomes following treatment with BCG. Correlates of remaining free from high-grade recurrences >2 years after BCG treatment included high expression of HLA class I histocompatibility antigens, granzyme B (GZMB), and inducible T cell co-stimulator (ICOS). Surprisingly, we found high expression of the immune checkpoint genes PD-L1 and LAG-3 to also be associated with remaining recurrence free after BCG (**Figure 4A**).

As this finding contradicts a prevailing hypothesis that immune checkpoint expression leads to BCG treatment failure, and there is growing interest in anti-PD-L1/PD-1 immunotherapy for patients with NMIBC, we further assessed the significance of expression of targetable immune checkpoints (PD-L1, PD-1, and CTLA-4) within our cohort. Expression of these immune inhibitory molecules was highest in UROMOL class 3 tumors and lowest in class 1 tumors (**Supplemental Figure 7**). Higher expression of PD-L1 ($p=0.058$), PD-1 ($p<0.01$), and CTLA-4($p<0.01$) in pretreatment NMIBC specimens was associated with lower recurrence rates after BCG immunotherapy in our cohort, suggesting that these molecules correspond to increased immune infiltration and are not associated with resistance to BCG (**Figure 4B, Supplemental Figure 7**). Examining pretreatment expression levels of these immune checkpoint genes in the 83-patient BCG-treated UROMOL cohort found no association with RFS in BCG-treated patients (**Figure 4C, Supplemental Figure 7**). Furthermore, expression of PD-L1, PD-1, and CTLA-4 in pre-treatment specimens from the Northwestern HGT1 cohort were also not associated with RFS after BCG (**Supplemental Figure 7)**.

Association between gene expression-based signatures and somatic DNA mutations

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As the UROMOL study inferred somatic mutations from RNA expression data alone(7), which can lead to false-positive deleterious mutation calls(24), we evaluated correlations between UROMOL subclasses and somatic DNA mutations using tumor-normal target panel data available for the MSK cohort. *FGFR3* mutations were enriched among luminal class 1 tumors, while infrequently altered in basal-like class 3 tumors (**Figure 1, Table 3**). Alterations in *ERBB2*, a gene known to be mutually exclusive with *FGFR3(12)*, were absent in class 1 tumors but seen in 20% of class 2 tumors and 36% of class 3 tumors (p=0.02) (**Table 3)**. Mutations in the chromatin remodeling gene *TET2* were enriched in basal-like class 3 tumors (**Table 3)**. Somatic mutations in the *TERT* promoter were highly enriched in UROMOL class 2 tumors, found to be present in all 20 samples (100%) (**Figure 1**). UROMOL class 2 tumors also had the highest fraction of the genome altered (**Supplemental Figure 8**).

Alterations in *TP53* and cell cycle genes are among the most common genetic events in MIBC and high-risk NMIBC, yet biomarker investigations into p53 status to guide clinical management have reported conflicting results(12,25). We explored a mutant p53-pathway gene signature, developed and validated in breast cancer(14), within our NMIBC cohort. This signature was recently shown to be associated with *TP53* status in MIBC(17), but has little overlap with the "p53-like" gene expression signature identified by Choi et al. in MIBC(6) (**Supplemental Figure 9**). The mutant p53-pathway gene signature was highest in class 2 tumors, followed by class 3 tumors; these two subclasses had the most TP53 mutations (**Figure 1, Supplemental Figure 9**). RNA-based p53 pathway assessment detected defects in the p53 pathway more than *TP53* DNA mutations alone and had fair correlation with DNA alterations in an expanded set of commonly altered cell cycle pathway genes (*TP53, MDM2, RB1, CDKN2A, CDKN1A, CCND1*) (**Supplemental Figure 10**). Neither p53 mutant-like gene expression nor *TP53* DNA alterations were associated with recurrence in the entire MSK cohort or those treated with BCG. Additionally, mutant p53-pathway gene signature tumors had high immune scores, whereas

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p53-wild-type-like tumors had intermediate immune scores (**Supplemental Figure 10)**. Higher mutant p53-pathway gene signatures also correlated with increased expression of proliferation and FOXM1 (**Supplemental Figure 11)**.

Finally, as the pretreatment TME inferred by immune score was associated with BCG response, we sought to determine whether there was a correlation between genomic alterations in individual genes and immune score. We found that *FGFR3* mutations by targeted panel sequencing and *FGFR3* overexpression by RNA profiling were both associated with lower immune scores (**Supplemental Table 3**, **Supplemental Figure 2**). *FGFR3* mutations remained significantly associated with low immune score even after adjusting for multiple comparisons. Conversely, *ERBB2* alterations were enriched in immune-score-high tumors (38% v. 0 v. 0, p=0.001) (**Supplemental Table 3**).

DISCUSSION

There are several barriers that must be overcome before molecular biomarkers identified within the context of retrospective clinical studies can be successfully translated to clinical practice. These include the limited quantity and often poor quality of tissue available for many patients with NMIBC. Here, we show that gene expression profiling by RNA counting is feasible using archival FFPE NMIBC clinical samples, despite relatively limited tumor tissue and low tumor cellularity.

To build upon prior work with the NMIBC-specific UROMOL gene expression classifier(7), we combined targeted panel sequencing of tumor-normal pairs along with transcriptomic profiling and observed that *TERT* promoter mutations were significantly enriched in UROMOL class 2 (100%) and class 3 (82%) tumors. This novel observation demonstrates the potential molecular insights that can be obtained by integrated RNA and DNA molecular profiling and supports the

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development of urine-based screening and surveillance biomarkers that detect *TERT* promoter mutations in urinary cell free DNA to identify aggressive bladder tumors earlier(26). We also found UROMOL molecular subtypes correlated with expected clinicopathologic characteristics but were not associated with BCG response. UROMOL subtypes can be recapitulated within bladder cancer cell lines, suggesting that these molecular subtypes are tumor cell “intrinsic” signatures(7). We hypothesize that molecular subtypes based on tumor cell phenotype, independent of signals from infiltrating stromal and immune cells, provide important biologic insights but are not likely to be robust predictive biomarkers for response to immunotherapy such as BCG.

To develop a better predictive biomarker for BCG response, we assessed immune cell infiltration and inflammation within the TME using a novel gene expression signature. We observed that a high immune score was associated with improved RFS after BCG immunotherapy in the MSK and UROMOL cohorts, demonstrating the importance of the pretreatment TME in determining BCG response, consistent with the work of several other groups(27,28). While BCG is a live, attenuated bacteria known to provoke an influx of innate and adaptive immune cells within the bladder wall, the recruitment of these immune cells appears unable to overcome an existing immunologically “cold” TME. Notably, no statistical difference in RFS after BCG was seen within the Northwestern cohort for the immune score. This may be due to it being a HGT1 only cohort compared to the MSK and UROMOL cohorts which also included LGTa and HGTa tumors. It is also possible that the addition of maintenance BCG may attenuate the negative effects of a “cold” pre-treatment TME, as 64% of the Northwestern cohort received maintenance BCG compared to only a few patients within the MSK and UROMOL cohorts. Maintenance BCG is recommended in multiple guidelines as it reduces the risk of recurrence by 19% at 5 years compared to induction BCG alone(29,30), but many patients do not receive it due to the persistent global BCG shortage and concerns over

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treatment-related toxicity(29-31). If prospectively validated, the immune score may provide a rational, biomarker-driven approach to selecting which patients would most benefit from induction and maintenance BCG. This could be invaluable in helping to alleviate the global BCG shortage.

Further investigation into the causes of a cold TME in NMIBC is needed, but our study suggests that combination strategies that modulate the TME to promote antitumor immune cell recruitment hold promise in NMIBC. Our finding that mutation and overexpression of *FGFR3* are associated with a low immune score in NMIBC is consistent with data from studies of MIBC, upper tract urothelial carcinoma, and mouse models of bladder cancer(23,32,33), providing further support for investigations into the potential immunomodulating benefits of *FGFR3*-targeted therapies. Interestingly, our data also suggest that *FGFR3* and *ERBB2* are not only mutually exclusive in NMIBC but result in contrasting differences in the TME. Both *FGFR3* and *ERBB2* are attractive “targets,” given the prevalence of these genomic alterations in patients with NMIBC. As more targeted therapies with demonstrable activity in metastatic urothelial cancer are evaluated in clinical trials for patients with NMIBC, these differences in the TME will likely become even more relevant.

Our analysis also found that high gene expression of PD-L1, PD-1, and CTLA-4 was not associated with worse BCG response in either the MSK, UROMOL, or Northwestern cohorts. While this may seem contrary to the prevailing theory that PD-1/PD-L1 expression is a mechanism of resistance to BCG(34,35), others have reported high PD-L1 gene expression to be associated with favorable outcomes with intravesical therapy in T1 NMIBC(36). These observations might be from differences in pre-analytical preparations or post-translational modifications between gene level and protein level expression of PD-1/PD-L1(37). However, IHC-based studies assessing the role of PD-1/PD-L1 expression in NMIBC by comparing BCG

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"responders" to "non-responders" have had conflicting results, varying considerably depending on the antibody and cut-off used, and studies supporting PD-1/PD-L1 as a mechanism of resistance to BCG generally do not adjust for differences in important clinical factors that are known to affect both BCG response and PD-L1 expression levels, such as concomitant CIS(34-36,38). The first and most highly cited study suggesting PD-L1 expression may be a mechanism of immune evasion to BCG was by Inman et al., where 11 of 16 patients with recurrent tumors had associated post-BCG granulomata with strong PD-L1 expression by IHC staining(35). However, the authors did not assess PD-L1 expression in post-BCG granulomata from patients without recurrence, which is particularly relevant as post-BCG granulomata are associated with a favorable response to BCG(39) and comprised of macrophages that often express PD-L1 even in healthy tissue(40). Pretreatment specimens from the prospective NMIBC cohort within Inman et al. showed no difference in recurrence rates after BCG by PD-L1 expression (PD-L1+ 30% [3/10] vs. PD-L1- 38% [13/34], p=0.2)(35). Taken together with our data, PD-1/PD-L1 expression is unlikely to mediate resistance to BCG immunotherapy in treatment-naïve NMIBC, and high expression of PD-L1, PD-1, or CTLA-4 should not preclude treatment with BCG. Our results raise concerns about ongoing trials combining BCG and immune checkpoint blockade, as they indicate that an immunologically "cold" TME may be a shared mechanism of resistance to both BCG and checkpoint inhibitors(23).

As p53 defects by IHC staining has been one of the most heavily investigated biomarkers in NMIBC with numerous conflicting reports of significance(25), we sought to investigate the additive value of DNA and RNA molecular profiling for p53-pathway alterations in NMIBC. While we found overlap between RNA-based p53 pathway assessment and genomic alterations in cell cycle pathway genes, this does not appear to be clinically relevant for recurrence after BCG. Whether defects in the p53-pathway are associated with progression to secondary MIBC remains unclear, but warrants further investigation(41).

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Limitations of our study include the use of targeted sequencing panels rather than whole exome/transcriptomic sequencing, and that clinical outcomes were available only for the MSK cohort. These limitations may have caused us to miss subtle but important associations with gene expression signatures and precluded our ability to perform robust multivariable analyses. Additional, large prospective validation that account for differences in tumor cell purity is required and ongoing at our center and elsewhere.

In sum, the current analysis demonstrates the feasibility of performing RNA-based subtyping on clinical NMIBC specimens, even those with relatively low tumor cellularity. Gene expression signatures can provide novel biologically and clinically relevant information on the TME (i.e., immune score) and tumor-intrinsic properties (i.e., mutant p53 pathway signature) that are additive and complementary to analysis of genomic DNA. The integration of transcriptomic tumor profiling with exome sequencing is a promising approach to improve risk stratification and treatment selection for patients with NMIBC.

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667 ***Table 1. Patient and tumor characteristics.*** Data are *presented as* mean (SD) or n (%).

	All n = 78	**UNC** n = 28	**MSK** n = 50	**MSK BCG-treated** n = 38
Age (years)	65.0 (12.5)	63.9 (12.1)	65.6 (12.7)	66.8 (11.4)
Male sex	59 (75.6%)	22 (78.6%)	37 (74.0%)	29 (76.3%)
Stage and grade[1]				
T1 high-grade	35 (46.1%)	18 (64.3%)	17 (34.0%)	14 (36.8%)
Ta high-grade	25 (32.0%)	0 (0.0%)	25 (50.0%)	23 (60.5%)
Ta low-grade	18 (21.1%)	10 (35.7%)	8 (16.0%)	1 (2.6%)
Carcinoma in situ (CIS)	22 (28.2%)	8 (28.5%)	14 (28.0%)	14 (36.8%)
Tumor cellularity[1]				
0-40	33 (43.4%)	13 (46.4%)	18 (36.0%)	17 (44.7%)
50-90	43 (56.7%)	11 (39.3%)	32 (64.0%)	21 (55.3%)
UROMOL subclass				
1/ Luminal	26 (33.3%)	7 (25.0%)	19 (38.0%)	10 (26.3%)
2/ CIS-like	28 (35.9%)	8 (27.6%)	20 (40.0%)	17 (44.7%)
3/ Early basal-like	24 (30.85%)	13 (46.4%)	11 (22.0%)	11 (30.0%)
Immune score[2]				
Low	20 (25.6%)	6 (21.4%)	14 (28.0%)	11 (30.0%)
Medium	20 (25.6%)	5 (17.9%)	15 (30.0%)	8 (21.0%)
High	38 (48.7%)	17 (60.7%)	21 (42.0%)	19 (50.0%)
Treatment				
BCG			38 (76%)	38 (100%)
Mitomycin			5 (10%)	0
TURBT only			6 (12%)	0
Cystectomy			1 (2%)	0
Tumor size ≥3 cm			26 (52%)	18 (47.4%)
Multiple tumors			19 (38%)	18 (47.4%)

668

669 [1]Missing values omitted

670 [2]Grouped into tertiles based on score rankBCG = bacillus Calmette-Guérin; CIS = carcinoma in

671 situ; MSK = Memorial Sloan Kettering Cancer Center; SD = standard deviation; TURBT =

672 transurethral resection of the bladder tumor; UNC = University of North Carolina.

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674 **Table 2. Descriptive statistics by UROMOL signature classification.** Data are presented as
675 mean (SD) or n (%).

	1/ Luminal n = 26	2/ Luminal CIS-like n = 28	3/ Early basal-like n = 24	p
Institution/cohort				0.08
UNC	7 (26.9%)	8 (28.6%)	13 (54.2%)	
MSK	19 (73.1%)	20 (71.4%)	11 (45.8%)	
Age (years)	61.6 (14.9)	65.3 (11.2)	68.3 (10.3)	0.159
Male sex	20 (76.9%)	20 (71.4%)	19 (79.2%)	0.797
Stage				0.001
T1	4 (15.4%)	15 (53.6%)	16 (66.7%)	
Ta	22 (84.6%)	13 (46.4%)	8 (33.3%)	
Grade[1]				< 0.001
High	12 (46.2%)	27 (96.4%)	21 (87.5%)	
Low	14 (53.8%)	0 (0.00%)	2 (8.33%)	
Carcinoma in situ	8 (30.8%)	2 (7.14%)	7 (29.2%)	0.063
Tumor cellularity[1]				0.004
0-40	6 (23.1%)	11 (40.7%)	16 (69.6%)	
50-90	20 (76.9%)	16 (59.3%)	7 (30.4%)	

676 [1] Missing values omitted

677 CIS = carcinoma in situ; MSK = Memorial Sloan Kettering Cancer Center; SD = standard
678 deviation; UNC = University of North Carolina.

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680 **Table 3. Gene alterations across UROMOL subclasses.** Bold indicates statistical
681 significance.

Gene	***1/ Luminal*** (n=19)	***2/ Luminal CIS-like*** (n=20)	***3/ Early basal-like*** (n=11)	p=
FGFR3	**17 (89%)**	**10 (50%)**	**2 (18%)**	**<0.001**
KRAS	1	2	0	0.8
HRAS	1	0	2	0.1
ERBB2	**0**	**4 (25%)**	**4 (36%)**	**0.02**
ERBB3	2	1	1	0.8
PIK3CA	5	3	4	0.4
TSC1	1	4	1	0.4
NF1	0	4	1	0.1
TP53	2	5	4	0.3
RB1	0	1	1	0.7
MDM2	1	3	0	0.5
CCND1	0	2	1	0.4
CDKN2A	3	7	2	0.3
CDKN1A	3	3	1	1
STAG2	4	4	2	1
KDM6A	9	6	7	0.2
ARID1A	2	5	2	0.6
KMT2A	2	1	0	0.6
KMT2C	2	3	0	0.5
KMT2D	6	5	1	0.4
EP300	4	1	2	0.3
CREBBP	1	5	1	0.2
TERT	**12 (63%)**	**20 (100%)**	**9 (82%)**	**0.007**
ERCC2	**2 (11%)**	**2 (10%)**	**5 (45%)**	**0.044**
ATM	0	4	2	0.1
BRCA1	0	2	0	0.3
BRCA2	0	1	1	0.7
TET2	**0**	**1 (5%)**	**3 (27%)**	**0.03**
MGA	1	3	0	0.5
BCOR	0	0	1	0.2
BRD4	0	0	0	1
DNAJB1	0	0	1	0.2
IDH1	0	0	0	1
JAK3	0	0	1	1
ARID1B	0	1	2	0.2
BAP1	1	3	2	0.6
PBRM1	2	2	0	0.6
NFE2L2	0	1	0	1
TBX3	1	0	0	0.6
NCOR1	1	0	1	0.5

682
683 CIS = carcinoma in situ
684

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685 **Figure Legends**

686

687 **Figure 1. Heatmap of patient and tumor genetic characteristics according to cohort and**
688 **UROMOL subclass.**

689

690 **Figure 2. Recurrence-free survival (RFS) stratified by UROMOL subclass. A**, entire MSK
691 cohort; **B**, MSK patients treated with BCG; **C**, entire UROMOL cohort; **D**, UROMOL patients
692 treated with BCG.

693

694 **Figure 3. RNA expression-based immune score differs among UROMOL subclasses and**
695 **is associated with recurrence-free survival (RFS) after BCG. A**, Average expression counts
696 for markers of various immune cell types for each patient. **B-E**, RFS by immune score in the **B**,
697 entire MSK cohort; **C**, MSK patients treated with BCG; **D**, entire UROMOL cohort, **E**, UROMOL
698 patients treated with BCG.

699

700 **Figure 4. Immune correlates of recurrence following BCG treatment. A**, Volcano plot
701 demonstrating correlation of expression of individual immune-related genes with probability of
702 remaining free of a high-grade recurrence beyond 24 months after BCG (only genes statistically
703 significant after Benjamini-Hochberg false discovery correction are in red and labeled). **B-C**,
704 RFS by PD-L1 (CD274) expression among BCG-treated (**B**) MSK patients and (**C**) patients in
705 the UROMOL cohort.

706

707

708

709

Figure 1

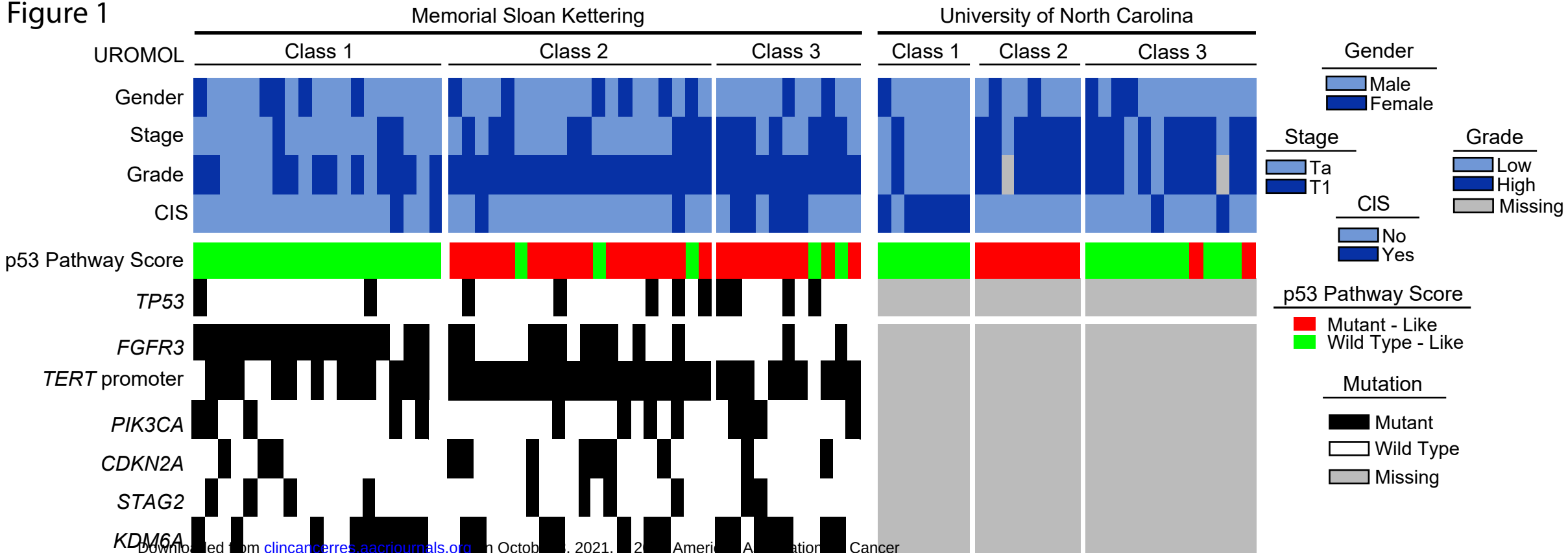

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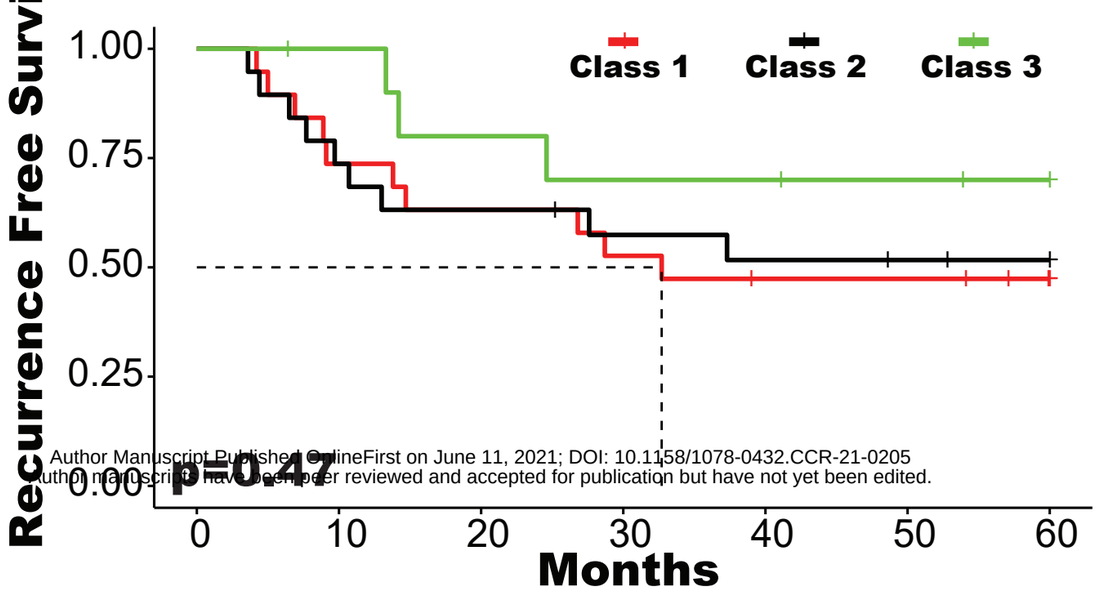
Figure 2

MSK COHORT

ALL PATIENTS



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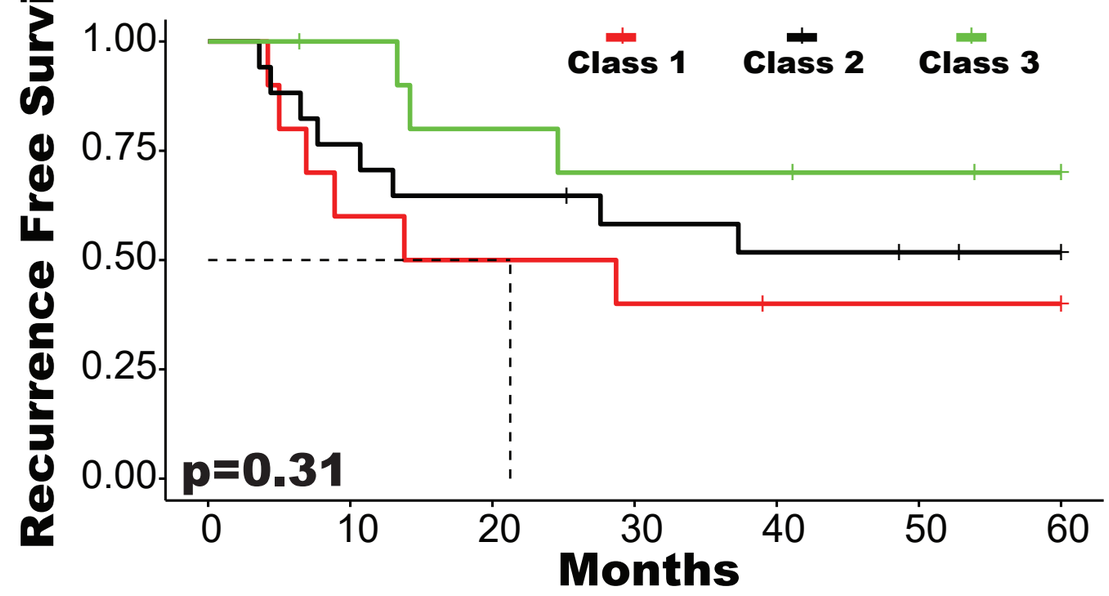
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Class 1	19	14	12	10	8	8	5
Class 2	19	14	12	10	9	8	7
Class 3	11	10	8	7	7	6	5

2A

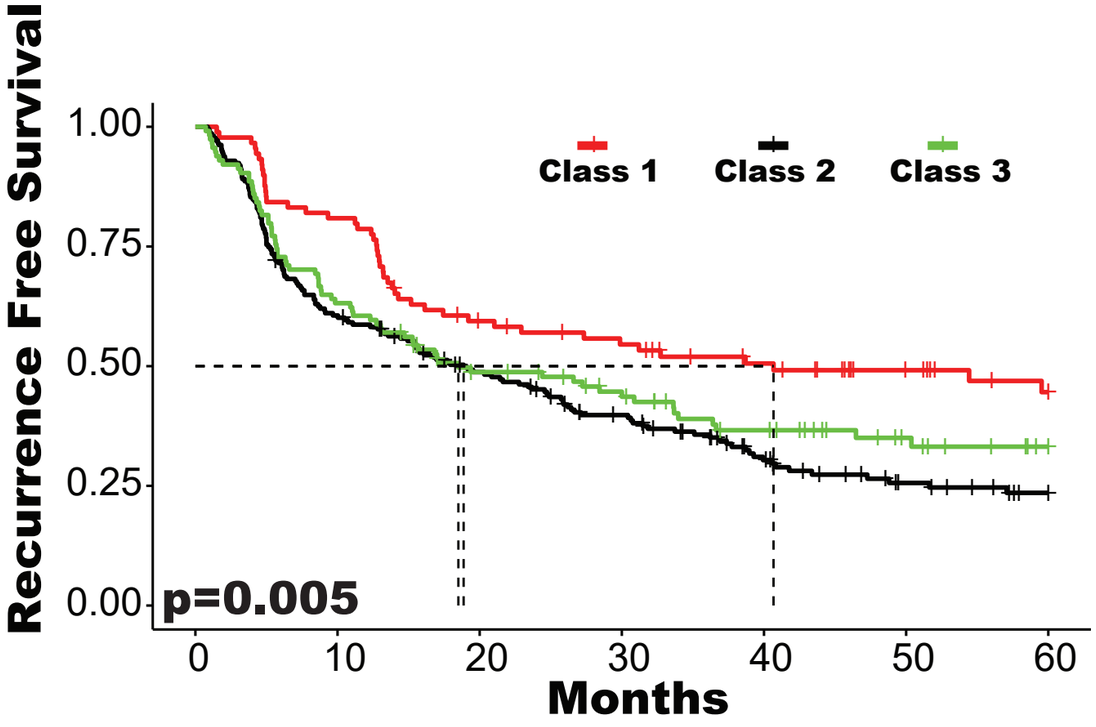
BCG TREATED PATIENTS



Class 1	10	6	5	4	3	3	3
Class 2	17	13	11	9	8	7	6
Class 3	11	10	8	7	7	6	5

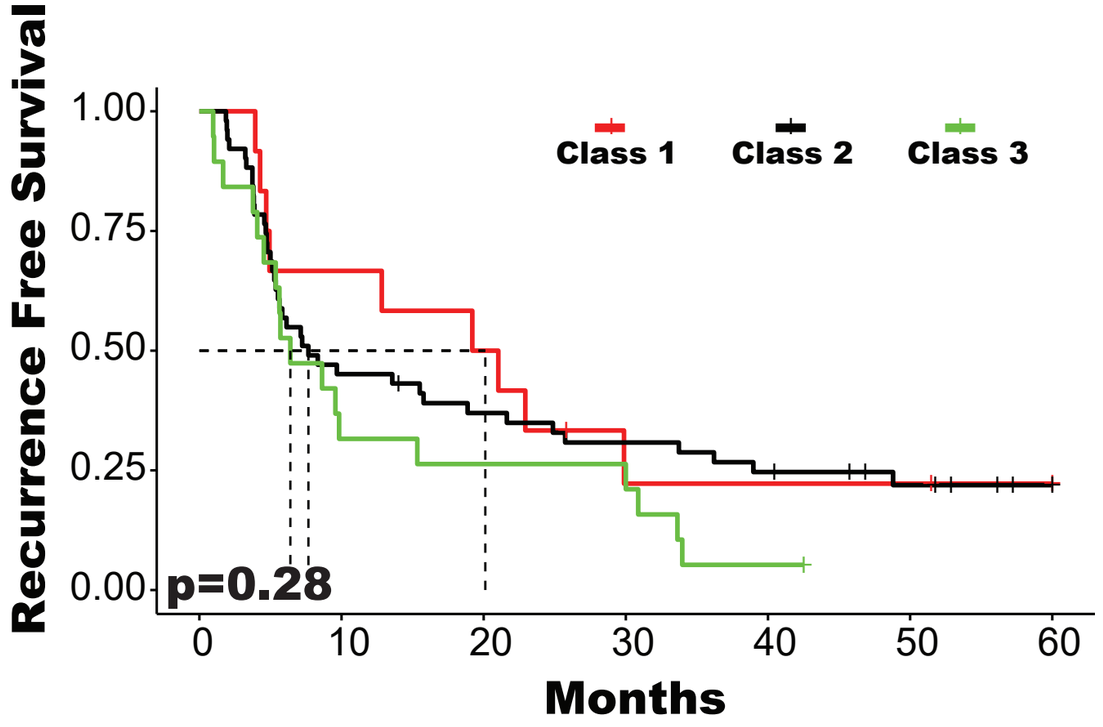
2B

UROMOL COHORT



Class 1	89	72	50	44	36	26	19
Class 2	211	127	95	70	44	27	18
Class 3	114	72	51	42	30	19	11

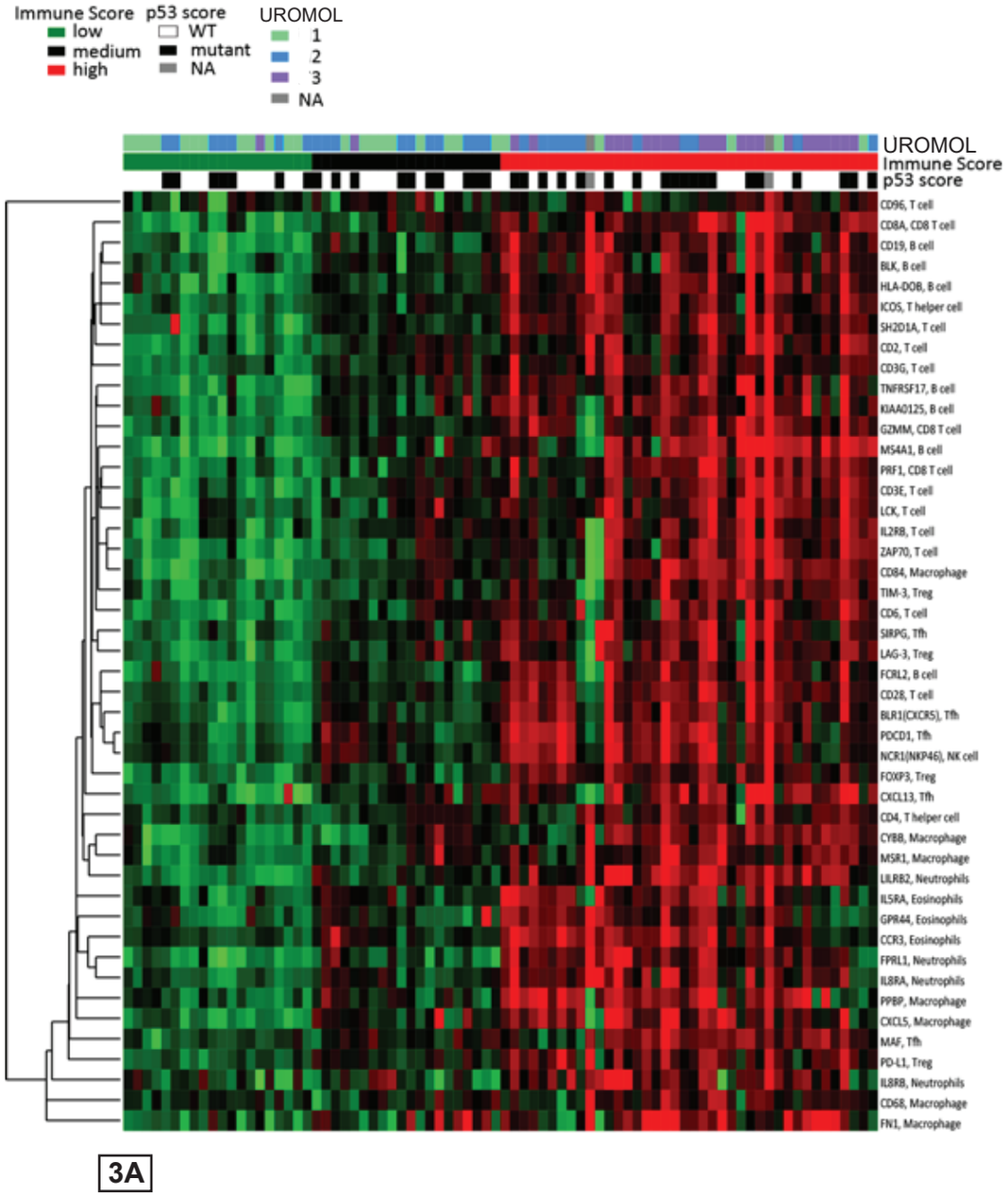
2C



Class 1	12	8	6	2	2	2	1
Class 2	51	23	18	15	12	8	4
Class 3	19	6	5	5	1	0	0

2D

Figure 3

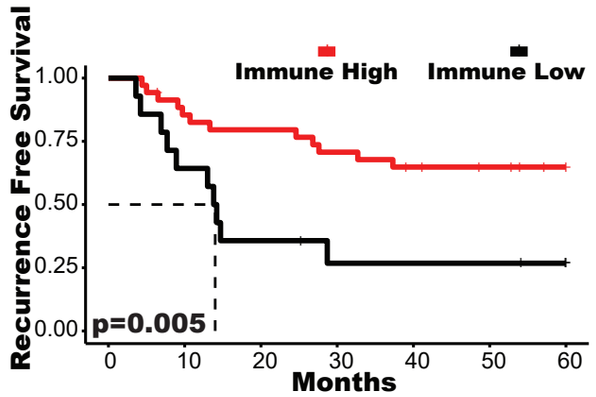


3A

ALL PATIENTS

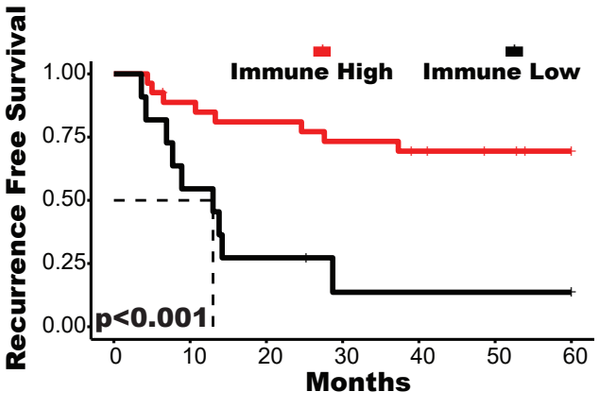
BCG TREATED PATIENTS

MSK COHORT



Immune High	35	29	27	24	21	19	16
Immune Low	14	9	5	3	3	3	1

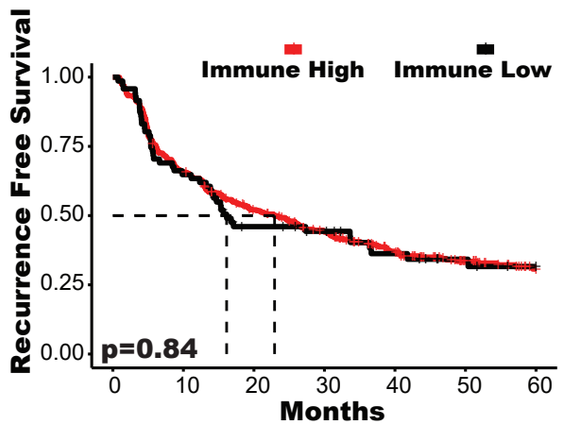
3B



Immune High	27	23	21	19	17	15	13
Immune Low	11	6	3	1	1	1	1

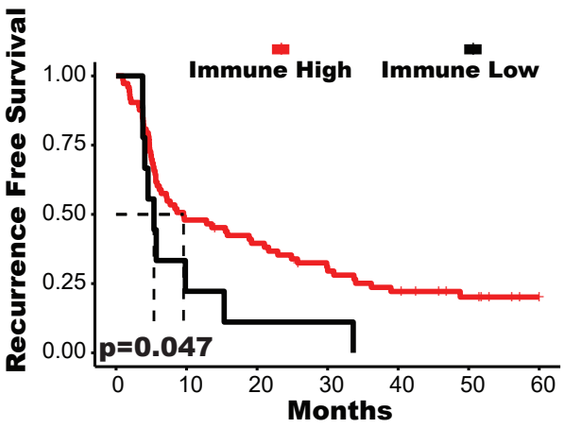
3C

UROMOL COHORT



Immune High	343	225	167	131	92	59	38
Immune Low	71	46	29	25	18	13	10

3D



Immune High	73	35	28	21	15	10	5
Immune Low	9	2	1	1	0	0	0

3E

Figure 4

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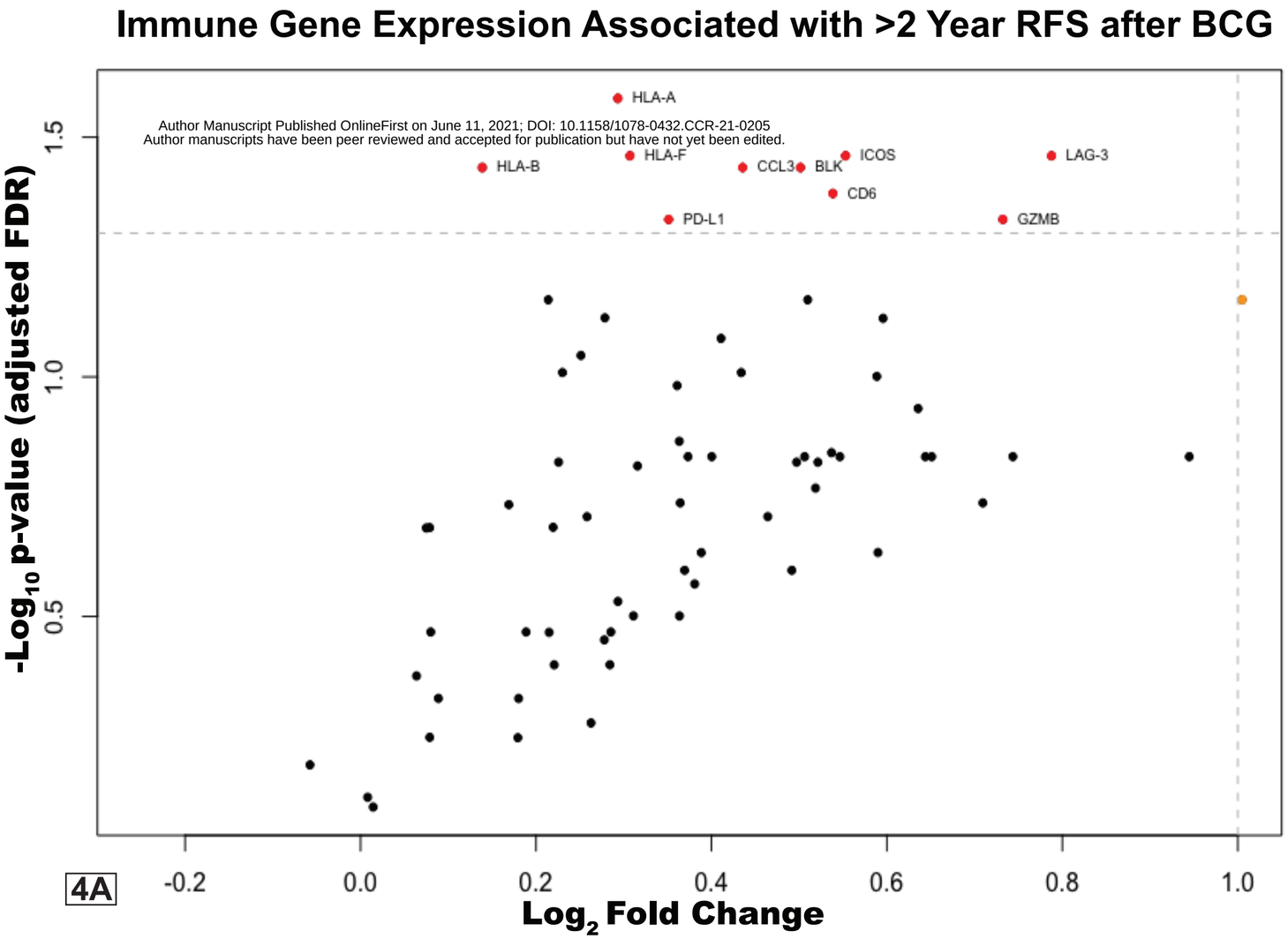
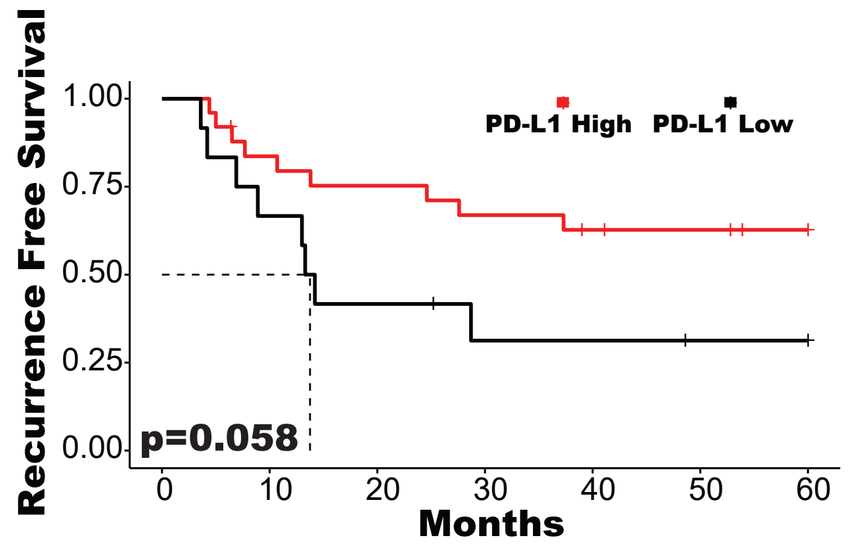


4A

BCG TREATED PATIENTS

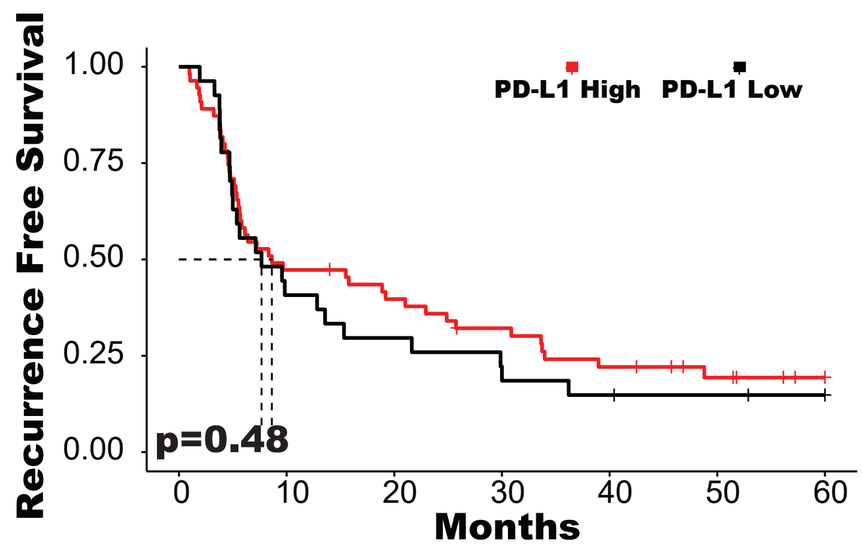
MSK COHORT



PD-L1 High	25	20	18	16	14	13	11
PD-L1 Low	12	8	5	3	3	2	2

4B

UROMOL COHORT



PD-L1 High	55	26	21	16	11	7	3
PD-L1 L ow	27	11	8	6	4	3	2

4C