Identification of The Immune Subtype Among Muscle-invasive Bladder Cancer Patients by Multiple Datasets

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ABSTRACT

Background: *Immunotherapies including PD-1/PD-L1 antibodies have been approved for the treatment of Muscle-invasive Bladder Cancer (MIBC) patients. However, immunotherapies could only be beneficial for about 20% MIBC patients. Thus, identification of the immune subtype is becoming increasingly important. This study aimed to explore the immune subtype by analyzing the gene expression profiles.* ***Methods****: A total of 6 datasets including (GSE13507, GSE31684, GSE32548, GSE32894, GSE69795, and TCGA-BLCA) were downloaded. The gene expression profiles from different datasets were combined since the batch effects were removed. We performed unsupervised clustering analysis to identify the immune subtype by the combined gene expression profiles. The tumor-infiltration levels of 22 immune cells, immune scores, and tumor purity were calculated, and the survival analysis was performed to investigate the prognosis difference between immune subtypes. The enriched pathways for each immune subtype were obtained.* ***Results:*** *We identified four novel immune subtypes (referred to S1, S2, S3, and S4) among MIBC patients. We found that S1 was enriched in immune scores had the best prognosis. In contrast, S3 was poor in immune scores and had the worst prognosis. Subtype S1, S2, S3, and S4 were enriched in immune-related pathways, extracellular matrix-related pathways, metabolism-related pathways, and cancer-related pathways, respectively.* ***Conclusion:*** *The current study suggests that the immune subtypes based on gene expression profiles could contribute to select the appropriate MIBC patient for immunotherapies.*

Keywords: *Molecular subtype, Immunotherapy, MIBC, Immunotype, TMB, Bioinformatics.*

INTRODUCTION

Bladder cancer (BC) is the most common genitourinary cancer of the urinary tract.[1,2]. A quarter of BLCA patients have muscle-invasive bladder cancer (MIBC), which has a higher risk of metastasis, or cancer cells migrating to regional pelvic lymph nodes and/or visceral regions, making the disease incurable.[3] Muscle-invasive bladder cancer (NMIBC) and non-muscle-invasive bladder cancer (NIBC) are the two kinds of BC (MIBC). Around a quarter of BC patients will develop MIBC, and more than half of MIBC patients will experience relapse and metastasis.[4] Radial cystectomy (RC) plus neoadjuvant cisplatin-based chemotherapy (NAC) is the standard first-line multimodal treatment for MIBC patients, however roughly 60% of MIBC patients do not exhibit a significant therapeutic response.[5] Furthermore, because of its toxicity, many people are unable or unwilling to accept cisplatin treatment.[6] The five-year survival rate for MIBC patients is as low as 50%.[7] There is also an urgent need for new treatment drugs. Immunotherapies, particularly immune checkpoint blockade (PD-1/PD-L1), have recently been licensed, improving the prognosis of MIBC patients significantly.[8] The practical use of immunotherapy, however, may be limited because only 20% of MIBC patients respond to treatment.[9] Tumor-infiltrating T cells.[10] PD-L1/PD-1 levels,[11] highly microsatellite instability (MSI-H),[12] tumor mutational burden (TMB),[13] and intestinal microbiota.[14] have all been found to be good indicators of immunotherapy efficacy. These potential markers were frequently unstable because numerous genes and pathways were involved in tumor immune evasion.[15] In the Checkmate025 research, for example, responses to Nivolumab (PD-1 antibody) exhibited no correlation with PD-L1 level, and patients with a high level of PD-L1 had a worse prognosis.[15] As a result, immunological subtypes established by clustering samples based on big genes from many datasets could be a good predictor of immunotherapy success.

According to multiple research,[16] patients with high tumor PD-L1 levels had better treatment response rates and lived longer. TIL density, especially CD8+ T cells, is a strong positive prognostic indicator, and immunotherapy works in part by reactivating a preexisting tumor immune response.[17] TMB stands for the amount of somatic mutations per million bases,[18] and tumor cells with a high TMB are more likely to generate neoantigens, which can be identified by T cells and trigger an anti-tumor response.[19] In 22 different tumor types, attempts to identify PD-1 antibody responders by combining TMB and tumor-infiltrating T cells have recently been published.[20] Apart from these biomarkers, other studies have advocated molecular subtype as a distinct technique for identifying immunotherapy candidates.[21–23] Based on RNA expression profiling, individuals with MIBC can be categorized into luminal and basal subtypes, with the basal subtype being more connected with the epithelial-mesenchymal transition (EMT), immune-related pathways, and worse prognosis than the luminal subtype.[24–26] However, more study is needed to confirm the role of molecular subtypes in predicting the therapeutic response of MIBC patients to immunotherapy.

In the age of precision immunotherapy, it's crucial to create an immunotype model that can predict immunotherapy response rates and identify mediators that are key determinants. Models and biomarkers could be utilized to influence immunotherapy response, adapt cancer treatment, cut costs, and avoid immune-related side effects.

In the current study, 683 samples from six separate cohorts were used to generate immunological subgroups. S1 was shown to have the best prognosis of the four immunological subtypes studied. Subtypes S1, S2, S3, and S4 were all enriched in immune-related, extracellular matrix-related, metabolism-related, and cancer-related pathways. Overall, our findings may aid researchers in better understanding the diversity of MIBC patients and identifying those who will benefit from immunotherapy.

METHODS

The expression matrix and clinical information of 6 bladder cancer datasets including GSE13507 (62 MIBC and 103 NMIBC samples),[27] GSE31684 (66 MIBC and 27 NMIBC

samples),[28] GSE32548 (38 MIBC samples and 93 NMIBC samples),[29] GSE32894 (93 MIBC and 215 NMIBC samples),[30] GSE69795 (20 MIBC samples and 18 NMIBC samples),[31] and TCGA-BLCA (404 MIBC and 4 NMIBC samples)[32] were downloaded. By using the SVA software[33] on the information from NMIBC and MIBC, these 6 datasets were merged into a single dataset, and batch effects were removed. Batch effects in datasets were detected using principal component analysis (PCA).

Identification of Immune Subtypes

The gene list and 736 immune-related genes were obtained from the Gene Expression Omnibus (GEO) under the entry 'GPL25507'. The 'ConsensusClusterPlus' program[34] used MIBC expression profiles of immune-related genes to identify the immunological subtype. The K-means technique was used to produce consensus clustering with 1,000 re-samplings.

Survival Analysis and Calculation of Immune Cell Proportions

To estimate survival distributions for each subtype, the overall survival data from these six datasets were merged, and Kaplan–Meier survival curves were displayed. Using the survival package in R, we did a log-rank test to see if differences between immune subtypes were significant. The CIBERSORT algorithm was used with 1000 permutations to compute immune cell proportions (such as B cells, dendritic cells, macrophages, neutrophils, NK cells, CD4+ T cells, and CD8+ T cells) against each sample. Using the estimate package, the ESTIMATE method[36] was used to determine immune scores, stromal scores, and tumor purity, and the Kruskal–Wallis test was chosen to compare the differences.

Functional Enrichment Analysis of Immune Subtypes

Subtype-specific pathways were discovered for each subtype by comparing samples from that subtype to the remaining samples using the GSEA approach. False discovery rate (FDR) 0.05 was used as the limit for subtype-specific pathways. The 'fGSEA' program was used to analyze differentially expressed genes among diffuse glioma subtypes using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

RESULTS

Removing the Batch Effects among Datasets

The "sva" program was used to normalize and remove batch effects from six datasets: GSE13507, GSE31684, GSE32548, GSE32894, GSE69795, and TCGA-BLCA. Before the batch effect was abolished, MIBC samples were mixed with NMIBC samples, and samples from other datasets were clearly segregated (**Figures 1A-B**). On the other hand, the PCA plot demonstrated that MIBC samples were segregated from NMIBC samples, and samples from different datasets were mixed (**Figures 1C-D**). The batch effects in six datasets were removed as a result of these findings. After batch effects were removed, the "sva" software produced the combined expression profiles of these six datasets. MIBC samples from the integrated expression profiles (a total of 683 samples) were kept for further study.

Identification of the MIBC Immune Subtypes

MIBC immune subtypes were identified using an expression matrix of 736 immune-related genes derived from merged expression data. To identify the distinct subtypes (K = 2, 3, 4, 5, and 6) among 683 MIBC samples, we used the 'ConsensusClusterPlus' program. Based on the CDF curves and Delta plots, the optimal division (k = 4) was chosen as the optimal number of clusters (**Figure 2A-B**). The heatmap's boundary remained pretty clear-cut at K = 4 (**Figure 2C**), indicating that the sample cluster was stable and robust. **Table 1** summarizes the distribution of immune subtypes among datasets.

We discovered substantial prognostic differences among the identified immunological subtypes using the previously described classification (log-rank test, p= 0.012, **Figure 2D**). Subtype 1 (S1) patients had a longer median survival time (67.3 months) than subtype 2 (S2) patients (35.9 months), subtype 3 (S3) patients (30.9 months), and subtype 4 (S4) patients (median survival: 30.9 months) (median survival: 16.9 months). Overall, we discovered four MIBC immunological subgroups that were linked to

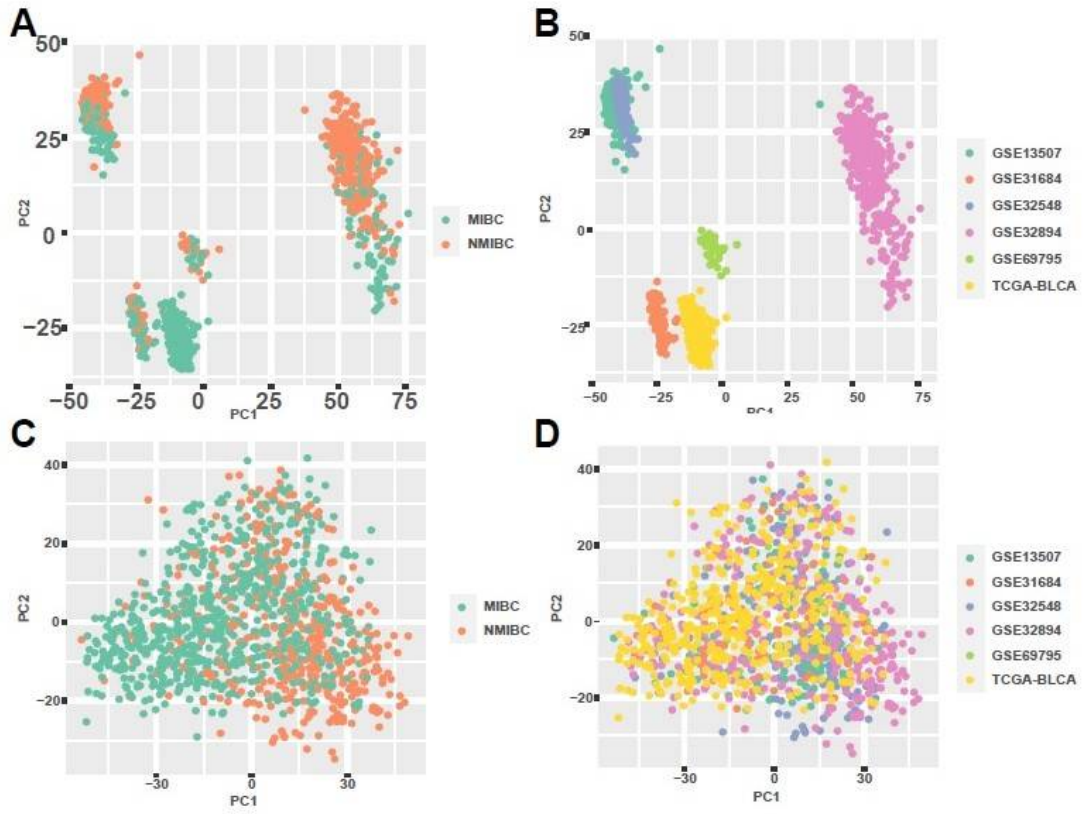


Figure 1. The normalization and batch effect removal from six datasets. (**a**) PCA plot illustrated the cluster of the samples by NMIBC/MIBC before batch effect removal. (**b**) PCA plot illustrated the cluster of the samples by datasets before batch effect removal. (**c**) PCA plot illustrated the cluster of the samples by NMIBC/MIBC after batch effect removal. (**d**) PCA plot illustrated the cluster of the samples by datasets after batch effect removal.

clinical outcomes based on gene expression profiles.

Correlation of MIBC Immune Subtypes with Tumor-infiltrating Immune Cells

The CIBERSORT technique was used to calculate tumor-infiltrating immune cells, and it revealed variances in immune cells among MIBC immune subtypes (**Figure 3**). (1) CD8 T cells, M1 macrophages, M2 macrophages, Monocytes, and Memory CD4 T cells were all greater in S1 samples. (2) In naive B cells and M0 macrophages, S2 samples were greater. (3) Resting NK cells, naive T cells, and Eosinophils were all greater in S3 samples. (4) In resting dendritic cells, active Mast cells, and neutrophils, S4 samples were greater.

Correlation of MIBC Immune Subtypes with Immune Scores and Molecular Subtypes

The immune subtypes' immunological scores, stromal scores, and tumor purity were calculated using the ESTIMATE technique. Immune and stromal scores were found to be

Table 1. The distribution of immune subtypes among datasets.

Subtype / Dataset	S1 N=149	S2 N=198	S3 N=195	S4 N=141
GSE13507	3 (2.01%)	20 (10.1%)	31 (15.9%)	8 (5.67%)
GSE31684	12 (8.05%)	18 (9.09%)	24 (12.3%)	12 (8.51%)
GSE32548	6 (4.03%)	13 (6.57%)	14 (7.18%)	5 (3.55%)
GSE32894	28 (18.8%)	21 (10.6%)	15 (7.69%)	29 (20.6%)
GSE69795	0 (0.00%)	8 (4.04%)	9 (4.62%)	3 (2.13%)
TCGA-BLCA	100 (67.1%)	118 (59.6%)	102 (52.3%)	84 (59.6%)

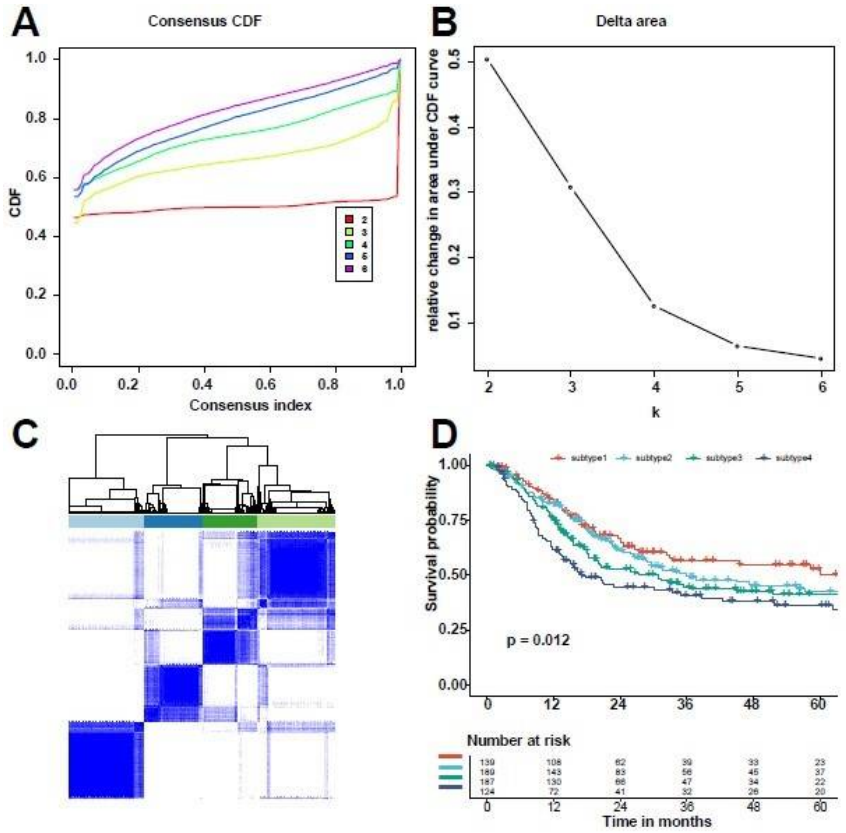


Figure 2. Identification of MIBC immune subtypes. (**a**) The cumulative distribution function (CDF) curves in consensus cluster analysis. (**b**) delta area plots in in consensus cluster analysis. Consensus scores for different subtype numbers (k = 2 to 6) are presented. (**c**) The heatmap illustrating the consensus matrix at k = 4. (**d**) Survival analysis of MIBC immune subtypes. The log-rank test was conducted to determine the significance of the differences.

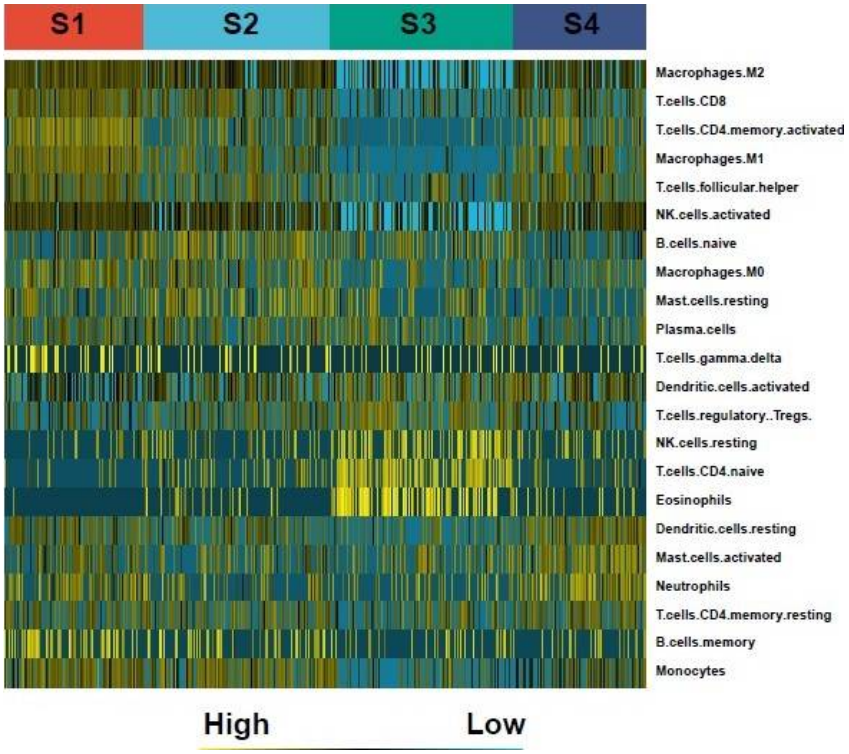


Figure 3. Immune characteristics of four MIBC immune subtypes. The heatmap showing the abundance of immune-cell populations calculated by CEBERSORT.

highest in S1, and lowest in S4 (immune scores: S1 > S4 > S2 > S3; stromal scores: S1 > S2 > S4 > S3). However, these immunological subtypes' tumor purity was in reverse order: (S3 > S4 > S2 > S1) (**Figure 4A-C**). S1 (Basal, N:80, P:82 percent ; Luminal, N:17, P:18 percent) and S4 (Basal, N:70, P:83 percent ; Luminal, N:14, P:17 percent) had different distributions of Basal and Luminal subtypes than S2 (Basal, N:43, P:36 percent ; Luminal, N:75, P:64 percent) and S3 (Basal (**Figure 4D**). It's worth noting that the Basal and Luminal subtype information was only accessible in the MINC samples from the TCGA-BLCA dataset.

Subtype-Specific Signaling Pathways among Immune Subtypes

GSEA analysis were used to uncover signaling pathways unique to the immunological subtypes observed (**Figures 5A, B**). Immune-related pathways including Cytokine-cytokine Receptor Interaction and Antigen Processing and Presentation were found to be overrepresented in subtype S1. Subtype S2 was shown to be particularly rich in extracellular matrix-related pathways such as Cell Adhesion Molecules (CAMs) and Vascular Smooth Muscle Contraction. Subtypes S3 and S4 were found to be associated with metabolism-related pathways (Metabolism of Xenobiotics by Cytochrome P450, Linoleic Acid Metabolism, and Fatty Acid Metabolism) and cancer-related pathways (Pathways in Cancer and Cell Cycle). Overall, we were effective in identifying immunological subtype characteristic signaling pathways.

DISCUSSION

There are two major molecular subgroups among MIBC patients, namely the Basal and Luminal subtypes, according to studies.[37,38] Because it is associated with a more aggressive

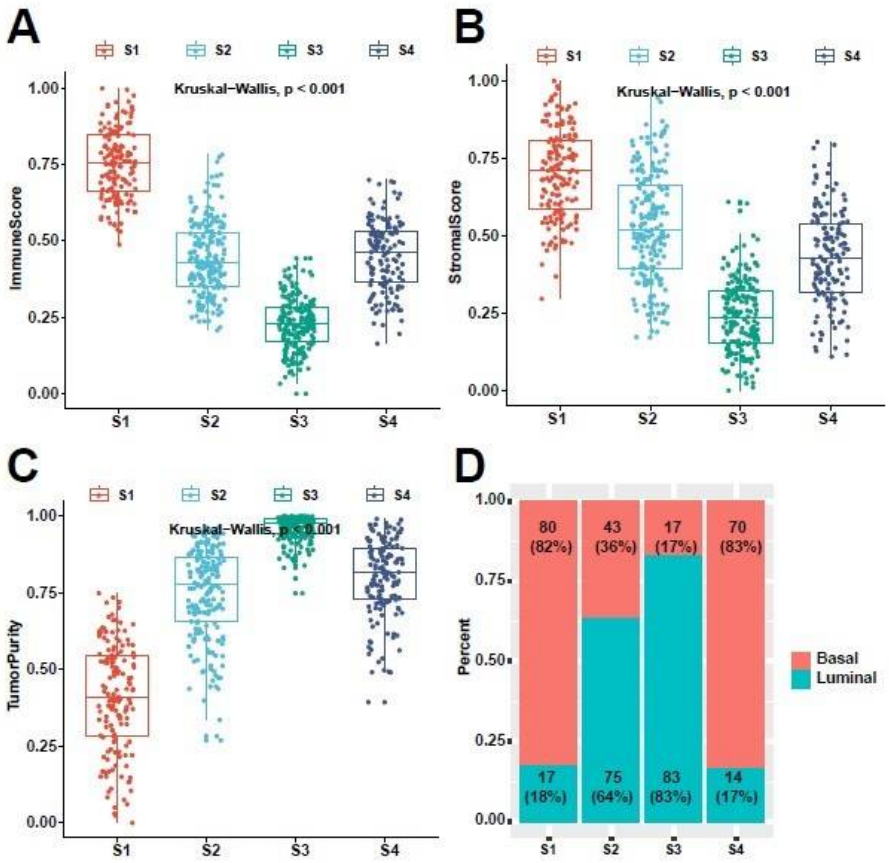
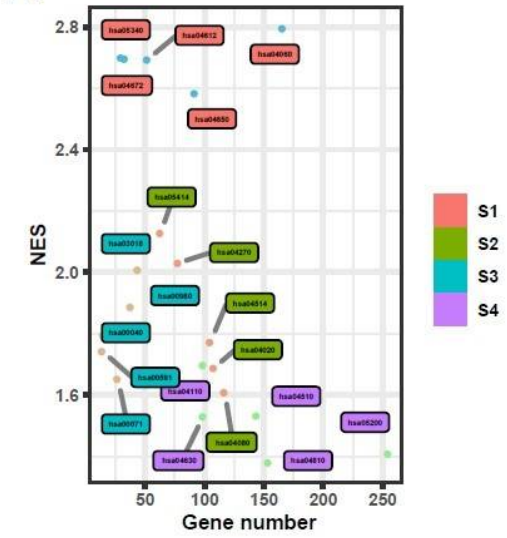


Figure 4. The correlation of stromal scores, immune scores, tumor purity, and molecular subtypes with the identified immune subtype. (**a-c**) Evaluation of stromal scores, immune scores, and tumor purity for the four immune subtypes by Kruskal-wallis test. (**d**) The distribution of molecular subtypes (Basal and Luminal subtype) in the four immune subtypes.

A



B

ID	Pathway
hsa04060	CYTOKINE-CYTOKINE RECEPTOR INTERACTION
hsa05340	PRIMARY IMMUNODEPICIENCY
hsa04672	INTESTINAL IMMUNE NETWORK FOR IGA PRODUCTION
hsa04612	ANTIGEN PROCESSING AND PRESENTATION
hsa04650	NATURAL KILLER CELL MEDIATED CYTOTOXICITY
hsa04080	NEUROACTIVE LIGAND RECEPTOR INTERACTION
hsa04020	CALCIUM SIGNALING PATHWAY
hsa04514	CELL ADHESION MOLECULES CAMS
hsa04270	VASCULAR SMOOTH MUSCLE CONTRACTION
hsa05414	DILATED CARDIOMYOPATHY
hsa03018	RNA DEGRADATION
hsa00980	METABOLISM OF XENOBIOTICS BY CYTOCHROME P450
hsa00040	PENTOSE AND GLUCURONATE INTERCONVERSIONS
hsa00591	LINOLEIC ACID METABOLISM
hsa00071	FATTY ACID METABOLISM
hsa05200	PATHWAYS IN CANCER
hsa04810	REGULATION OF ACTIN CYTOSKELETON
hsa04510	FOCAL ADHESION
hsa04110	CELL CYCLE
hsa04630	JAK STAT SIGNALING PATHWAY

Figure 5. Bubble plots for 5 enriched KEGG pathways with the lowest p.value in each immune subtype. (**a**) The plot of KEGG pathways. (**b**) The annotation of KEGG pathways.

phenotype and a higher risk of distant metastasis than the Luminal subtype, the Basal subtype has gotten a lot of attention.[38] Although significant progress has been made in the MIBC molecular subtype, more study into the MIBC immunological subtype is required. The identification of immunological subtypes is becoming increasingly important since it may aid in the selection of suitable candidates for immunotherapies.

TMB, which is independent of PD-L1 expression, is a powerful predictor of tumor behavior and immunotherapy response in patients with small-cell lung cancer.[39] On the other hand, TMB criteria for predicting response in a variety of different malignancies aren't well established.[40] Apart from limited correlation research, the mechanism by which TMB predicts immunotherapy sensitivity is mainly unknown.[34] Furthermore, molecular subtypes may provide additional information for predicting immunotherapy response. The basal and luminal subtypes are derived from separate progenitor cells, according to various studies, and the basal subtype has a higher ORR in immunotherapy treatment.[09,41,42] Immunotype A patients exhibited the best ORR and had the most immunological checkpoints, TMB, and CD8+ T cells, indicating that immunotherapy was highly recommended for them. It's because immunotype A corresponds to previously identified "hot tumors".[43] Patients with Immunotype B exhibited a lower ORR, a lower level of immunological checkpoints and CD8+ T cells, and a moderate number of TMB. More research is needed to establish if this tendency is analogous to "cold tumors," which are characterized by insufficient T cell priming (low tumor mutational load, poor antigen presentation, and intrinsic T cell death insensitivity).[43–45] To increase T cell responses and turn cold tumors into "hot tumors," treatment techniques include cancer stem cell (CSC) vaccination or adoptive T cell transfer.[43, 46] Immunotype C patients, on the other hand, had the lowest ORR. They had strong immunological checkpoints, intermediate CD8+ T cells, and low TMB, implying that immunotherapy may not be suited for this patient population. TTN, TP53, KMT2D, MUC16, ARID1A, KDM6A, and SYNE1) were identified as cancer risk genes after they were found to be changed often among three immunotypes. Seven more genes are as important: PIK3CA, RB1, FGFR3, KMT2C, MACF1, RYR2, and EP300. Three immunotypes have varied mutation rates for these genes, allowing for a more thorough and comprehensive understanding of MIBC immunotype mutation rates. Individual genes

in a co-expression network are less stable than modules because the overall function of a module can be maintained when individual gene expression can be replaced by other genes with similar redundant functions [02]. Network analysis revealed eight hub genes for the MIBC immunotype-related module (ACTA2, ACTA1, COL1A1, COL1A2, COL5A1, DCN, SPARC, VIM). The disease stage-related hub gene involvement of COL1A1, COL1A2, and COL5A1 was previously discovered by another group,[47] which is compatible with our findings. Multiple datasets should be used to find the robust immune subtype among MIBC patients. When merging disparate datasets, the batch effect will be a key stumbling block for researchers. Fortunately, 'sva' package[33] has been shown the ability to remove the batch effect in studies.[48,49] According to the PCA results, the batch effect was successfully removed. Because we analyzed 683 samples from six separate cohorts, the four immunological subgroups we discovered may be more robust than a single dataset. Among the four immune subtypes, S1 received the highest immunological and stromal evaluations, whereas S3 had the lowest. The ESTIMATE approach did not produce the same findings as the CIBERSORT approach. S1 has a lot of CD8 T cells, M1 macrophages, M2 macrophages, Monocytes, and Memory CD4 T cells. As a result, S1 patients should receive immunotherapy, but S3 patients should not. Based on the distribution of immunological scores and molecular subtypes in these four immune subtypes, we could determine that 1) S1 was the Basal subtype with more immune cells and S4 was the Basal subtype with fewer immune cells. 2) Tumor cells were lower and higher in the Luminal subtypes S2 and S3, respectively.

CONCLUSION

Finally, the findings of this study improved immunological subtype research in MIBC samples by identifying four immune subtypes with varying immunological scores. Immune subsets revealed may aid doctors in deciding on treatment for MIBC patients. These findings will pave the way for new immunotherapy approaches in the future.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics 2020. CA Cancer J Clin. 2020; 70:7-30.
2. Chen Z, Liu G, et al. A co-expression network for differentially expressed genes in bladder cancer and a risk score model for predicting survival. Hereditas. 2019;156:24.
3. Kim J, Akbani R, Creighton CJ, et al. Invasive bladder cancer: Genomic insights and therapeutic promise. Clin Cancer Res. 2015;21:4514-24.
4. Bognar Z, Fekete K, Antus C, et al. Desethylamiodarone-A metabolite of amiodarone-Induces apoptosis on T24 human bladder cancer cells via multiple pathways. PLoS One. 2017; 12(12):e0189470.
5. Seiler R, Ashab H, Erho N, et al. Impact of molecular subtypes in muscle-invasive bladder cancer on predicting response and survival after neoadjuvant chemotherapy. Eur Urol. 2017;72:544–54.
6. Raggi D, Miceli R, Sonpavde G, et al. Second-line single-agent versus doublet chemotherapy as salvage therapy for metastatic urothelial cancer: a systematic review and meta-analysis. Ann Oncol. 2016;27:49–61.
7. Stenzl A, Cowan NC, De Santis M, et al. Treatment of muscle-invasive and metastatic bladder cancer: update of the EAU guidelines. Eur Urol. 2011;59:1009-18.
8. Chen Z, Liu G, Liu G, et al. Defining muscle-invasive bladder cancer immunotypes by introducing tumor mutation burden, CD8+ T cells, and molecular subtypes. Hereditas. 2021;158(1):1.
9. Sharma P, Retz M, Siefker-Radtke A, et al. Nivolumab in metastatic urothelial carcinoma after platinum therapy (CheckMate 275): a multicentre, single-arm, phase 2 trial. Lancet Oncol. 2017;18:312-22.
10. Spencer KR, Wang J, Silk AW, Ganesan S, Kaufman HL, Mehnert JM. Biomarkers for immunotherapy: Current developments and challenges. Am Soc Clin Oncol Educ Book. 2016;35:e493-e503.
11. Davis AA, Patel VG. The role of PD-L1 expression as a predictive biomarker: an analysis of all US Food and Drug Administration (FDA) approvals of immune checkpoint inhibitors. J Immunother Cancer. 2019; 7:278.

12. Ciardiello D, Vitiello PP, Cardone C, et al. Immunotherapy of colorectal cancer: Challenges for therapeutic efficacy. Cancer Treat Rev. 2019;76:22-32.
13. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. Science. 2015;348:69-74.
14. Dai Z, Zhang J, Wu Q, et al. Intestinal microbiota: a new force in cancer immunotherapy. Cell Commun Signal. 2020;18:90.
15. Shen X, Zhao B. Efficacy of PD-1 or PD-L1 inhibitors and PD-L1 expression status in cancer: meta-analysis. BMJ. 2018;362:k3529.
16. Davis AA, Patel VG. The role of PD-L1 expression as a predictive biomarker: an analysis of all US Food and Drug Administration (FDA) approvals of immune checkpoint inhibitors. J Immunother Cancer. 2019; 7:278.
17. Havel JJ, Chowell D, Chan TA. The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. Nat Rev Cancer. 2019;19:133-50.
18. Lv J, Zhu Y, Ji A, Zhang Q, Liao G. Mining TCGA database for tumor mutation burden and their clinical significance in bladder cancer. Biosci Rep. 2020; 40:BSR20194337.
19. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. Science. 2015;348:69-74.
20. Cristescu R, Mogg R, Ayers M, et al. Pan-tumor genomic biomarkers for PD-1 checkpoint blockade-based immunotherapy. Science. 2018;362:eaar3593.
21. Becht E, de Reyniès A, Giraldo NA, et al. Immune and stromal classification of colorectal cancer is associated with molecular subtypes and relevant for precision immunotherapy. Clin Cancer Res. 2016;22:4057-66.
22. Lim J, Poulin NM, Nielsen TO. New strategies in sarcoma: Linking genomic and immunotherapy approaches to molecular subtype. Clin Cancer Res. 2015;21:4753-9.
23. Todenhöfer T, Seiler R. Molecular subtypes and response to immunotherapy in bladder cancer patients. Transl Androl Urol. 2019;8:S293-S295.
24. Choi W, Porten S, Kim S, et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell. 2014;25:152-65.
25. Dadhania V, Zhang M, Zhang L, et al. Meta-analysis of the luminal and basal subtypes of bladder cancer and the identification of signature immunohistochemical markers for clinical use. EBio Med. 2016;12:105-17.
26. Liu G, Chen Z, Danilova IG, Bolkov MA, Tuzankina IA, Liu G. Identification of miR-200c and miR141-mediated lncRNA-mRNA crosstalks in muscle-invasive bladder cancer subtypes. Front Genet. 2018;9:422.
27. Kim WJ, Kim EJ, Kim SK, et al. Predictive value of progression-related gene classifier in primary non-muscle invasive bladder cancer. Mol Cancer. 2010;9:3.
28. Riester M, Taylor JM, Feifer A, et al. Combination of a novel gene expression signature with a clinical nomogram improves the prediction of survival in high-risk bladder cancer. Clin Cancer Res. 2012; 18:1323-33.
29. Lindgren D, Sjödahl G, Lauss M, et al. Integrated genomic and gene expression profiling identifies two major genomic circuits in urothelial carcinoma. PLoS One. 2012;7:e38863.
30. Sjödahl G, Lauss M, Lövgren K, et al. A molecular taxonomy for urothelial carcinoma. Clin Cancer Res. 2012;18:3377-3386.
31. McConkey DJ, Choi W, Shen Y, et al. A prognostic gene expression signature in the molecular classification of chemotherapy-naïve urothelial cancer is predictive of clinical outcomes from neoadjuvant chemotherapy: A phase 2 trial of dose-dense Methotrexate, Vinblastine, Doxorubicin, and Cisplatin with Bevacizumab in urothelial cancer. Eur Urol. 2016;69:855-62.
32. Robertson AG, Kim J, Al-Ahmadie H, et al. Comprehensive molecular characterization of muscle-invasive bladder cancer. Cell. 2018;174:1033.
33. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. Bioinformatics. 2012;28:882-3.
34. Wilkerson MD, Hayes DN. Consensus Cluster Plus: a class discovery tool with confidence assessments and item tracking. Bioinformatics. 2010;26:1572-3.
35. Newman AM, Liu CL, Green MR, et al. Robust enumeration of cell subsets from tissue expression profiles. Nat Methods. 2015;12:453-7.
36. Yoshihara K, Shahmoradgoli M, Martínez E, et al. Inferring tumour purity and stromal and immune cell admixture from expression data. Nat Commun. 2013;4:2612.
37. Liu G, Chen Z, Danilova IG, Bolkov MA, Tuzankina IA, Liu G. Identification of miR-200c and miR141-mediated lncRNA-mRNA crosstalks in muscle-invasive bladder cancer subtypes. Front Genet. 2018; 9:422.
38. Choi W, Czerniak B, Ochoa A, et al. Intrinsic basal and luminal subtypes of muscle-invasive bladder cancer. Nat Rev Urol. 2014;1:400-10.
39. Boumber Y. Tumor mutational burden (TMB) as a biomarker of response to immunotherapy in small cell lung cancer. J Thorac Dis. 2018;10:4689-93.
40. Chan TA, Yarchoan M, Jaffee E, et al. Development of tumor mutation burden as an immunotherapy biomarker: utility for the oncology clinic. Ann Oncol. 2019;30:44-56.
41. Choi W, Porten S, Kim S, et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell. 2014;25:152-65.
42. Dadhania V, Zhang M, Zhang L, et al. Meta-analysis of the luminal and basal subtypes of bladder cancer and the identification of signature immunohistochemical markers for clinical use. EBio Med. 2016;12:105-17.

43. Galon J, Bruni D. Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. Nat Rev Drug Discov. 2019; 18:197-218.
44. Camus M, Tosolini M, Mlecnik B, et al. Coordination of intratumoral immune reaction and human colorectal cancer recurrence. Cancer Res. 2009;69:2685-93.
45. Sanmamed MF, Chen L. A Paradigm shift in cancer immunotherapy: From enhancement to normalization. Cell. 2018;175:313-26.
46. Shi X, Zhang X, Li J, et al. PD-1 blockade enhances the antitumor efficacy of GM-CSF surface-modified bladder cancer stem cells vaccine. Int J Cancer. 2018; 142:2106-17.
47. Di Y, Chen D, Yu W, Yan L. Bladder cancer stage-associated hub genes revealed by WGCNA co-expression network analysis. Hereditas. 2019;156:7.
48. Tan TZ, Rouanne M, Tan KT, Huang RY, Thiery JP. Molecular subtypes of urothelial bladder cancer: Results from a meta-cohort analysis of 2411 tumors. Eur Urol. 2019;75:423-32.
49. Abbas-Aghababazadeh F, Li Q, Fridley BL. Comparison of normalization approaches for gene expression studies completed with high-throughput sequencing. PLoS One. 2018;13:e0206312.