LETTER TO EDITOR

Multi-omics consensus ensemble refines the classification of muscle-invasive bladder cancer with stratified prognosis, tumour microenvironment and distinct sensitivity to frontline therapies

Dear Editor,

The molecular classification of muscle-invasive bladder cancer (MIBC) based on transcriptomic signatures has been extensively studied.1−4 The complementary nature of information provided by different molecular profiles motivated us to refine MIBC classification by aggregating multi-omics data. The entire workflow is outlined in Figure 1A, information on the eight datasets is summarized in Tables S1−S2, and technical details are listed in the Supporting Materials and Methods.

A consensus ensemble was generated through 10 multi-omics integrative clustering approaches on five omics datasets of 396 MIBCs from the TCGA database.5 We identified four robust integrative consensus subtypes (iCSs; Figure S1A,B), which showed distinctive molecular patterns and were significantly associated with clinico-pathological features and previously identified molecular classifications (Figure 1B); iCS1 and iCS4 dramatically overlapped with basal-like subtypes, whereas iCS2 and iCS3 were enriched for luminal-like subtypes (p < 0.001; Figure 1C, Table S3). We relabelled iCS1 to iCS4 as iBS1, iLS2, iLS3, and iBS4, respectively. Our classification system was tightly associated with prognosis (Figure 1D,E), showing superior performance than PAM, oneNN, and Lund classifications, but inferior than TCGA and consensus molecular subtype (CMS) classifications regarding overall stratification (Figure S2); iBS4 with the worst outcome that was refined from basal-like subtypes caught our attention.

Regulon analysis strongly manifested the biological pertinency of a four-classification where differentiated activity of cancerous chromatin remodelling regulons highlighted other possible differential regulatory patterns among iCSs (Figure 2A), indicating that epigenetically driven transcriptional networks might be important differentiators of these subtypes.

We observed that iLS2 showed a higher tumour mutation burden (TMB, p = 0.002; Figure 2B,C) and harboured more mutations in APOBEC-relevant signatures (p = 0.007; Figure 2D). Of the frequently mutated genes (>5%), iBS1 harboured more mutations of TP53, RB1 and KMT2A, while iBS4 was enriched for mutations in NFE2L2 (also known as NRF2) and TRANK1; KIAA0947, MED12, COL6A6, and ARID2 were mutated more frequently in iLS2, whereas iLS3 was enriched for mutations in FGFR3, STAG2 and SPEN (Table S4). iBS1 had better chromosomal stability with lower copy number alterations (CNAs) (p < 0.001; Figure 2E). Ch9p21.3 was susceptible to inactivation in cell immortalization and diseases; within this region, interferon-alpha (IFNA) genes, MTAP and CDKN2A/B were differentially altered in iBS1 compared to others, which may contribute to shaping different basal-like subtypes (Figure 2B).

We generated a 120-gene signature to predict iCSs in external cohorts with 403 MIBCs (Figures S3A,B and S4A-C, Table S5). The signature-predicted iCSs highly overlapped with the CMS but further refined the basal-like classification (p < 0.001; Figure S4D,E). Consistently, iBS4 showed the most unfavourable prognosis (Figure S4F-G).

Since cancer immunity plays a critical role in tumour progression, tumour microenvironment deconvolution suggested that immunocyte infiltration was dramatically higher in both iBS1 and iLS2 than in the other subtypes (Figure 3A, Table S6); iBS1 had relatively higher expression of genes that represent potential targets for immunotherapy. The immune landscape was validated (Figure S5A,B). Additionally, we found a similar immunologic profile from the IMvigor210 cohort (n = 298), in which the clinical effect of PD-L1 blockade with atezolizumab was evaluated in metastatic MIBC patients (Figure 3B), and a higher proportion of iBS1 achieved a complete response (p = 0.024;
The multi-omics consensus ensemble identifies four molecular subtypes of MIBC. (A) Design overview. This study enrolled a total of 1097 muscle-invasive bladder cancer (MIBC) cases and identified four MIBC subtypes under multi-omics framework that stratify prognosis, tumour microenvironment and distinct sensitivity to frontline therapies. An R package ‘refineMIBC’ was provided for MIBC refinement in clinical setting. (B) Comprehensive heatmap showing the molecular landscape of four integrative consensus subtypes (iCSs) of MIBC (n = 396). Other previously defined gene expression-based MIBC subtypes were annotated at the top of the heatmap, including prediction analysis of microarrays-based (PAM), one nearest neighbour (oneNN) prediction model-based, Lund, TCGA and consensus molecular subtypes (CMS). (C) Pie charts showing the proportion of other gene expression-based MIBC subtypes in the current iCS. Kaplan–Meier curves of (D) progression-free survival and (E) overall survival with log-rank test for 396 MIBC patients stratified by iCS
Figure 2 Molecular landscape of four MIBC iCSs. (A) Heatmap showing profiles of regulon activity for 23 regulator (top panel), and potential regulators that are relevant with chromatin remodelling (middle panel); Similar patterns were shared by iBS1 and iBS4, but iBS1 differed with high activity of GATA6, FGFR1 and ESR1 for regulon activity, while iBS4 was distinctly associated with high activity of TP63. A total of 296 unique differentially methylated promoters derived from each iCS versus adjacent normal samples (bottom panel); the iLS3 (265 vs. 45 in iLS2) and iBS4 (191 vs. 26 in iBS1) had more hypermethylated promoters (296 unique loci) than the 21 normal samples had. (B) Genomic alteration landscape according to iCS. Samples are sorted in descending order according to the cumulative contribution of APOBEC-relevant mutational signatures (i.e., SBS2 + SBS13) within each iCS. TMB, relative contribution of four mutational signatures, selected differentially mutated genes (>5%) and broad-level CNAs (>20%), and selected genes located within Chr9p21.3 are shown from the top to the bottom panels. Of note, iBS1 harboured more mutations of TP53 (74%; p < 0.001), RBL (39%; p < 0.001) and KMT2A (18%; p = 0.033) than others, while iBS4 was enriched in mutations of NFE2L2 (16%; p = 0.001; p = 0.005 compared to iBS1 [3%]) and TRANK1 (10%; p = 0.06; p = 0.001 compared to iBS1 [0%]); KIAA0947 (11%; p = 0.005), MED12 (11%; p = 0.005), COL6A6 (13%; p = 0.008), and ARID2 (14%; p = 0.01) were mutated more frequently in iLS2, whereas iLS3 was enriched in mutations of FGFR3 (34%; p < 0.001), STAG2 (22%; p = 0.006), and SPEN (11%; p = 0.05). The proportion of iCSs in each alteration is presented in the right bar charts. The distributions of TMB and APOBEC contributions are shown in (C) and (D), respectively. (E) Distribution of fraction genome altered (FGA) and fraction genome gain/loss (FGA/FGG). Bar charts are presented as the mean ± standard error of the mean. Statistical p values were calculated by Kruskal–Wallis rank sum test for multiple comparison.

Figure 3C). The predicted iBS4 showed poorer outcome than immune-hot iCSs (i.e., iBS1 and iLS2; p < 0.05 at 6 months, p < 0.1 at 12 months) and had a segregated survival curve compared to immune-cold iLS3 (Figure S6A). Due to the potentially delayed clinical effect of immunotherapy, we also compared the long-term overall survival (OS) rates after three months of treatment, leading to the observation that iBS4 was associated with poorer long-term OS than iBS1 (p = 0.046), iLS2 (p = 0.027) and iLS3 (p = 0.095), suggesting its high malignancy and potential resistance to immune checkpoint inhibitors (ICIs) (Figure S6B).
Mariathasan et al. reported that the clinical effect of anti-PD-L1 blockade may be influenced by tumour immune and immune cell phenotypes. In this context, we renamed iBS1 'basal-inflamed', iBS4 'basal-noninflamed', iLS2 'luminal-excluded', and iLS3 'luminal-desert' according to the phenotype proportions (Figure 3D,E). SubMap revealed that only the basal-inflamed subtype showed a high likelihood of responding to ICIs (FDR < 0.01; Figures 3F and S7), which indicated that the current classification may be useful to identify ideal candidates for immunotherapy, especially for basal-like MIBC.

Recently, Hao et al. suggested that Chr4 loss could induce an unfavourable 'immune-cold' phenotype. We demonstrated that only the basal-noninflamed subtype showed significant Chr4p and 4q deletions (FDR < 0.25; Table S7), and Chr4p loss was tightly linked to unfavourable OS (HR = 1.56, 95% CI: 1.001–2.437, p = 0.0496). Chr4 harbours several immune regulatory genes and genes that encode chemokines which are crucial for T cell recruitment. Additionally, Chr4 deletion is genetically linked with immune deficiency syndromes. Integrative analysis of RNA-Seq and CNA indicated that...
Oncogenic pathways, replication stress and targeted inhibitors in MIBC as well as predictive performance of random forest basal-classifier and its application in pan-carcinoma investigation. Dysfunctional oncogenic pathways quantified by single-sample gene set enrichment analysis are presented in (A) a heatmap and (B) a boxplot for the MIBC-TCGA cohort. Relevant mutations involved in several oncogenic pathways are annotated at the top. The cell cycle oncogenic pathway was significantly activated in basal-inflamed/noninflamed MIBC; the luminal-desert and basal-noninflamed subtypes showed relatively higher activation of the oncogenic NRF2 pathway; the luminal-excluded subtype was poorly enriched in the WNT pathway, while the luminal-desert subtype showed the lowest enrichment in angiogenesis genes but the highest activation of the TGF-β pathway. (C) Enrichment heatmap showing the signalling pathways (Reactome...
Chrs deletion in the basal-noninflamed subtype may drive the decreased expression of key immune markers and their key mediator, NFKB1 (Figure 3G-H).

Enrichment analysis revealed that the cell cycle oncogenic pathway was activated in basal-inflamed/noninflamed MIBC \((p < 0.001; \text{Figures } 4A,B \text{ and } S8A,B)\). Activation of the cell cycle pathway induces cell cycle checkpoint regulatory proteins (e.g., ataxia telangiectasia and Rad3-related [ATR] and WEEl) involved in replication stress, which are associated with DNA damage responses that contribute to cisplatin resistance; such an association mirrored a potentially higher sensitivity of responding to ATR and WEEl inhibitors in basal-inflamed/noninflamed MIBC (Figures 4D,E and S8C,D). A recent study reported that NRF2 enables an immune-cold microenvironment by inducing COX2/PGE2 and inhibiting the DNA-sensing innate immune response\(^9\); consistently, the luminal-desert/basal-noninflamed subtypes with activated NRF2 oncogenic pathway had dramatically higher COX2 expression levels than others in both the TCGA and IMvigor210 (Figures 4F and S9A-C) cohorts, suggesting that dysfunction of oncogenic pathways might drive the low immune infiltration of these MIBCs. Unfortunately, it is not feasible to combine currently unavailable NRF2 inhibitors with ICIs.

Nevertheless, preclinical models have demonstrated that COX inhibitors and anti-PD-1 immunotherapy have a synergistic effect\(^10\); therefore, targeting NRF2 downstream markers provides a therapeutic opportunity for immune-cold MIBCs. Additionally, the different biological characteristics of the four subtypes may indicate the need for different targeted therapeutic interventions (Table S8).

Given the distinct molecular and prognostic characteristics of basal-inflamed/noninflamed MIBC compared with traditional basal-like classifications, we developed a random forest predictor, which is superior than decision tree, to refine basal-like MIBC (Table S9-10, Figure S10). The final basal classifier contained five immune-related genes, C3AR1, CCL8, FCGR3A, LILRB2 and PDCDILG2, and the predictor achieved superior performance (Figure 4G) and showed the capability of prognostic stratification in refining different basal-like classifications (Figure 4H-L). Strikingly, almost all kinds of epithelial tumours could be refined into immune-hot or immune-cold basal-like phenotypes (Figure 4M), suggesting a global immunological divergence across basal-like carcinomas.

We acknowledge the limitations of our study, where signals quantified by bulk RNA-Seq and microarray profiles were confounded from mixed cell populations; thus, incorporating these findings with multiplex immunohistochemistry assays to investigate intrinsic tumour cell variation and their crosstalk with the tumour microenvironment, which interferes with the therapeutic response is warranted.

In summary, we identified four MIBC subtypes with distinct landscapes using a multi-omics approach that stratifies prognosis, tumour microenvironment characteristics and distinct sensitivity to frontline therapies. Additionally, we offered the R package “\texttt{refineMI BC}” as a translational research tool to refine MIBC classification from a single-sample perspective in retrospective or prospective studies.

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**CONFLICT OF INTEREST**

The authors have no conflict of interest.
DATA AVAILABILITY STATEMENT
We developed the R package, “refineMIBC”, which is documented and freely available at https://github.com/xlucpu/refineMIBC. This package implements a 120-gene template that assigns subtype labels according to the multi-omics consensus ensemble of muscle-invasive bladder cancer (MIBC) using nearest template prediction. The consensus ensemble identifies 4 integrative consensus subtypes: basal-inflamed, basal-noninflamed, luminal-excluded, and luminal-desert. This package also deploys a 5-immune-gene classifier to refine each basal-like MIBC as either basal-inflamed or basal-noninflamed by a random forest classifier if basal-like classification has already been identified by other approaches (e.g., CMS, PAM, oneNN, Lund, etc.).

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