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Original Article

Genomic and Transcriptomic Profiling of High-risk Bladder Cancer Reveals Diverse Molecular and Microenvironment Ecosystems

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Abstract

Background and objective: High-risk bladder cancer recurs in 30% of cases and causes fatal outcomes in 10% within 2 yr despite surgical resection, endoscopic surveillance, and bacillus Calmette-Guérin (BCG) immunotherapy. The global shortage of BCG highlights the urgent need for alternative or complementary strategies. This study aimed to identify molecular subtypes and develop a precision framework to predict recurrence risk and guide treatment.

Methods: Transcriptomic profiling and targeted genomic sequencing were performed with validation by single-cell RNA sequencing and spatial transcriptomics. A machine learning model incorporating genomic and transcriptomic features was developed to predict recurrence risk.

Key findings and limitations: Four subtypes were identified, and an inflamed tumor subtype with high endogenous retroelement expression and increased commensal bacterial presence demonstrated the highest responsiveness to BCG therapy. The predictive model achieved high accuracy (area under the curve = 0.87, 95% confidence interval: 0.72–1.0) for recurrence risk. Findings are limited by sample size, necessitating validation in larger cohorts.

Conclusions and clinical implications: We describe a new conceptual framework for T1 tumors. Those with increased immune cells (subtype 2) have a better response to BCG, which may be secondary to enhanced baseline immune activity.

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ADVANCING PRACTICE

What does this study add?
This study identifies four molecular subtypes of high-risk (T1) bladder cancer. The subtype most responsive to bacillus Calmette-Guérin (BCG) therapy displays an inflamed phenotype, reactivation of silenced retroelements, and increased density of commensal bacteria. Additionally, we present a precision framework that integrates molecular features to predict recurrence risk with high accuracy (area under the curve = 0.87).

Clinical Relevance
This study provides a comprehensive genomic and transcriptomic characterization of high-risk T1 bladder cancer, identifying four molecular subtypes with distinct biological pathways and therapeutic vulnerabilities. Notably, an inflamed subtype enriched with immune infiltration, retroelement activation, and commensal bacteria demonstrated superior responsiveness to BCG, while a machine learning-based framework accurately predicted recurrence risk. These findings support a precision-medicine strategy to optimize BCG allocation in the context of global shortages, while simultaneously informing the development of alternative targeted therapies for patients who do not respond to BCG. Associate Editor: Gianluca Giannarini, MD.

Patient Summary
There is a global shortage of bacillus Calmette-Guérin (BCG). Our study identifies a tumor subtype with a low recurrence risk after BCG treatment, characterized by immune infiltration and increased commensal bacteria. Targeting BCG use in these patients can reduce strain on supply and guide the development of alternative therapies in the BCG-unresponsive population.

1. Introduction

Nearly 80% of bladder cancer diagnoses are non–muscle-invasive (non–muscle-invasive bladder cancer [NMIBC], stage T1 or less), treated with surgical resection and intravesical immunotherapy for 36 mo [1]. Yet, 26% of T1 tumors will recur within 2 yr, and 10% will progress to metastasis. Bacillus Calmette-Guérin (BCG), the tuberculosis vaccine, is the most effective agent to decrease recurrence and progression and has been the primary oncological treatment of T1 cancers for over 50 yr [2]. However, production shortages have produced a global scarcity of BCG, with no foreseeable improvement in supply. Consequently, there is an urgent need to identify alternative therapeutic targets that can effectively augment or replace BCG in patients with limited access or poor response [3].

Despite its long-term therapeutic benefit in bladder cancer, the mechanisms of response to BCG therapy are not known. Most T1 tumors have limited immune infiltration, and mechanistically, it is unclear whether the immune response to BCG resulting in recurrence-free survival is anti-BCG (a tuberculosis-specific response), antitumor, or both. However, despite multiple attempts to classify tumors based on transcriptomic subtypes, a comprehensive atlas of actionable characteristics encompassing the observed heterogeneity in T1 NMIBC is lacking [4–8]. In this study, we identify four distinct molecular subtypes based on expression profiling of T1 tumors characterized by unique features of transcriptomic and genomic profiles. By integrating these diverse clinical and molecular features, we identify molecular subtypes exhibiting distinctive biology and predict response to BCG.

2. Patients and methods

2.1. Patient cohorts, institutional review board approval, and clinical data

Approval and permission to waive the requirement for informed consent was obtained from the Northwestern Institutional Review Board (STU00204352). All patients were newly diagnosed, had not received prior intravesical treatment, and completed at least the initial phase of BCG, which consisted of six doses at full strength. Recurrence was defined as a high-grade bladder tumor after the last transurethral resection. Response was defined as recurrence after 24 mo of disease-free status. Further details regarding the analysis can be found in the Supplementary material.

3. Results

3.1. Unsupervised clustering identifies drivers of heterogeneity in T1 NMIBC

We profiled 106 stage T1 tumors and sequenced 92 using a 194-gene panel for T1-stage bladder cancers. We identified four tumor subtypes through unsupervised clustering of the 1500 most variable genes (Supplementary Fig. 1A). Comparing these clusters with those from the study of Robertson et al [4], the only other stage-specific profiling, we found an adjusted mutual information score of 0.29, indicating distinct differences in expression profiles (Supplementary Fig. 1B). Classifying the tumors in our cohort using The Cancer Genome Atlas (TCGA) [9] expression classification for muscle-invasive bladder cancer, we found that 88% of tumors were classified as luminal papillary (93/106; Fish-

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er's exact test $p < 0.001$). The remaining tumors were distributed among luminal infiltrated (six of 106), luminal (five of 106), and few basal squamous (two of 106) tumors. In comparison with other modules, 66% of tumors were UROMOL [5] class 2a (70/106; Fig. 1A).

We analyzed recurrence-free survival among tumor subtypes after BCG treatment to see whether molecular differences affected response. Three of the four transcriptomic subtypes had poor outcomes: 13/32 patients in subtype 1 (median survival 20.1 mo), five of 12 in subtype 3 (median survival 22.6 mo), and 14/37 in subtype 4 (median survival 17.7 mo) had recurrences at 24 months. Subtype 2 showed improved recurrence-free survival: 19/22 remained free of recurrence at 24 mo (median survival 34.9 mo; log-rank $p = 0.023$; Fig. 1B).

3.2. Transcriptomic diversity in NMIBC

We investigated gene expression programs for each subtype (Fig. 2A). Subtype 1 tumors showed downregulated immune signatures, and enriched pathways for mRNA splicing and fatty acid metabolism. Subtype 2 tumors enriched immune, interleukin, and cytokine signaling pathways, with a higher RNA-based immune score by an ESTIMATE analysis [10]. These findings align with enhanced immune infiltration in subtype 2 tumors, highlighted by CD45+ immunohistochemistry (Supplementary Fig. 2A). Subtype 3 tumors were enriched in cell cycle regulation and DNA damage response pathways. Subtype 4 tumors had enhanced FGF signaling, protein synthesis, and RNA metabolism pathways (Fig. 2A). We explored regulon networks within each subtype (Fig. 2B) to dissect transcriptional regulatory networks driving these gene programs. Upregulated inflammatory regulons within the STAT and IRF protein families were identified in subtype 2. A notable feature of subtype 2 tumors was significant downregulation of the PPARG regulon (Fig. 2B) [11]. Subtype 3 showed enrichment for transcription factors in DNA damage response and cell cycle regulation, including BRCA1. Subtype 4 tumors displayed significantly higher MYC regulon activity. We validated the biology identified by our classification in a previously reported cohort of non–muscle-invasive bladder tumors (Zuiverloon 2023 cohort [8]; Supplementary Fig. 2B).

3.3. Genomic diversity in high-risk bladder cancer

To complement the transcriptional profiling, we expanded our analysis by investigating the somatic mutational profiles of 92 tumors. We identified mutations in at least one chromatin modifier gene in 93% of tumors (Fig. 3). We then profiled the mutations for each subtype. Subtype 1 tumors had the highest frequencies of mutations in ERBB2 (45%; Fisher's exact test, $p < 0.001$) and ERBB3 (48%; Fisher's exact test, $p < 0.01$; Supplementary Fig. 3D). ERBB2 and ERBB3 are members of the EGFR family of tyrosine kinases that activate downstream pathways such as RAS-ERK and PI3K-AKT. Directed overexpression of ERBB2 was sufficient to induce APOBEC expression, and APOBEC signatures (S2 and S13) were enriched in subtype 3 (Supplementary Fig. 5). Subtype 2 tumors were characterized by mutations in genes implicated in immune suppression. PPARG mutations were detected in 31% of subtype 2 tumors (Fisher's exact test $p = 0.045$), along with XPO1 mutations in 38% of tumors (Fisher's exact test $p < 0.001$; Supplementary Fig. 3D). Subtype 2 tumors also had lower levels of PPARG mRNA and PPARG regulon activity relative to tumors in other subtypes (Fig. 2B and Supplementary Fig. 3A). We found that subtype 3 tumors had a higher frequency of mutations in RB1 (55%; Fisher's exact test $p < 0.01$) and TP53 (64%; Fisher's exact test $p = 0.074$; Supplementary Fig. 3D). Consistent with previous reports of TP53 mutations describing a higher frequency in carcinoma in situ (CIS) tumors, we found tumors within subtype 3 to have elevated CIS scores relative to other clusters (Supplementary Fig. 3B).

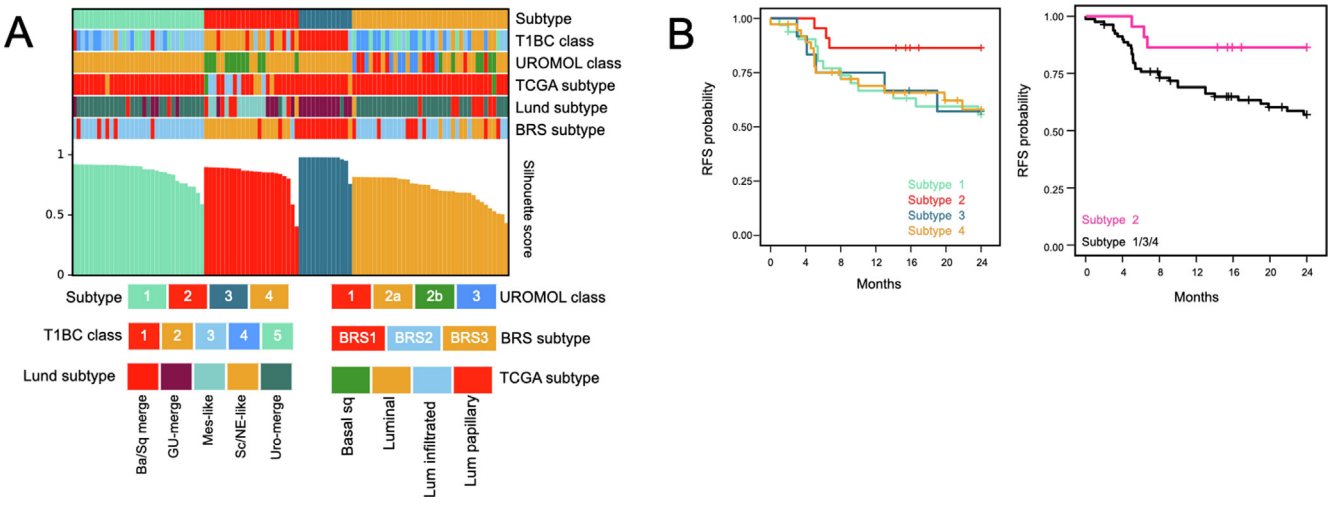

Fig. 1 – Unsupervised clustering reveals four molecular groups of high-risk bladder cancers. (A) Covariate tracks displaying the predicted subtypes of 106 T1 tumors using the T1BC, UROMOL, TCGA, and Lund classifiers. (B) Kaplan-Meier curves of recurrence-free survival for patients defined by transcriptomic subtypes with a log-rank *p* value for the highlighted comparisons (left) and for patients comparing subtype 2 versus subtype 1 + 3 + 4 with a log-rank p value (right). Ba/Sq = basal squamous; GU = genitourinary; Lum = luminal; Mes = mesenchymal; RFS = recurrence-free survival; Sc/NE = small cell/neuroendocrine; TCGA = The Cancer Genome Atlas.

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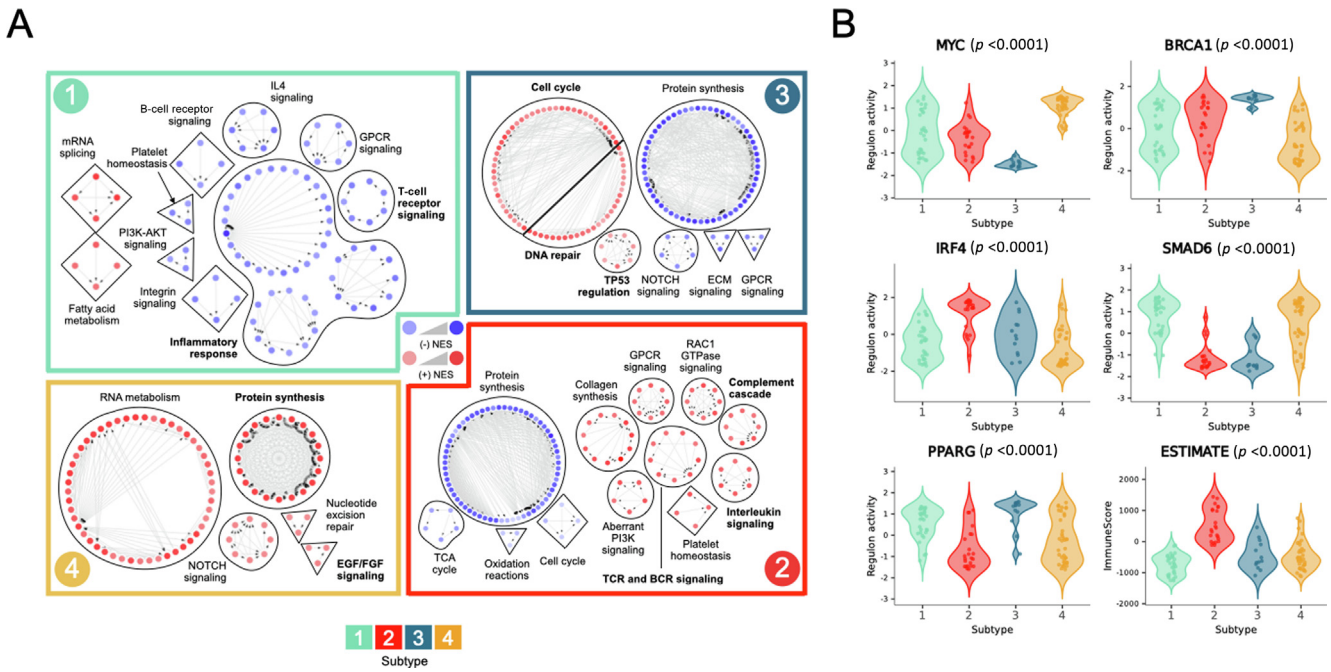

Fig. 2 – Distinct transcriptomic programs distinguish identified NMIBC subtypes. (A) Distinguishing gene program networks in each subtype visualized as a Cytoscape network. Each node represents a pathway, and edges represent shared genes between the connecting pathways. Nodes colored in red are upregulated and nodes colored in blue are downregulated gene sets in each subtype group. (B) Violin plots showing distribution of regulon activity across identified subtypes. BCR = B-cell receptor; ECM = extracellular matrix; IL4 = interleukin 4; TCR = T-cell receptor.

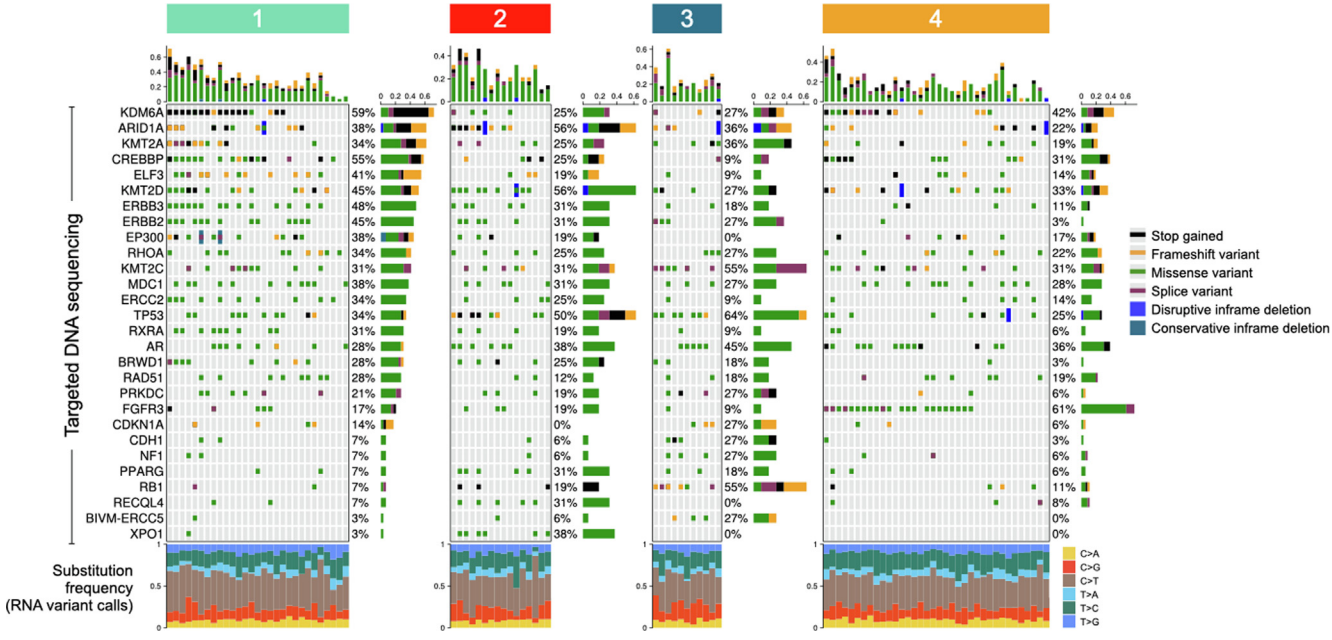

Fig. 3 – Genomic diversity in identified NMIBC subtypes. Oncoprint depicting the recurrent somatic mutations identified in tumors within each subtype (top). Relative proportions of six possible base-pair substitutions identified by RNA variant calling (bottom).

FGFR3 mutations were significantly enriched in subtype 4 tumors (61%) compared with other groups (Fisher's exact test $p < 0.001$; Supplementary Fig. 3D). A feedback loop between FGFR3 and MYC that supports the oncogenic dependency of FGFR3-mutated cell lines has been described in bladder cancer [12], and we found that subtype 4 tumors also exhibited high activity of the MYC regulon and significant enrichment of metabolic programs (Fig. 2B and Supplementary Fig. 3C). Directed overexpression of FGFR3 activating mutations was sufficient to cause sensitivity to

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MEK, EGFR, and Myc inhibitors in cells with normal FGFR3 expression (Supplementary Fig. 4).

3.4. Multiomics transcriptional validation of subtypes by spatial and single-cell profiling to investigate heterogeneity

Bulk RNA sequencing may limit resolution of tumor heterogeneity by averaging expression profiles across individual cells and tissues, particularly rare cells. Our focus on T1 tumor heterogeneity led to the validation of an expression-based subtyping method in two sample types: similar stage (T1) tumors profiled by spatial analysis and single cell RNA sequencing, the first cross-omics evaluation of bulk RNA sequencing–derived gene signatures in bladder cancer. We employed digital spatial profiling using the NanoString GeoMx human whole transcriptome atlas to distinguish tumor-specific epithelial expression from the tumor microenvironment (stroma). By analyzing 129 pan-cytokeratin–positive tumor areas of interest (AOIs) from 43 treatment-naive T1 tumors (Fig. 4A), we carefully selected each PanCK+ AOI to minimize stromal contamination, avoiding tumor AOIs with immune infiltration that could be classified as subtype 2. We gathered a median of three AOIs (range: one to five) per patient and restricted the analysis to those with more than one profiled AOI. Of the patients, 51% (18/35) showed no heterogeneity in subtype expression, with 44% classified as subtype 4, 28% as subtype 3, and 28% as subtype 1 (Fig. 4A). The remaining 17/35 patients displayed heterogeneous subtype expression across tumor AOIs (Fig. 4A). Comparisons of gene expression programs between AOIs in each class preserved biological processes identified from bulk RNA sequencing. For instance, subtype 1 exhibited increased fatty acid metabolism, subtype 3 tumors showed an elevated CIS score (Supplementary Fig. 6A and 6C) and high expression of cell cycle markers (E2F targets and G2M checkpoint), and subtype 4 tumors had enhanced hypoxia pathway and MYC targets, as well as elevated levels of FGFR3 signaling and TGFB1 mRNA (Supplementary Fig. 6A, 6B, 6D, and 6E).

We further validated our subtyping at the single-cell level by single-cell RNA sequencing from eight high-grade tumors. A large fraction of cells in each sample were epithelial (Fig. 4B and 4C). We extracted and reclustered the epithelial population, identifying 11 unique clusters that expressed luminal markers (Fig. 4B and Supplementary Fig. 6F–I). Next, we assigned each cell an identity based on the subtype gene signatures. Six of the eight analyzed tumors had >50% of cells assigned as subtype 4, with one tumor sample classified as subtype 3 (Fig. 4C). One tumor sample had a heterogeneous profile, with 40% of cells belonging to subtype 4 and 45% belonging to subtype 1. A small fraction of cells in two samples was classified as subtype 2 (Fig. 4B). In summary, each subtype's unique gene expression programs and pathways identified using bulk RNA sequencing were validated through isolated interrogation of the epithelium by digital spatial profiling and single-cell RNA sequencing, highlighting heterogeneous gene expression profiles of bladder cancer that appear to be retained across cell compartments and at single-cell resolution.

3.5. Differentiating characteristics of subtype 2 tumors and BCG response

Subtype 2 tumors exhibited the longest recurrence-free survival to BCG, showing enhanced antitumor immunity markers, including higher immune cell infiltration by B cells, NK cells, CD8+ T cells, macrophages, and myeloid dendritic cells (Supplementary Fig. 7), confirmed by histology (CD45+; Supplementary Fig. 2A, bottom panel). Their higher baseline immune infiltration before BCG prompted an investigation into mechanisms enhancing immunity. We noted a significant positive correlation (Pearson coefficient = 0.46, $p < 0.001$) between tumor neoepitope count and immune cell fraction via ESTIMATE (Supplementary Fig. 7). Subtype 2 tumors had more neoantigens (Supplementary Fig. 7C). Given the enhanced immunity noted in skin and renal cancers from local ERV activation, we hypothesized that reactivation of endogenous retroelements or bladder exposure to bacteria/viruses before BCG may aid immune cell recruitment [13]. We compared transposable element expression (LINE and ERV) in tumors with above-median immune scores, finding elevated expression of several retroelements in subtype 2 tumors (Supplementary Fig. 8A). Metatran-

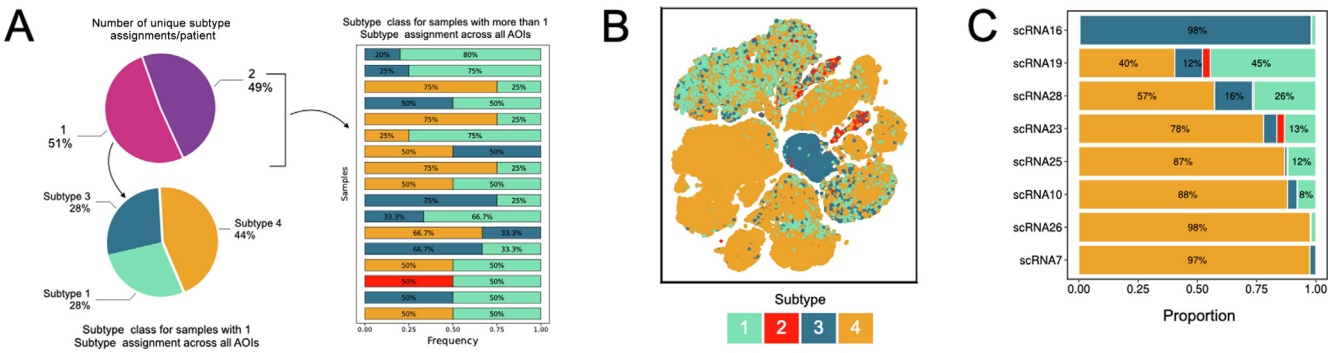

Fig. 4 – Heterogeneity in identified programs dissected using spatial and single-cell transcriptomic profiling. (A) Pie chart showing the frequency of intratumor heterogeneity in subtype expression in a digital spatial profiling dataset of non–muscle-invasive tumors (top, left). Pie chart showing the frequency of subtype classification for patients with homogenous subtype classification across all AOIs (bottom, left). Bar chart showing the subtype assignments for patients with greater than one subtype assignments across all AOIs (right). (B) T-SNE plot showing the results of epithelial subset reclustering colored by subtype assignments. (C) Stacked bar plot indicating the frequency of assigned subtypes for each sample. AOI = area of interest.

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Table 1 – Relative importance of each predictor in the final random forest model

Features in the random forest model	Variable importance score
Bacterial read count	7.73
Neoantigen count	7.22
KMT2A mutation	2.43
RHOA mutation	1.74
KMT2D mutation	1.54
Subtype 2	1.28
FGFR3 mutation	1.10
ARID1A mutation	1.09
ERCC2 mutation	1.00
Subtype 4	0.84

scriptomic deconvolution showed significant upregulation of bacterial species linked to urinary tract infections in subtype 2 tumors (Supplementary Fig. 8B and 8C) [14]. Overall, our results suggest that NMIBC tumors with strong preexisting antitumor immunity may be good candidates for BCG.

3.6. *Integrated multifactorial model of response to BCG immunotherapy*

Our study identified multiple factors that can influence BCG response in T1 tumors. The ability to predict BCG response holds significant clinical implications, prompting us to explore various machine learning algorithms for developing a predictive model, each presenting unique strengths and tradeoffs. We constructed a random forest model for BCG response prediction (Table 1). The best model prediction was obtained using a combination of nine features, which included density of bacterial read count, neoantigen count, subtype 4 and subtype 2 status, and mutations in KMT2A, RHOA, KMT2D, ARID1A, ERCC2, and FGFR3 (area under the curve = 0.87, 95% confidence interval: 0.72–1.0 on the test data; Table 1).

4. Discussion

BCG has been the primary immunotherapy for high-risk bladder cancer for 50 yr, but identification of response features is challenging since antitumor activity is not directly linked to genomic traits. We describe four targetable metaclusters through harmonizing transcriptomic analysis and matched targeted sequencing data (Supplementary Fig. 9). Our new clustering subtyping retains some features of our prior work; for instance, S3 largely represents the T1-LumGU cluster. However, most clusters are novel, aggregating tumors from various subtypes based on molecular characteristics such as FGFR3 expression. Finally, we develop a machine learning model to identify individual features related to clinical recurrence.

Subtype 1 is characterized by mutations in ERBB2/ERBB3 and some tumors with APOBEC mutation signatures. Tan et al found that high HER2 expression in a retrospective analysis of 454 NMIBC patients correlated with a poor BCG response [15]. Antibody-drug conjugates for targeted bladder delivery present a potential treatment for this subtype. Subtype 3 features genomic instability, a higher prevalence of TP53/RB1 mutations, increased activity in cell cycle and DNA repair pathways, and heightened APOBEC mutagenesis. DNA-damaging agents such as mitomycin C, gemcitabine/TAR200, or docetaxel may be most effective due to replication stress. The distinct signature of subtype 4 includes enriched FGFR3 mutations and increased MYC regulon activity. In addition to approved FGFR3 inhibitors such as erdafitinib or TAR210 device, we identify therapies targeting MYC and MEK as potential treatments for subtype 4, either alone or combined with FGFR3 inhibitors [16].

Subtype 2 tumors showed increased immune infiltration and inflammatory gene sets before BCG. Patients with subtype 2 tumors had the best prognosis, with 86% being recurrence free at 12 mo. We also identified reactivated retroelements in tumors with higher immune infiltration and increased bacterial and viral expression. Previous studies link asymptomatic bacteria to better BCG therapy responses [17–19]. Patients with asymptomatic bacteriuria before BCG have longer recurrence-free survival [18] Conversely, antibiotics before or during BCG or systemic therapy correlate with worse recurrence-free survival [17,20]. These findings emphasize the need to investigate the role of bladder microbiota in immunotherapy response.

Our study has several limitations. First, our cohort's sample size is limited. Since many analyses, including the final random forest model, require raw sequencing data, we could not validate our predictive model with other datasets. We tried to validate the prognostic impact of subtyping but could not show that S2 had better outcomes in the Erasmus cohort. As S2 was enriched in immune and epithelial cells, we could not identify it in epithelial tumors from sc-RNA-seq and pan-cytokeratin components via a spatial analysis. Additionally, we could not conduct formal metagenomics or obtain urine cultures for our cohort. We used RNA sequencing to identify neoantigens and mutation signatures; however, errors in translation could affect final expression. Lastly, our machine learning was validated internally, but prospective evaluation would enhance our findings.

5. Conclusions

We identify a new expression-based system to target unique drivers in high-risk bladder cancer and validate the expression of these subtypes spatially and at a single cell resolution.

***Author contributions*:** Joshua J. Meeks 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: Meeks, Meghani.
Acquisition of data: Meghani, Yu, Frydenlund, Li, Choy, Abdulkadir, Meeks.
Analysis and interpretation of data: Meghani, Yu, Frydenlund, Li, Choy, Abdulkadir, Meeks.
Drafting of the manuscript: Meghani, Yu, Frydenlund, Li, Choy, Abdulkadir, Meeks.
Critical revision of the manuscript for important intellectual content: Meghani, Yu, Frydenlund, Li, Choy, Abdulkadir, Meeks.
Statistical analysis: Meghani, Frydenlund, Li, Meeks.

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Supplementary data

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