

The Intratumoral Microbiome Landscape of Bladder Cancer: Ecological Remodeling and Clinical Prognosis

Deyun Shen

The First Affiliated Hospital of USTC, University of Science and Technology of China

Yawei Shu

The First Affiliated Hospital of USTC, University of Science and Technology of China

Jun Xiao

ahx.jmnwk@126.com

The First Affiliated Hospital of USTC, University of Science and Technology of China

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Abstract

Background

The progression of bladder cancer (BCa) from non-muscle-invasive (NMIBC) to muscle-invasive (MIBC) stages is characterized by high aggressiveness and poor outcomes, driving an urgent need for robust prognostic biomarkers. While the intratumoral microbiome is increasingly recognized as an active participant in the tumor microenvironment, its dynamic alterations across BCa stages and potential clinical translational value remain poorly understood.

Methods

We performed 16S rRNA sequencing on 106 BCa tumor tissues to delineate the intratumoral microbiome landscape in NMIBC and MIBC. Alpha and beta diversity analyses, Spearman correlation networks, and Linear discriminant analysis Effect Size (LEfSe) were utilized to evaluate community structure, microbial interactions, and keystone taxa. Univariate and multivariate Cox regression analyses were applied to identify independent prognostic factors and develop a nomogram for predicting overall survival (OS).

Results

Alpha and beta diversity revealed no significant global structural differences between NMIBC and MIBC. However, network analyses demonstrated profound intra-community rewiring; the MIBC network was highly fragmented, lacking positive correlations among beneficial *Lachnospiraceae* and exhibiting competitive exclusion between *Pseudomonas* and *Lactobacillus*. LEfSe identified a microbial shift from a commensal-dominated state (enriched with *Klebsiella* in NMIBC) to a pathogen-associated state in MIBC, marked by opportunistic pathogens like *Streptococcus*, *Ralstonia*, and *Enterobacter*. Through multivariate Cox regression, 11 genera were identified as independent OS predictors, including risk factors (*Finegoldia*, *Aliidiomarina*, *Coprococcus*, *Negativicoccus*, *Stenotrophomonas*, *Ralstonia*) and protective factors (*Paracoccus*, *Comamonas*, *Enterococcus*, *Klebsiella*, *Leptolyngbyaceae_uncl*). The 11-genus-based prognostic nomogram achieved AUC values of 0.784, 0.801, and 0.858 for 1-, 2-, and 3-year OS prediction, respectively, with excellent calibration.

Conclusions

BCa progression to MIBC is driven by the severe remodeling of microbial networks and enrichment of specific opportunistic pathogens rather than a global ecological collapse. The novel 11-genus-based nomogram provides a highly accurate, microbiome-derived prognostic tool for personalized risk stratification in BCa.

Introduction

Bladder cancer (BCa) is the most common malignant tumor of the urinary system, and its morbidity and mortality rate are at the forefront of urinary system tumors[1]. At present, surgical resection combined with postoperative chemotherapy is the main method for BCa patients[2, 3]. Clinically, BCa is primarily classified into non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) based on the depth of tumor invasion[4]. Although patients with NMIBC generally exhibit a relatively high early survival rate, they are highly prone to postoperative recurrence, and a substantial proportion inevitably progress to MIBC, which is highly aggressive, prone to distant metastasis, and associated with a dismal prognosis. Currently, challenges of BCa include highly heterogeneous, with variations in genetic mutations, histological subtypes, and responses to treatment[5]. Reliance on traditional pathological grading and the TNM staging system exhibits notable limitations in precisely predicting the overall survival (OS) of patients[6, 7]. Therefore, there is an urgent need to explore the underlying mechanisms driving the malignant progression of BCa from novel biological dimensions and to identify more robust prognostic biomarkers to guide individualized clinical interventions.

Emerging evidence implicates the tumor-associated microbiome as a critical regulator of cancer progression[8–10]. A variety of bacteria have been shown to promote the malignant progression of a variety of tumors, including colorectal cancer, lung cancer, gastric cancer, prostate cancer, liver cancer, breast cancer, et al[11–14]. In recent years, the rapid advancement of high-throughput sequencing technologies (such as 16S rRNA sequencing and metagenomics) has fundamentally overturned the traditional dogma of a "sterile" urinary tract[15]. Accumulating evidence confirms that the unique intratumoral microbiome ecosystems colonize the urinary system and tumor tissues. These microbial communities, deeply embedded within the tumor microenvironment (TME), are not merely innocent "bystanders" but active participants in tumorigenesis and tumor progression[16–18]. To explore the types of microbiomes that may play a potential role in BCa, several studies performed 16S rRNA sequencing of urine and tumor tissue from BCa patients. A variety of bacteria were found to have significant changes in the tumor tissue or urine of BCa patients. Including *Lactobacillus*, *Ruminococcaceae*, *Acinetobacter*, *Ralstonia*, *Proteus*, *Staphylococcus* and *Enterobacteriaceae*, et al [7, 19–24]. Although studies have revealed dysbiosis in the tissue or urinary microbiome between BCa patients and healthy individuals, systematic investigations into the dynamic alterations of specific microbial communities within tumor tissues across different disease stages, and their clinical translational value remain highly limited[19–21, 25, 26]. During the process of tumor cells breaching the basement membrane and invading the muscularis, drastic alterations in the local microenvironment are frequently accompanied by the aberrant enrichment and metabolic reprogramming of specific microbial communities (such as opportunistic pathogens like *Klebsiella* and *Streptococcus*)[27–31]. These stage-specific differential microbes are highly likely to act as key drivers of malignant progression. However, previous studies predominantly focusing on the diagnostic capacity of the urinary/tissue microbiome[24, 32, 33], in-depth studies focusing on the construction of robust clinical survival prediction models based on the distinct microbial community signatures are still lacking.

In light of this, the present study aims to comprehensively delineate the topographical landscape of microbial communities within NMIBC and MIBC tumor tissues. By deeply analyzing the significantly differential microbial taxa between the two groups and integrating long-term clinical follow-up data, we intend to construct and validate a novel microbiome-based prognostic signature. Furthermore, this study will preliminarily explore the potential metabolic networks and microenvironmental regulatory mechanisms driven by these key differential microbes during BCa progression. This research will not only provide a novel "host-microbiome" interaction perspective for understanding the evolution of BCa but also promises to offer a robust molecular tool for precise risk stratification and survival prediction in patients with bladder cancer.

Patients and methods

Patient recruitment and sample collection

This study was approved by the Ethics Committee of The First Affiliated Hospital of University of Science and Technology of China (USTC) (Approval No.2020-H(N)-135), and written informed consent was obtained from all participants prior to surgery. A total of 106 patients with histologically confirmed BCa, who underwent transurethral resection of bladder tumor (TURBT) or radical cystectomy at our institution between January 2021 and December 2024, were included in this study.

To minimize potential confounding effects on the intratumoral microbiota, strict exclusion criteria were implemented. Patients were excluded if they met any of the following criteria: (1) use of systemic antibiotics, probiotics, or prebiotics within three months prior to surgery; (2) history of preoperative neoadjuvant chemotherapy, intravesical instillation (e.g., BCG), or pelvic radiotherapy; (3) presence of active acute urinary tract infections or other systemic infectious diseases; and (4) presence of other concurrent primary malignancies. To prevent environmental microbial contamination, fresh BCa tumor tissue samples were collected immediately post-excision under strictly sterile conditions in the operating room. The obtained tissue specimens were rapidly rinsed with sterile phosphate-buffered saline (PBS) to remove residual blood and urine, snap-frozen in liquid nitrogen, and subsequently stored at -80°C until total genomic DNA extraction for 16S rRNA sequencing. All relevant clinicopathological characteristics and medium- to long-term follow-up survival data were systematically retrospectively collected from the electronic medical record system. **Table S1** summarized the clinicopathological features of all BCa cases.

16S rRNA gene sequencing and data processing

Total genomic DNA was extracted from the BCa tumor tissue samples using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocols. The concentration and purity of the extracted DNA were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and DNA integrity was evaluated by 1% agarose gel electrophoresis. To profile the intratumoral microbiome, the V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified by polymerase chain reaction (PCR).

Total genomic DNA was extracted from the collected samples, and the integrity and size of the extracted DNA were verified using agarose gel electrophoresis. To profile the microbial communities, the V3-V4 hypervariable region of the bacterial 16S rRNA gene was targeted for amplification. The initial round of PCR was conducted using region-specific primers (341F and 806R), followed by purification of the amplicons with magnetic beads to eliminate unspecific products and impurities. The size and purity of these first-round PCR products were subsequently confirmed via agarose gel electrophoresis.

To construct the sequencing libraries, a second round of PCR was performed to attach sample-specific barcode sequences and sequencing adapters to the ends of the amplicons. Following another round of magnetic bead purification, the resulting libraries were rigorously quantified and normalized. The normalized libraries were then equimolarly pooled and utilized to generate DNA Nanoballs (DNBs). Finally, the prepared DNBs were loaded onto the DNBSEQ-G99RS high-throughput sequencing platform (MGI Tech Co., Ltd., Shenzhen, China) and subjected to paired-end 300 bp (PE300) sequencing according to the manufacturer's standard protocols.

16S rRNA gene sequencing data processing and taxonomic annotation

Raw sequencing data were first subjected to rigorous quality control. Trimmomatic (v0.39) was utilized for quality filtering, followed by the identification and excision of primer sequences using Cutadapt (v4.9). The quality-controlled sequences were subsequently imported into the QIIME 2 pipeline (v2024.10.1) for denoising, error correction, and amplicon sequence variant (ASV) generation via the DADA2 algorithm. To eliminate rare variants and potential sequencing artifacts, ASVs with a relative abundance of less than 0.005% of the total sequences were strictly filtered out. Taxonomic assignment of the remaining high-quality ASVs was conducted using a Naive Bayes classifier trained on the latest versions of the SILVA, Greengenes, and UNITE reference databases.

Microbial diversity analysis

Alpha diversity, which reflects the intra-habitat community richness and evenness, was evaluated using the Observed species, Shannon, Gini-Simpson, and Pielou indices. The differences in alpha diversity between the NMIBC and MIBC groups were analyzed using the Wilcoxon rank-sum test. Beta diversity, designed to assess the inter-habitat structural variation of microbial communities, was calculated based on Bray-Curtis, Jaccard, and Weighted/Unweighted UniFrac distance matrices. Principal Coordinate Analysis (PCoA) was subsequently performed to visualize the separation of the microbial community structures between different clinical stages, and the statistical significance of sample groupings was tested using Analysis of Similarities (ANOSIM) or Permutational Multivariate Analysis of Variance (PERMANOVA). All diversity analyses and taxonomic visualizations were performed using the vegan and ggplot2 packages in R software (version V4.2.1).

Differential abundance and co-occurrence network analysis

To identify specific microbial taxa associated with disease stages, differential abundance analyses were performed using appropriate statistical methods (including Student's t-test, Wilcoxon rank-sum test, and Metastats) based on data distribution. Furthermore, Linear discriminant analysis Effect Size (LEfSe) was utilized to rigorously estimate the effect size of species abundance on the differential outcomes, thereby identifying keystone species with significant inter-group variations. Finally, to explore the complex intra-community interactions across different samples, microbial co-occurrence networks were constructed based on Spearman correlation analysis. Strong and significant interactions, characterized by a P -value < 0.05 and a large absolute correlation coefficient, were selected to map the network topologies.

Identification of Stage-Specific Keystone Taxa

To identify high-dimensional microbial biomarkers with statistically significant differential abundances between the NMIBC and MIBC tissues, the Linear discriminant analysis Effect Size (LEfSe) method was employed. Briefly, the non-parametric factorial Kruskal-Wallis sum-rank test was first used to detect features with significant differential abundance, followed by the Wilcoxon rank-sum test to investigate biological consistency among subclasses. Finally, Linear Discriminant Analysis (LDA) was utilized to estimate the effect size of each differentially abundant taxon. In this study, an LDA score threshold of 2.0 and a P -value < 0.05 were considered to indicate statistically significant enrichment.

Survival Analysis and Prognostic Feature Selection

OS was defined as the time from the date of surgery to death from any cause or the last follow-up. All microbial-related factors (diversity indicators, bacterial abundance) were grouped using the median. Groups with relative bacterial abundance greater than the median were designated as High group, and those with abundance less than the median were designated as Low group. Microbial-related factors median values for each factor are shown in **Table S2**. To identify microbial taxa and clinical variables associated with OS, univariate Cox proportional hazards regression analysis was initially performed. Genera and clinical features that demonstrated a significant prognostic value ($P < 0.1$) in the univariate analysis, as well as clinically significant factors, were subsequently enrolled into a multivariate Cox regression model to adjust for potential confounding effects. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated. Variables retaining statistical significance ($P < 0.05$) in the multivariate model were defined as independent prognostic factors. These survival analyses were executed using the survival and survminer packages in R.

Construction and Validation of the Prognostic Nomogram

Based on the independent microbial prognostic factors identified from the multivariate Cox regression analysis, a comprehensive prognostic nomogram was established to predict the 1-, 2-, and 3-year OS probabilities of BCa patients using the rms package in R. To quantitatively assess the discriminative ability and predictive accuracy of the constructed nomogram, time-dependent receiver operating characteristic (ROC) curves were generated, and the Area Under the Curve (AUC) was calculated for 1-, 2-, and 3-year survival using the timeROC package. Furthermore, calibration curves were plotted to

visually evaluate the concordance between the nomogram-predicted survival probabilities and the actual observed outcomes, with a 45-degree diagonal line representing perfect prediction.

Results

Taxonomic landscape and microbial diversity of the BCa intratumoral microbiome

To investigate the dynamic alterations of the local microbiome during the malignant progression of BCa, we initially evaluated the overall community ecology based on 16S rRNA sequencing data from the 106 BCa tumor tissues (Fig. 1A). The clinicopathological characteristics of the 106 patients were shown in **Table S1**. The 20 microbiomes, including *Pseudomonas*, *Acinetobacter*, *Advenella*, and *Vibrio*, had the highest overall abundance among all tumor samples (Fig. 1B). To investigate the global landscape of the local BCa microbiome, we initially evaluated the diversity between the NMIBC and MIBC groups. Principal coordinate analysis (PCoA) based on Weighted/Unweighted UniFrac, Bray-Curtis, and Jaccard distance matrices for beta diversity demonstrated extensive spatial overlap between the two cohorts, lacking a distinct separation pattern (Fig. 1C). Consistently, alpha diversity analyses revealed no statistically significant differences between the NMIBC and MIBC groups in terms of community richness (Observed ASVs) and evenness (Shannon, Simpson, and Pielou indices) (Fig. 1D, $P > 0.05$).

Taxonomically, the dominant microbial composition at the phylum level was primarily constituted by *Proteobacteria*, *Firmicutes*, and *Bacteroidota* across both groups (**Fig. S1A**). The *Gammaproteobacteria*, *Clostridia*, *Bacteroidia*, *Bacilli*, *Alphaproteobacteria* and *Actinobacteria* were the dominant class level microbial composition (**Fig. S1B**). The *Pseudomonadales*, *Burkholderiales*, *Vibrionales*, *Enterobacterales*, *Bacteroidales*, *Lactobacillales* and *Lachnospirales* were the dominant order level microbial composition (**Fig. S1C**). Further, the dominant Family level microbial composition was *Pseudomonadaceae*, *Moraxellaceae*, *Alcaligenaceae*, *Vibrionaceae*, *Comamonadaceae*, *Enterobacteriaceae* and *Lachnospiraceae* (**Fig. S1D**). At the genus level, *Pseudomonas*, *Acinetobacter*, *Advenella*, *Vibrio* and *Comamonas* maintained high preponderance irrespective of the tumor stage (Fig. 1E). These findings indicate that the progression from NMIBC to MIBC is not accompanied by a global ecological collapse or massive structural remodeling of the intratumoral microbiome, as the baseline community architecture remains relatively stable.

Intratumoral microbial communities lose commensal interactions and internal coordination in MIBC

Although the overall community architecture remained stable, the complex intra-community microbial interactions may exert profound effects on the tumor microenvironment. Therefore, we constructed Spearman correlation networks for the top 30 genera in both NMIBC and MIBC tissues (Fig. 2A and 2B). Overall, the MIBC network demonstrated weaker and fewer significant correlations compared to NMIBC, indicating a more disrupted microbial co-occurrence pattern in advanced disease. Genera belonging to

the family *Lachnospiraceae* (e.g., *Lachnospiraceae_NK4A136_gp* and *Unassigned_Lachnospiraceae*) were positively correlated in NMIBC but not in MIBC, suggesting that muscle invasion may disrupt commensal interactions among butyrate-producing bacteria. A positive correlation between *Gardnerella* and *Staphylococcus* was uniquely observed in NMIBC, which might reflect a uro-genital microbial signature that disappears upon disease progression to MIBC. Notably, in MIBC, most pairwise with no correlations, implying that the microbial community loses internal coordination, potentially due to tumor microenvironment remodeling, antibiotic pressure, or urinary tract obstruction.

In addition, the network analysis unmasked extensive co-occurrence (synergistic) and mutually exclusive (competitive) relationships within the communities (Fig. 2C and 2D). A comparison of the network topologies between NMIBC and MIBC indicated that with increasing tumor malignancy, the correlation intensities and connectivity patterns among core taxa (e.g., *Pseudomonas* and *Streptococcus*) underwent a degree of rewiring. In contrast to NMIBC, the MIBC co-occurrence network exhibited a more fragmented topology, fewer positive correlations, and a higher proportion of environment-derived genera (*Vibrio*, *Marinobacter* and *Enhydrobacter*), suggesting a loss of commensal interactions during muscle invasion. Genera such as *Lachnospiraceae_uncl* and *Muribaculaceae* were present in both networks but showed lower degree centrality in MIBC, implying a reduced ecological role in advanced disease. Positive correlations among putative butyrate-producing bacteria (*Lachnospiraceae* and *Muribaculaceae*) observed in NMIBC were largely absent in MIBC, consistent with a shift toward a pro-inflammatory or dysbiotic microbial community. A negative correlation between *Pseudomonas* and *Lactobacillus* was apparent only in MIBC, potentially reflecting competitive exclusion between opportunistic pathogens and beneficial.

Identification of specific differential taxa associated with muscle-invasive progression

Although the global microbiome structure was comparable between the two groups, we hypothesized that specific keystone taxa might play a pivotal role in the local microenvironmental reprogramming. Therefore, Linear discriminant analysis Effect Size (LEfSe) was employed to identify microbial biomarkers with statistically significant differential abundances between NMIBC and MIBC. The analysis unmasked multiple distinct taxa with strong group preferences (Fig. 3A-B). Notably, the MIBC tissues exhibited a significant enrichment of several opportunistic pathogens and specific metabolic bacteria, including *Streptococcus*, *Ralstonia*, *Corynebacterium*, *Enterobacter*, *Neisseria*, and *Enterococcus*. Conversely, *Klebsiella* showed a higher relative abundance predominantly in the NMIBC tissues. LEfSe cladogram revealed distinct taxonomic enrichments between MIBC and NMIBC (Fig. 3C). In MIBC, 6 opportunistic genera including *Streptococcus*, *Ralstonia*, *Corynebacterium*, *Enterobacter*, *Enterococcus*, and *Neisseria* were significantly enriched, whereas *Klebsiella* was more abundant in NMIBC, suggesting a shift from a commensal-dominated to pathogen-associated microbiota during muscle invasion. Figure 3D shows the comparison results of abundance plot of six bacteria that were significantly enriched in MIBC. These specific microbial taxa, significantly enriched during the muscle-invasive stage,

not only constitute a crucial microbial signature for distinguishing disease stages but also strongly imply their potential involvement.

Univariate and multivariate Cox regression analysis of microbial factors associated with BCa overall survival

To determine whether OS was associated with microbial indices, we first included some routine microbial indices in addition to general clinicopathological characteristics for univariate and multivariate Cox regression analysis. Univariate Cox regression analyses were performed for general clinicopathological characteristics, Preoperative urine bacterial count, Preoperative urine Nitrite and diversity indices (observed species, Shannon, Gini simpson, Pielou and goods coverage). Pathological N stage (Nx vs. N0), MIBC (vs. NMIBC), Preoperative urine bacterial count and goods coverage were significantly associated with OS (**Fig. S2A**, $P < 0.05$).

In multivariate analysis, incorporated α -diversity indices (Gini simpson, goods coverage) and Preoperative urine bacterial count. Pathological N stage (Nx vs. N0, HR = 8.32, $P < 0.05$) and MIBC (vs. NMIBC) remained significant (HR = 8.85, $P < 0.05$). Further, Preoperative urine bacterial count (HR = 1.001, $P < 0.05$), high Gini simpson (HR = 7.8, $P < 0.05$) and high goods coverage (HR = 26.3, $P < 0.05$) were significantly associated with OS, suggesting these microbial indices were independent predictor of OS, each 1% increase in the relative value of Preoperative urine bacterial count was associated with a 1% higher risk of death, high Gini simpson was associated with a 680% higher risk of death, and high level of goods coverage was associated with a 2530% higher risk of death(**Fig. S2B**).

Having established that OS in BCa patients is associated with certain routine microbial indices, we further explored whether the abundance level of specific bacterial genera was an independent predictor of OS. To further investigate whether the abundance of specific bacterial genera independently predicts overall survival (OS), we focused on two categories of genera: (i) those significantly enriched in NMIBC vs MIBC (*Streptococcus*, *Ralstonia*, *Corynebacterium*, *Enterobacter*, *Enterococcus*, *Neisseria*, and *Klebsiella*), and (ii) 40 additional high-abundance genera in tumor tissues (e.g., *Anoxybacillus*).

Univariate Cox regression analyses revealed that high abundance of several genera was significantly associated with worse OS (Fig. 4, $P < 0.05$). The highest HR were observed for *Aliidiomarina* (HR = 4.55, 95% CI: 2.43–8.51, $P < 0.001$), *Coproccoccus* (HR = 3.84, 95% CI: 2.01–7.37, $P < 0.001$), *Ralstonia* (HR = 3.73, 95% CI: 2.00–6.95, $P < 0.001$), *Enterobacter* (HR = 3.62, 95% CI: 1.90–6.91, $P < 0.001$), and *Streptococcus* (HR = 3.28, 95% CI: 1.73–6.20, $P < 0.001$). High abundances of *Neisseria* (HR = 3.15), *Vibrio* (HR = 2.99), *Fingoldia* (HR = 2.58), and *Methyloversatilis* (HR = 2.56) were also significantly associated with poor OS (all $P < 0.05$). Of note, a high abundance of *Advenella* was associated with favorable OS (HR = 0.376, $P = 0.027$), suggesting a potential protective role. Other genera, including *Klebsiella*, *Enterococcus*, *Pseudomonas*, and *Lactobacillus* showed no significant association with OS in univariate analysis (Fig. 4, $P > 0.05$).

To identify bacterial genera that independently predict overall survival (OS) in bladder cancer patients, we performed multivariate Cox regression analysis incorporating all genera that were significant in univariate analysis. As shown in Fig. 5, high abundance of several genera remained independent risk factors for OS: *Finegoldia* (HR = 7.95, 95% CI: 2.10–30.1, $P = 0.002$), *Aliidiomarina* (HR = 6.69, 95% CI: 1.46–30.8, $P = 0.015$), *Coprococcus* (HR = 5.93, 95% CI: 1.38–25.5, $P = 0.017$), *Negativicoccus* (HR = 14.1, 95% CI: 2.60–76.5, $P = 0.002$), *Stenotrophomonas* (HR = 13.6, 95% CI: 1.32–141, $P = 0.029$), and *Ralstonia* (HR = 326, 95% CI: 2.38–44600, $P = 0.021$). In contrast, high abundance of other genera was associated with better OS (protective factors): *Paracoccus* (HR = 0.0458, 95% CI: 0.00466–0.449, $P = 0.008$), *Comamonas* (HR = 0.0938, 95% CI: 0.0104–0.847, $P = 0.035$), *Enterococcus* (HR = 0.0937, 95% CI: 0.0172–0.511, $P = 0.006$), *Klebsiella* (HR = 0.173, 95% CI: 0.0425–0.705, $P = 0.014$), and *Leptolyngbyaceae_uncl* (HR = 0.183, 95% CI: 0.0459–0.731, $P = 0.016$). Notably, several genera that were significant in univariate analysis, including *Streptococcus*, *Enterobacter*, and *Neisseria*, lost statistical significance after multivariate adjustment ($P > 0.05$), suggesting that their prognostic value may be confounded by other clinical variables.

Development and performance of a nomogram for predicting 1-, 2-, and 3-year overall survival

Based on the 11 bacterial genera that were identified as independent prognostic factors in the multivariate Cox regression analysis (*Finegoldia*, *Aliidiomarina*, *Coprococcus*, *Negativicoccus*, *Stenotrophomonas*, *Ralstonia*, *Paracoccus*, *Comamonas*, *Enterococcus*, *Klebsiella*, and *Leptolyngbyaceae_uncl*), we constructed a nomogram to predict OS at 1, 2, and 3 years in bladder cancer patients (Fig. 6A). The discriminative ability of the nomogram was assessed using time-dependent receiver operating characteristic (ROC) curves. The area under the curve (AUC) values were 0.784 at 1 year, 0.801 at 2 years, and 0.858 at 3 years, indicating good to excellent predictive accuracy, with the best performance at 3 years (Fig. 6B).

Calibration plots for 1-, 2-, and 3-year survival demonstrated good agreement between the nomogram-predicted probabilities and the actual observed survival outcomes, as the calibration curves were close to the ideal 45-degree diagonal line (Fig. 6C). These results suggest that the microbial genera-based nomogram provides well-calibrated and clinically useful predictions of medium-term survival in bladder cancer patients.

Discussion

In this study, we comprehensively delineated the dynamic alterations of the intratumoral microbiome during the malignant progression of BCa from NMIBC to MIBC. While previous studies have primarily focused on the diagnostic capacity of urinary or tissue microbiomes[22, 24], our research extends this paradigm by systematically investigating the intratumoral microbial landscape across different disease stages to construct a robust prognostic signature. We demonstrated that the progression to MIBC is accompanied by a profound remodeling of microbial interactions and the specific enrichment of

opportunistic pathogens, rather than a global ecological collapse. Furthermore, we successfully constructed an 11-genus-based nomogram that provides highly accurate predictions for medium-term OS.

Interestingly, the alpha and beta diversity analyses revealed no significant differences in global community structure between the NMIBC and MIBC cohorts. This observation is highly noteworthy when compared to previous studies characterizing the BCa urobiome[34–36], where authors reported significantly inconsistent alterations in diversity indices. Our findings align with recent tumor tissue-based profiling studies that found no significant alterations in alpha or beta diversities between NMIBC and MIBC stages[37, 38]. These discrepancies often stem from variations in different sample types (urine vs. tissue) and external confounding factors. By focusing strictly on the changes of microbe-types, our results suggest that malignant progression to MIBC is not necessarily accompanied by a global ecological collapse. Instead, we identified a profound rewiring of intra-community interactions. Network analysis indicated that the MIBC microenvironment exhibits a more fragmented microbial topology with weaker positive correlations. Notably, the synergistic interactions among putative butyrate-producing bacteria-*Lachnospiraceae*[39] which were prominent in NMIBC, were largely absent in MIBC. The loss of these commensal networks may reflect a critical shift toward a dysbiotic, pro-inflammatory tumor microenvironment (TME) that facilitates tumor invasion[40]. Additionally, the emergence of negative correlations between beneficial bacteria (e.g., *Lactobacillus*, *Akkermansia*) and opportunistic pathogens (e.g., *Pseudomonas*) in MIBC further supports the hypothesis of competitive exclusion during tumor progression.

Furthermore, the localized microenvironmental reprogramming during muscle invasion was further characterized by the aberrant enrichment of specific keystone taxa. the specific pathogenic shifts identified in our LEfSe analysis are strongly supported by our emerging independent cohorts. The LEfSe analysis revealed a significant transition from a commensal-dominated microbiota in NMIBC (enriched in *Klebsiella*) to a pathogen-associated microbiota in MIBC. We observed a marked accumulation of opportunistic genera in MIBC tissues, including *Streptococcus*, *Ralstonia*, *Enterococcus*, *Enterobacter*, and *Neisseria*. Previous studies have identified *Streptococcus*, *Enterococcus* and *Enterobacter* as the general dominant taxon in BCa compared to controls[27, 31, 41–44]; however, our findings refine this by linking its enrichment specifically to the muscle-invasive stage. Other researchers have also identified these opportunistic pathogens as hallmark taxa in highly invasive urological malignancies. The reproducible enrichment of these specific genera across different independent cohorts strongly implies that they are not merely transient passengers but potentially universal drivers orchestrating the malignant progression of bladder cancer, warranting further functional exploration. Furthermore, previous reports by Hussein et al. and Wang et al. noted a significant reduction of beneficial *Lactobacillus* in the urine of BCa patients[45, 46]. Concordantly, our network analysis uniquely captured a negative correlation between *Pseudomonas* and *Lactobacillus* specifically in MIBC, highlighting the competitive exclusion of beneficial microbes by opportunistic pathogens during tumor progression.

A primary objective of this study was to translate these microbiome signatures into a robust clinical tool. While recent prognostic models for BCa predominantly rely on host transcriptomic data (e.g., TCGA immune-related gene signatures) and previous microbiome studies mainly sought early diagnostic markers[47–49], we identified 11 bacterial genera as independent prognostic factors for overall survival directly from 16S rRNA sequencing. Through univariate and multivariate Cox regression analyses, we identified 11 bacterial genera as independent prognostic factors for OS. High abundances of *Finegoldia*, *Aliidiomarina*, *Coprococcus*, *Negativicoccus*, *Stenotrophomonas* and *Ralstonia* emerged as significant independent risk factors, while genera such as *Paracoccus*, *Comamonas*, *Enterococcus*, *Klebsiella* and *Leptolyngbyaceae_uncl* acted as protective factors. It is worth noting that while several pathogens like *Streptococcus* and *Enterobacter* showed strong risk associations in univariate analysis, they lost statistical significance after multivariate adjustment, underscoring the complex confounding effects of clinical variables on microbial prognostic value. Integrating these 11 independent genera, our novel nomogram demonstrated excellent discriminative ability, yielding AUC values of 0.784, 0.801, and 0.858 for predicting 1-, 2-, and 3-year OS, respectively. The strong agreement in calibration plots further confirms that this microbiome-based signature provides well-calibrated and clinically useful survival predictions. This strongly underscores those specific intratumoral taxa, such as the high-risk genera *Finegoldia* and *Ralstonia*, provide substantial independent prognostic value beyond traditional clinical variables.

Despite these promising findings, our study has limitations. As a retrospective analysis based on 16S rRNA sequencing, our taxonomic resolution is largely restricted to the genus level, limiting direct metabolic pathway inferences. While we established strong statistical associations between the intratumoral microbiota and clinical outcomes, the precise "host-microbiome" crosstalk requires rigorous *in vitro* and *in vivo* functional validation. Future studies employing single-cell transcriptomics alongside spatial microbiology will be vital to unraveling these complex mechanisms.

In conclusion, our study maps the topographical landscape of the intratumoral microbiome in BCa and uncovers the critical remodeling of microbial networks during muscle invasion. The development of an 11-genus-based prognostic nomogram offers a highly accurate, microbiome-derived molecular tool to guide individualized clinical interventions and risk stratification in patients with bladder cancer.

Abbreviations

<i>ANOSIM</i>	Analysis of Similarities
<i>ASV</i>	Amplicon sequence variant
<i>AUC</i>	Area Under the Curve
<i>BCa</i>	Bladder cancer
<i>BCG</i>	Bacillus Calmette-Guérin
<i>CI</i>	Confidence interval
<i>DNBs</i>	DNA Nanoballs
<i>HR</i>	Hazard ratio
<i>LDA</i>	Linear Discriminant Analysis
<i>LEfSe</i>	Linear discriminant analysis Effect Size
<i>MIBC</i>	Muscle-invasive bladder cancer
<i>NMIBC</i>	Non-muscle-invasive bladder cancer
<i>OS</i>	Overall survival
<i>PBS</i>	Phosphate-buffered saline
<i>PCoA</i>	Principal Coordinate Analysis
<i>PCR</i>	Polymerase chain reaction
<i>PERMANOVA</i>	Permutational Multivariate Analysis of Variance
<i>ROC</i>	Receiver operating characteristic
<i>TCGA</i>	The Cancer Genome Atlas
<i>TME</i>	Tumor microenvironment
<i>TNM</i>	Tumor, Node, Metastasis
<i>TURBT</i>	Transurethral resection of bladder tumor

Declarations

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Data availability

The datasets generated and analyzed during the current study are available in the NCBI BioProject repository-PRJNA1468207, <http://www.ncbi.nlm.nih.gov/bioproject/1468207> . All other data relevant to the study are included in the article or uploaded as supplementary information files.

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Contributions

D.S. (Deyun Shen) designed the study, analyzed the data, and drafted the manuscript; **Y.S.** (Yawei Shu) was responsible for sample collection and processing; **J.X.** (Jun Xiao) acquired the funding, supervised the overall project, and critically reviewed the final manuscript. All authors read and approved the final manuscript.

Ethics declarations Ethics approval and consent to participate

Informed written consent was obtained from all patients prior to sample collection. The study was conducted in strict accordance with the ethical principles outlined in the Declaration of Helsinki and received approval from the Ethics Committee of The First Affiliated Hospital of University of Science and Technology of China (USTC) (Approval No. 2020-H(N)-135).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

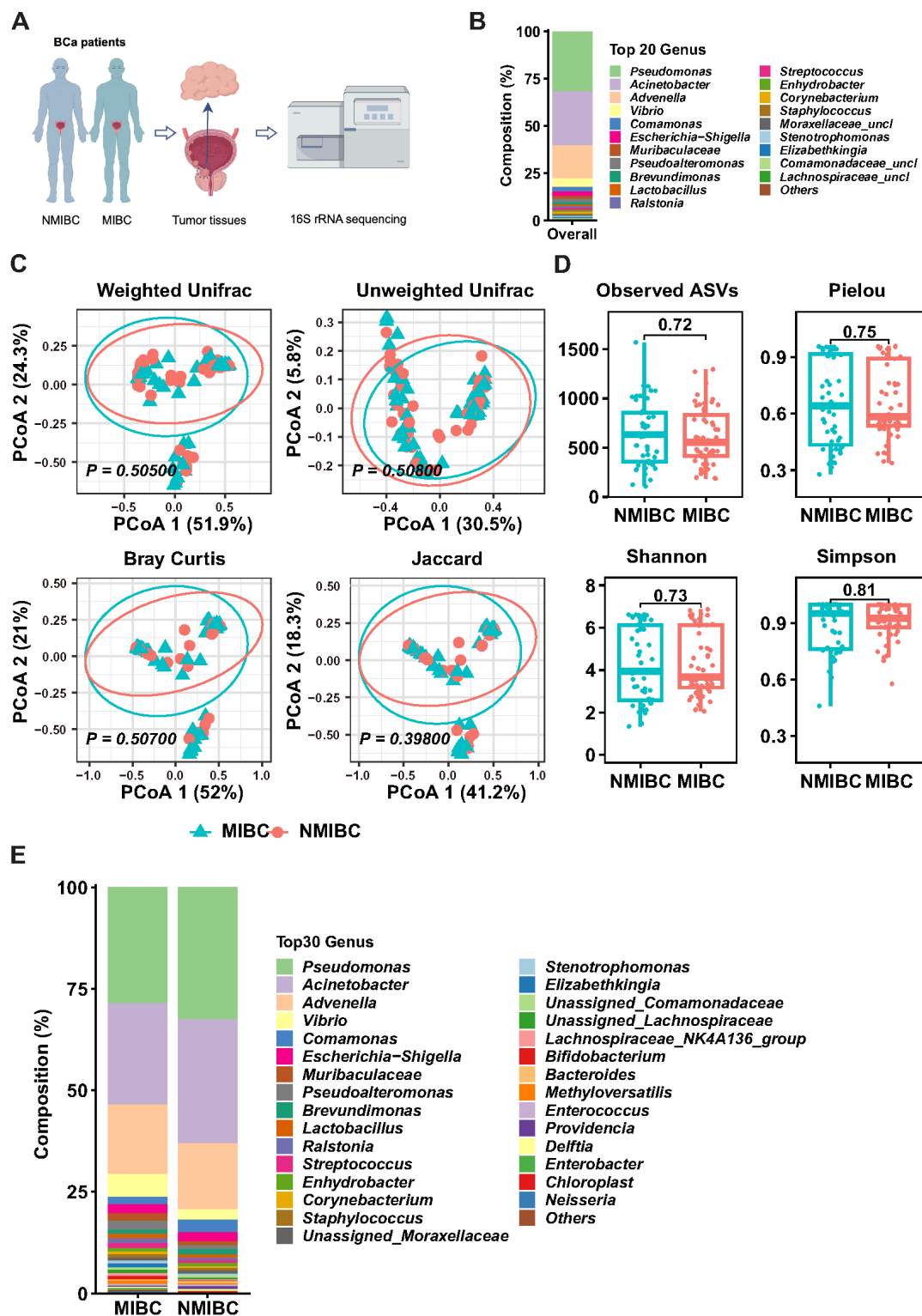


Figure 1

Taxonomic landscape and microbial diversity of the BCa intratumoral microbiome. (A) Schematic illustration of the overall study design. Fresh BCa tumor tissues were collected from patients with non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) and subsequently subjected to 16S rRNA gene sequencing. (B) Stacked bar plot showing the overall relative abundance of the top 20 genera across all tumor tissues. (C) Principal coordinate analysis (PCoA) plots depicting the

beta diversity based on Weighted UniFrac, Unweighted UniFrac, Bray-Curtis, and Jaccard distance matrices. **(D)** Comparison of alpha diversity indices (Observed ASVs, Pielou, Shannon, and Simpson) between the NMIBC and MIBC cohorts. $P > 0.05$ indicate no statistically significant differences in global diversity. **(E)** Stacked bar plot illustrating the relative abundance of the top 30 most abundant genera compared between the NMIBC and MIBC cohorts.

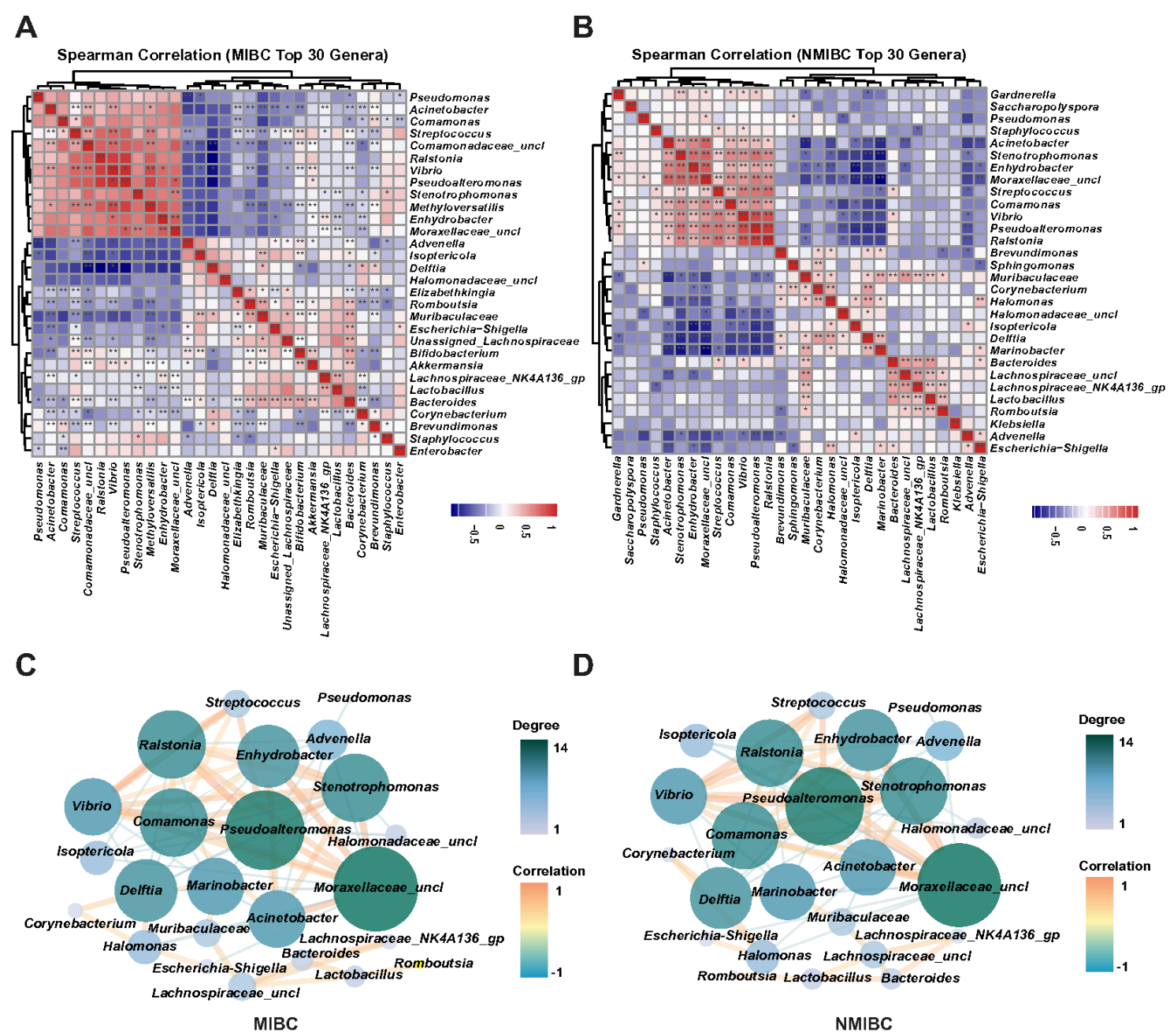


Figure 2

Intratumoral microbial communities lose commensal interactions and internal coordination in MIBC. **(A), (B)** Heatmaps of Spearman correlation coefficients among the top 30 most abundant bacterial genera within the MIBC **(A)** and NMIBC **(B)** tumor microenvironments. Red and blue indicate positive

(synergistic) and negative (competitive) correlations, respectively. Asterisks denote statistical significance (* $P < 0.05$, ** $P < 0.01$). **(C), (D)** Topologies of the microbial co-occurrence networks in the MIBC **(C)** and NMIBC **(D)** cohorts. Each node represents a specific bacterial genus. The color gradient and size of the nodes are proportional to their degree centrality in the network. The edges connecting the nodes represent significant correlations, with edge colors reflecting the Spearman correlation coefficients. The network in MIBC exhibits a more fragmented topology with weaker positive correlations compared to NMIBC.

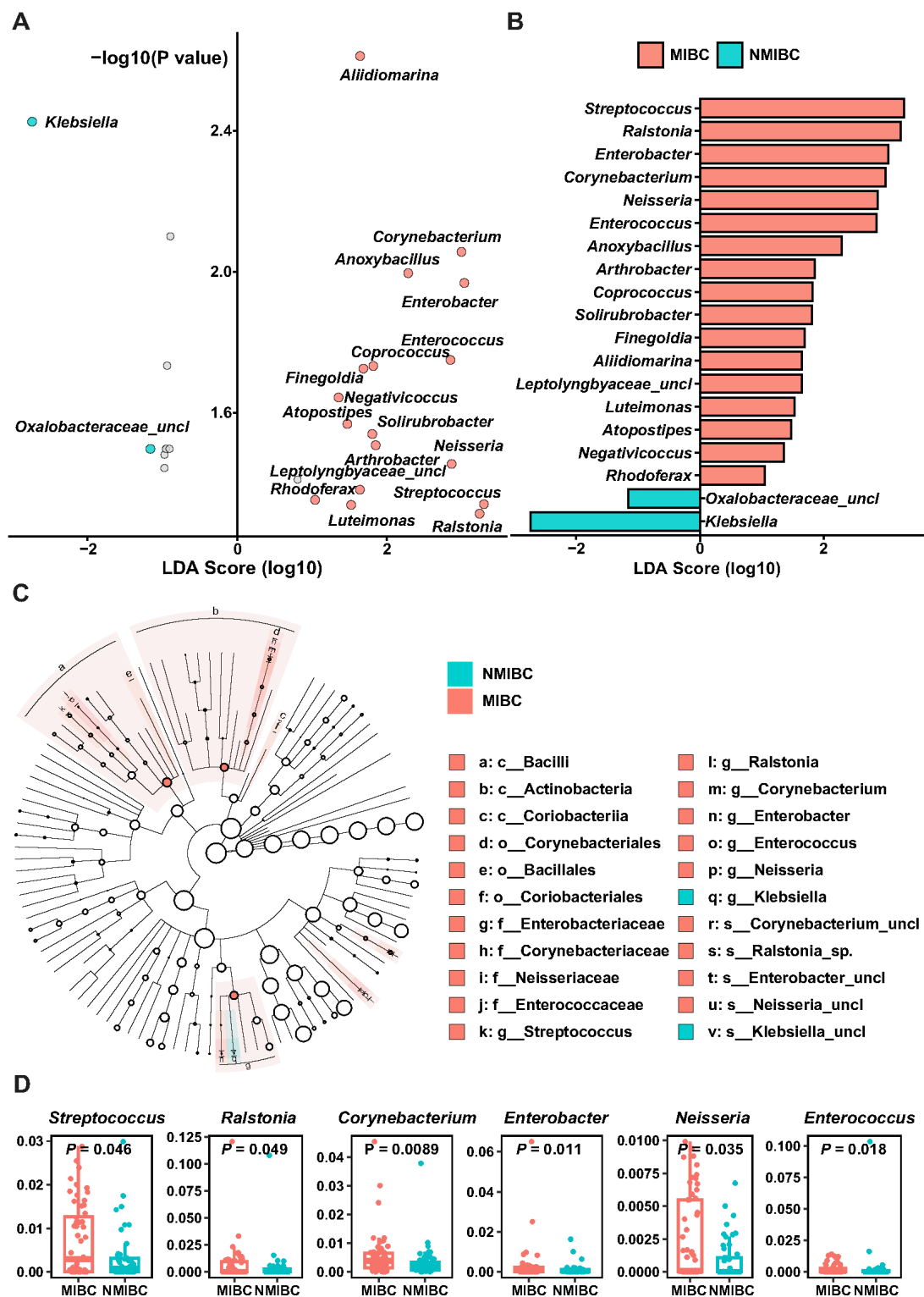


Figure 3

Identification of specific differential taxa associated with muscle-invasive progression. (A)(B)(C)(D)(E)(F)(G)(H)(I)

(A) Scatter plot illustrating the differentially abundant taxa between the MIBC and NMIBC groups, showing the LDA score on the x-axis and statistical significance ($-\log_{10}(P \text{ value})$) on the y-axis. **(B)** Bar chart of the LDA scores representing specific microbial taxa significantly enriched in the MIBC (red) and NMIBC (blue) cohorts. Only taxa meeting the significant LDA threshold are shown. **(C)** LEfSe cladogram showing the phylogenetic distribution and structural representation of the differential microflora. The concentric circles represent taxonomic levels from phylum to genus (from inner to outer). Red and blue nodes indicate microbial taxa significantly enriched in MIBC and NMIBC, respectively. **(D)** Boxplots comparing the relative abundances of six opportunistic genera (*Streptococcus*, *Ralstonia*, *Corynebacterium*, *Enterobacter*, *Neisseria*, and *Enterococcus*) that are significantly enriched in the MIBC (red). *P*-values were calculated using the Wilcoxon rank-sum test.

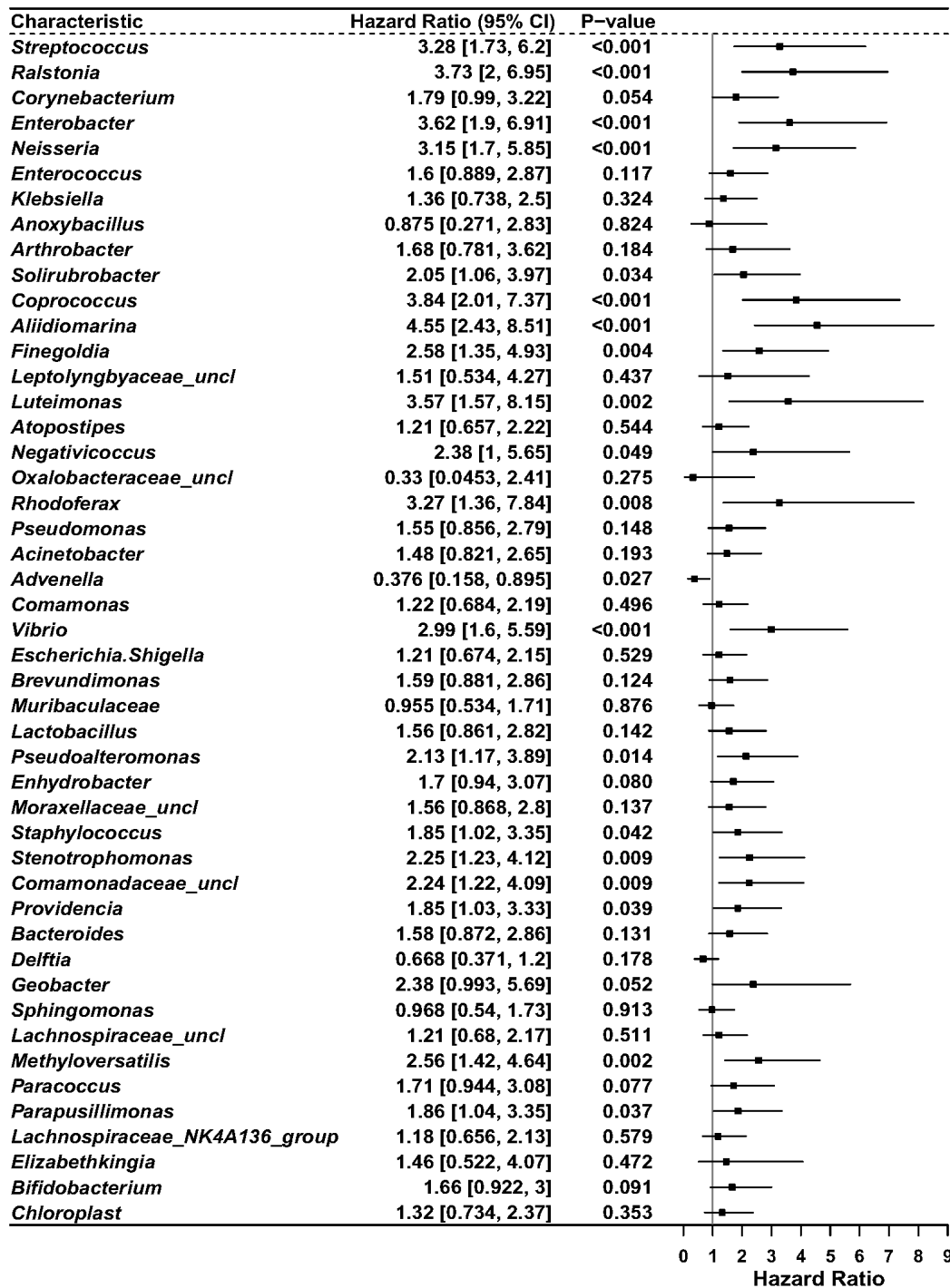


Figure 4

Univariate Cox regression analysis identifying specific bacterial genera associated with overall survival in BCa patients. Forest plot illustrating the prognostic value of specific intratumoral bacterial genera for OS. For each evaluated genus, patients were stratified into high-abundance and low-abundance groups based on its median relative abundance across the cohort. The squares represent the Hazard Ratio (HR) of the high-abundance group relative to the low-abundance group, and the horizontal lines denote the

95% confidence intervals (CIs). HR > 1 indicates a higher risk of death associated with a high abundance of the respective genus.

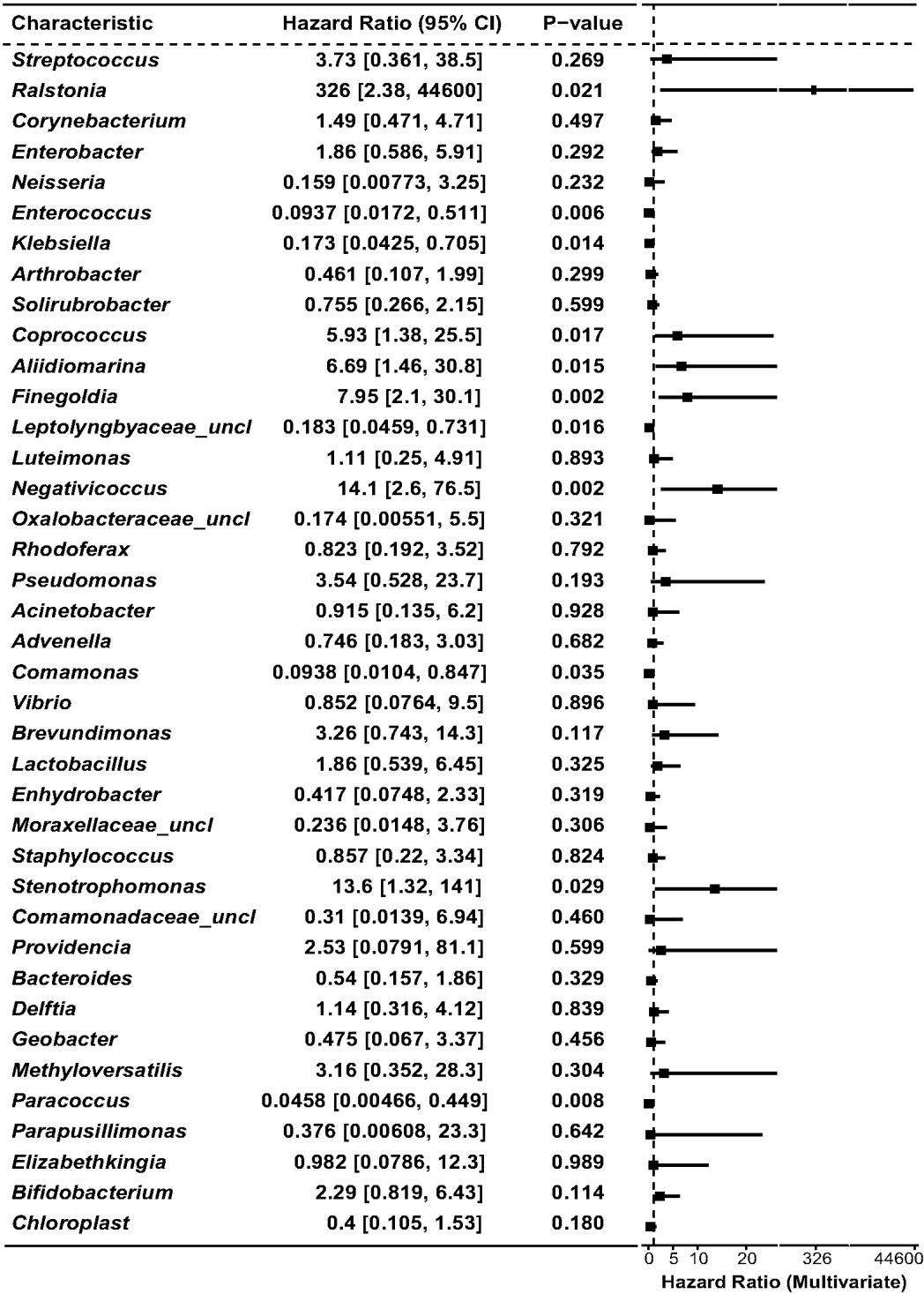


Figure 5

Multivariate Cox regression analysis identifying independent microbial prognostic factors for overall survival in BCa patients. Forest plot displaying the adjusted HRs and 95% CIs for specific bacterial

genera. Genera that demonstrated potential prognostic value in the univariate analysis were incorporated into this multivariate model. For each evaluated genus, patients were stratified into high-abundance and low-abundance groups based on its median relative abundance across the cohort.

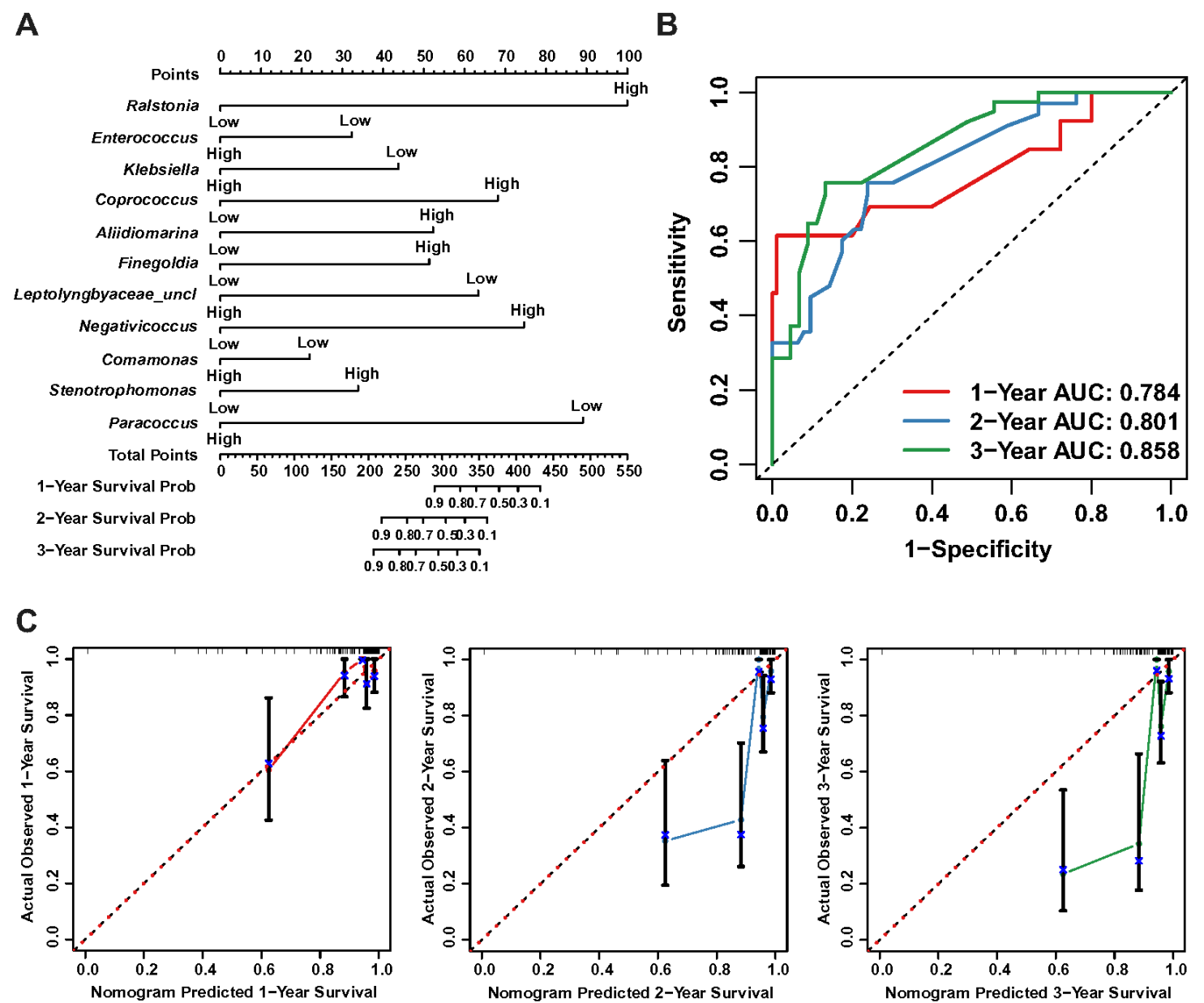


Figure 6

Development and performance of a nomogram for predicting 1-, 2-, and 3-year overall survival. (A) A nomogram constructed to predict the 1-, 2-, and 3-year OS probabilities of BCa patients. The model incorporates the 11 independent bacterial prognostic factors identified via multivariate Cox regression. For each genus, patients are stratified into "High" or "Low" abundance groups. (B) Time-dependent receiver operating characteristic (ROC) curves evaluating the predictive accuracy of the nomogram. The area under the curve (AUC) values for 1-year, 2-year, and 3-year survival predictions are 0.784, 0.801, and

0.858, respectively. **(C)** Calibration plots for the nomogram-predicted versus actual observed survival probabilities at 1, 2, and 3 years. The diagonal dashed line represents the performance of an ideal predictive model.

Supplementary Files

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