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ORIGINAL PAPER

Predictive role of lactylation-related gene signature in the prognosis and immunotherapy response in bladder cancer

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Summary ***Objective: Lactylation is a type of chemical modification involving the introduction of lactyl groups to a molecule which can affect the interactions between tumor cells and their microenvironment.***
This study aims to evaluate the possible role of lactylation-related gene signature in the prediction of both prognosis and immunotherapy response in bladder cancer (BLCA).
Methods: Lactylation-related genes were obtained from the published work and two subtypes (cluster A and B) were identified through unsupervised clustering. The differences including clinical features, differentially expressed genes (DEGs), pathways, and immune cell infiltration between these two clusters were thoroughly examined.
Results: By utilizing the DEGs between the two clusters, a lactylation score was identified to predict the overall survival status and the response of BLCA patients receiving immunotherapy. Our results demonstrated that patients with a high lactylation score tended to have a worse survival period and increased immune cell infiltration level. Further analysis showed that high lactylation score may be associated with higher sensitivity to immune checkpoint inhibitor (ICI) treatment which is crucial in the identification of the suitable candidates for ICI therapy.
Conclusions: Our results emphasize the possible predictive role of lactylation-related gene signature both in the survival rates of BLCA and its implications for treatment strategies.

KEY WORDS: Lactylation; Gene signature; Bladder cancer; Prognosis; Immunotherapy.

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INTRODUCTION

Bladder cancer (BLCA) is one of the most common malignant pathologies of the genitourinary tract, with approximately 573,000 new cases and 212,000 deaths per year worldwide. The incidence of BLCA is still increasing owing to population growth and ageing (1-3). In recent years, *immune checkpoint inhibitors* (ICIs) took attention as an effective alternative for advanced BLCA due to their better tolerance especially by older patients with more comorbidities. However, despite durable responses shown in a subset of patients with BLCA, the overall response rate of ICI's is only 15%~25%, which increases the demand for biomarkers of response and therapeutic strategies that can overcome resistance to ICIs (4, 5).

Lactylation modification has been well studied for its potential role in cancer progression (6-8). The tumor microenvironment is characterized by low pH, hypoxia, and increased lactate production. Lactate, a product of pyruvate metabolism, is known to play an important role in promoting tumor growth and metastasis by modulating the extracellular matrix, angiogenesis, and immune evasion (9-11).

Lactylation has also been found to alter the activity and stability of intracellular proteins, leading to changes in certain cellular processes including glycolysis (12, 13) and apoptosis (14). In addition, lactylation can further affect the interactions between tumor cells and their microenvironment by promoting or inhibiting tumor progression (15-17).

These findings suggest that lactylation may serve as a potential target for the development of novel cancer therapeutics to interfere with lactate signaling pathways and disrupt tumor progression. However, the role of lactylation in BLCA remains still unclear.

In this present study, we aimed to identify lactylation-related gene subtypes of BLCA through unsupervised clustering in order to compare clinical features, *differentially expressed genes* (DEGs), pathways and immune cell infiltration between the lactylation-related gene subtypes identified.

Furthermore, by establishing a special lactylation score we also aimed to predict the overall survival of BLCA patients. Lastly, we investigated the correlation between the lactylation score and the tumor immune microenvironment, as well as the potential for immunotherapeutic efficacy.

Materials and methods

Sources of sample

The Cancer Genome Atlas Urothelial Bladder Carcinoma (TCGA-BLCA) is a data collection initiative that is part of a larger effort to connect cancer phenotypes to genotypes. The data includes clinical, genetic, pathological, and radiological information of 412 tumor samples and 19 normal samples. GSE13507 is a public gene expression dataset that identifies prognosis-related gene signatures in BLCA. It contains 165 primary tumors, 23 recurrent tumors, 58 normal bladder mucosae surrounding cancer and 10 normal bladder mucosae, the clinical characteristics of included samples are attached as well.

Data collection

The mRNA expression profile and clinical data for TCGA-BLCA (412 tumor tissues) were downloaded from the *University of California Santa Cruz* (UCSC)-Xena database (https://xenabrowser.net/datapages/), while GSE13507 (165 tumor tissues) was obtained from the *Gene Expression Omnibus* (GEO) database (*https://www.ncbi.nlm.nih.gov/geo/*). The clinical characteristics and survival outcomes of BLCA patients from these two datasets were shown in supplementary Table S1. R package "*limma*" was executed to normalize the expression data for nonuniform matrix distribution. To improve the reliability, the two datasets were merged and batch effects were removed using the R package "*sva*". The gene set for lactylation was acquired from the published work (18).

Online analysis

The genetic alteration of selected genes, including *copy number variant* (CNV), methylation and mutation, were conducted using *Gene Set Cancer Analysis* (GSCA) database (*http://bioinfo.life.hust.edu.cn/GSCA/#/*), which is an integrated platform for genomic, pharmacogenomic, and immunogenomic gene set cancer analysis. The platform has four main functional modules for cancer GSA to explore, analyze and visualize expression, genomic variations, tumor immune infiltration, drug sensitivity and their associations with clinical outcomes.

Enrichment analysis

Gene Ontology (GO) analysis and *Kyoto Encyclopedia of Genes and Genomes* (KEGG) analysis are two common functional annotation methods, which are mainly used to study the functions of genes and proteins. GO analysis is performed by classifying the function of a gene or protein into three distinct levels: Molecular Function, Cellular Component and Biological Process to describe their different functions and interactions within cells. KEGG analysis is a functional annotation method based on metabolic pathways and biological signaling networks. By correlating genes or proteins with metabolic pathways and signaling networks in KEGG databases, it is possible to understand their functional roles and interrelationships in the cells. To obtain the pathways used in *Gene Set Variation Analysis* (GSVA), the HALLMARK, Reactome, and KEGG pathways were downloaded from version 7.5.1 of the Msigdb database. The R package "GSVA" was used to calculate the pathway score for each sample. The R package "*clusterprofiler*" was used to carry out GO and KEGG analysis of DEGs.

Immune cell infiltration analysis

The R package "ESTIMATE" was used to assess the BLCA samples' tumor microenvironment, including stromal score, immune score, and ESTIMATE score. In order to measure the 23 immune cell infiltrations, the GSVA R package's ssGSEA function was used. Following that, the various groups were compared based on their immune cell level.

Construction of Lactylation score

Using the R package "*limma*", we identified 275 DEGs distinguishing between cluster A and B with $|logFC| > 0.5$ and $p < 0.05$. Subsequently, we conducted univariate regression analysis, which revealed 11 DEGs significantly associated with overall survival at $p < 0.001$. Based on these 11 DEGs, we performed principal component analysis (PCA). Using principal components 1 and 2, we constructed the lactylation score (19).

Statistical analysis

All data are presented as the mean ± *Standard Deviation* (SD). Statistical analysis was performed using R software (https://www.r-project.org/, version:4.1.1). $P < 0.05$ (two-tailed) was considered statistically significant: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

Results

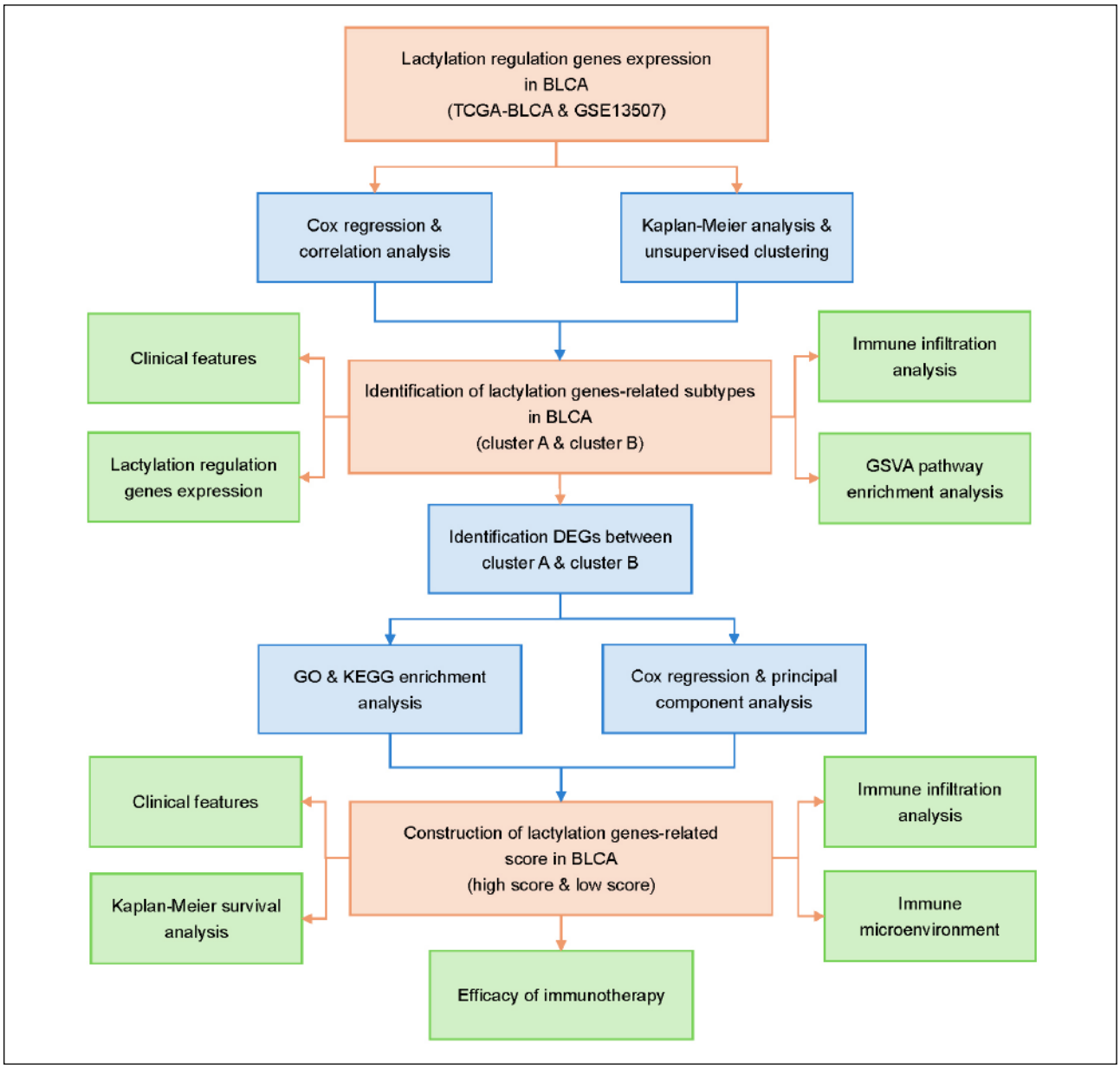
Identification of lactylation genes-related subtypes of BLCA

The flow chart of the study is show in Figure 1. At first, we combined the expression profiles of tumor tissues from TCGA-BLCA and GSE13507, and then used univariate Cox regression and Kaplan-Meier analysis to evaluate the prognostic significance of lactylation genes (***Supplementary Table S2***). Figure 2A depicts the results of univariate Cox regression and correlation analysis of lactylation genes. 10 lactylation genes were revealed to have some correlation with each other. Among them, 9 genes were identified as risk factors and one was favorable factor. While Figure 2B illustrates 7 lactylation genes that were significantly associated with the prognosis of BLCA patients revealed by Kaplan-Meier analyses. Next, we divided BLCA into two subtypes, cluster A and B, based on the expression pattern of lactylation genes (Figure 3A), and found that there was a clear distinction between cluster A and B according to the PCA plot (Figure 3B). We also presented the distribution of clinical features and lactylation gene expression in each cluster (Figure 3C, D).

Furthermore, we assessed the immune infiltration in BLCA samples, and found that the stromalscore, immunescore, and ESTIMATEscore were all higher in cluster A (Figure 3E), and most immune cell levels were more highly infiltrated in cluster A than in cluster B (Figure 3F).

We also investigated the differences between the two clusters in terms of various pathways using GSVA. We discovered that most of the HALLMARK pathways, KEGG pathways, and Reactome pathways had higher scores in cluster B (***Supplementary Figure S1***). For HALLMARK pathways, the scores TGF BETA signaling pathway and MTORC1 signaling pathway were higher in cluster B. For KEGG, the scores of TGF BETA signaling pathway, WNT signaling

Figure 1.
Flow chart of the study.

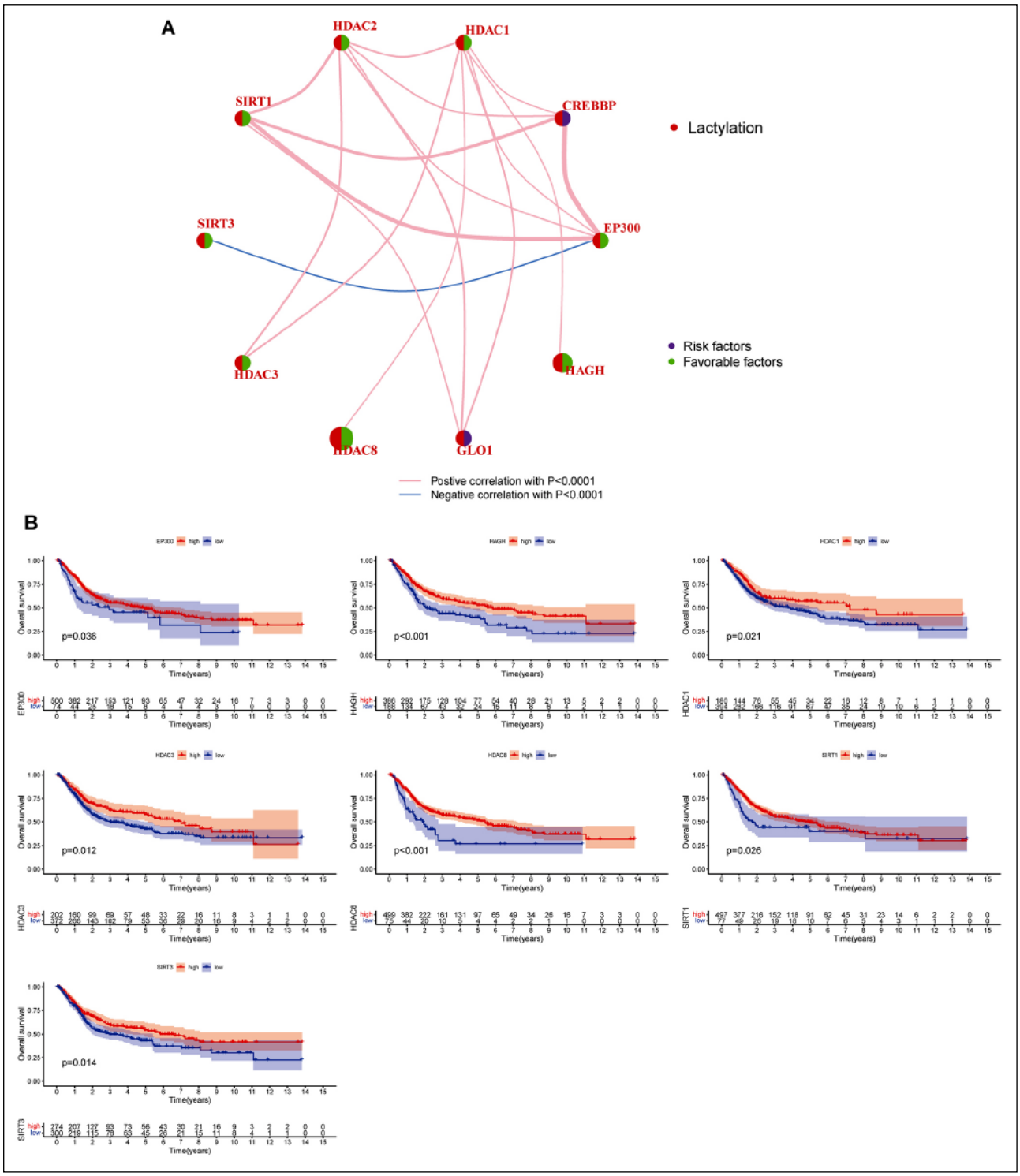


pathway, and MTOR signaling pathwaywere higher in cluster B. For Reactome results, most pathways were observed to be higher in cluster B. These findings suggested that the biggest difference between the two subtypes was in the pathways associated with carcinogenesis.

Identification of gene subtypes and construction of lactylation score

By employing the "*limma*" package, 275 DEGs were screened between cluster A and cluster B (Figure 4A). The GO enrichment analysis highlighted that these DEGs were enriched in cell growth and G1/S transition of mitotic cell cycle in *Biological Process* (BP), cell leading edge and nuclear speck in *Cellular Component* (CC), and DNA–binding transcription factor binding in *Molecular Function* (MF) (Figure 4B-D). In terms of KEGG, the DEGs were predominantly enriched in Cell cycle, p53 signaling pathway, and Nicotinate and nicotinamide metabolism (Figure 4E). Furthermore, we utilized univariate Cox regression analysis to determine the prognostic significance of the 275 DEGs and identified 11 genes associated with overall survival ($p < 0.001$) (Figure 5A, ***Supplementary Table S3***). To confirm above findings, unsupervised clustering separated patients into two subtypes based on these 11 prognostic genes, geneCluster A-B (Figure 5B). Patients categorized in geneCluster A had worse survival rates compared to those in geneCluster B (Figure 5C). The expression of the 11 lactylation-related genes was depicted in Figure 5D, while Figure 5E showed the distribution of clinical features and expression of the 11 prognostic genes in the two geneClusters. Using the PCA algorithm, the 11 prognostic genes were used to establish the lacty-

Figure 2.
Correlation and prognostic value of lactylation genes in BLCA:
A. Network diagram showing the interaction of lactylation genes in BLCA. The line connecting the lactylation genes represents their correlation, with the line thickness indicating the strength of the correlation between lactylation genes. Blue and pink represent negative and positive correlations, respectively. B. Kaplan-Meier analysis of indicated genes.



lation score. Figure 5F suggested that patients with a high lactylation score have worse survival. The correlation between cluster, geneCluster, lactylation score, and survival status is depicted in Figure 5G using a Sankey diagram. We analyzed the correlation of lactylation score with immune cell infiltration and found that patients with high lactylation score have elevated immune cell infiltration level (Figure 5H).

Figure 3.
Identification of lactylation genes-related subtypes of BLCA:
A. Consensus matrix heatmap defining various clusters and their correlation area. B. PCA plot of BLCA patients in two clusters. C. Distributions of clinical features and expression levels of lactylation genes between two clusters. D. The expression of lactylation genes in cluster A and cluster B. E. The tumor microenvironment difference in two clusters. F. The difference of immune cell infiltration level in two clusters.

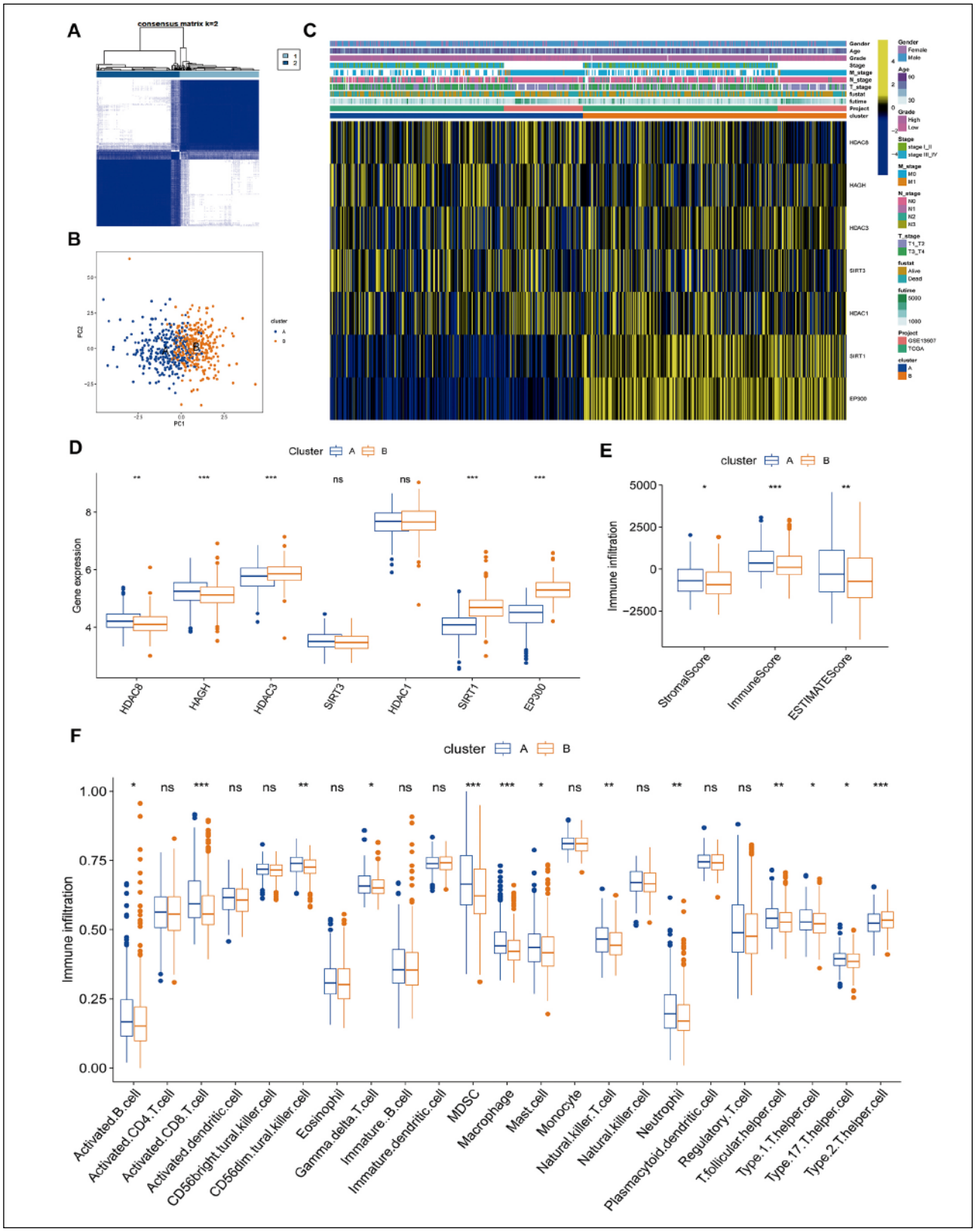


Figure 4.
Enrichment analysis of DEGs between two lactylation subtypes:
A. Volcanic map of DEGs between two clusters. B-D. GO enrichment analysis of DEGs, including BP, CC, and MF.
E. The correlation of DEGs with top5 terms of KEGG results.

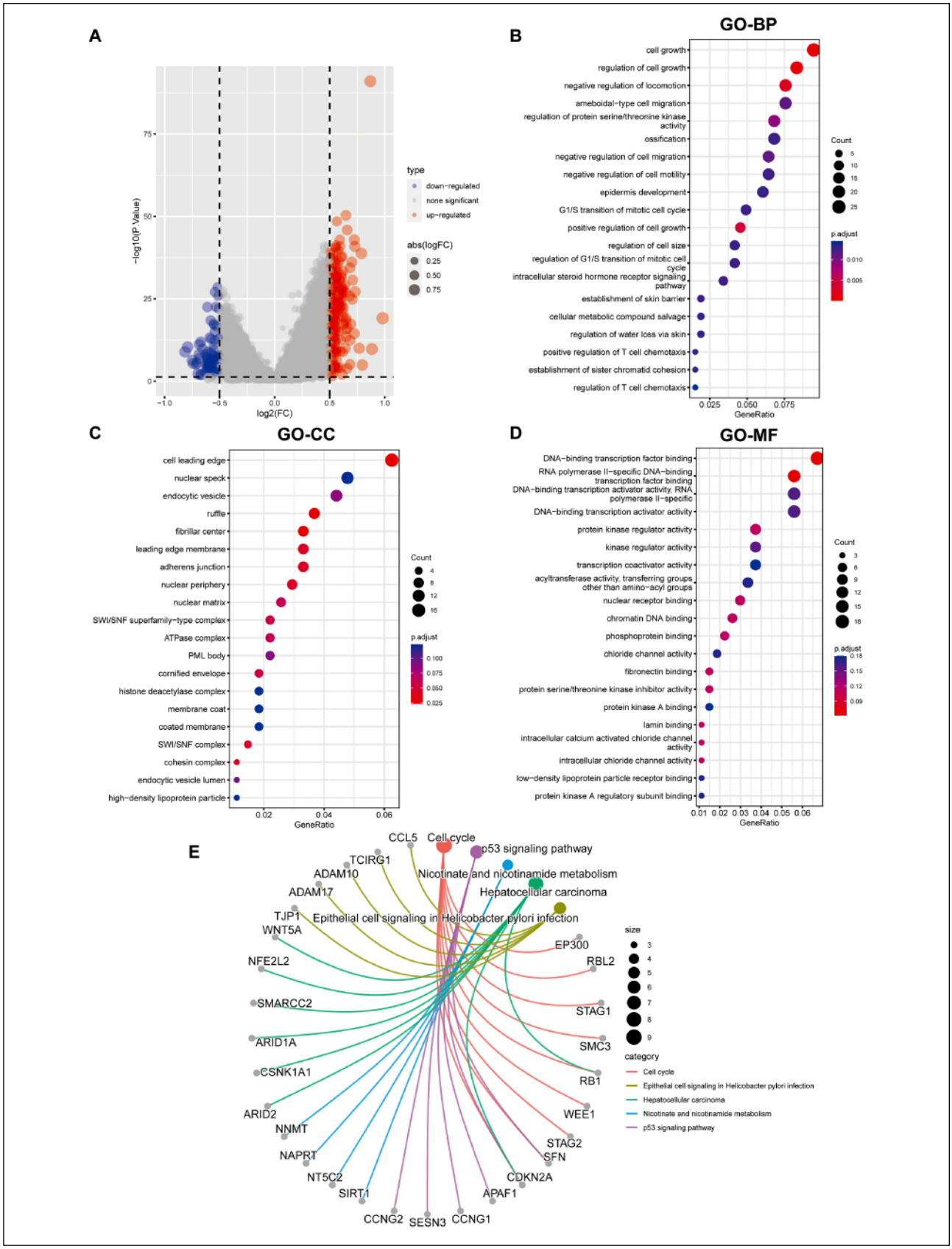
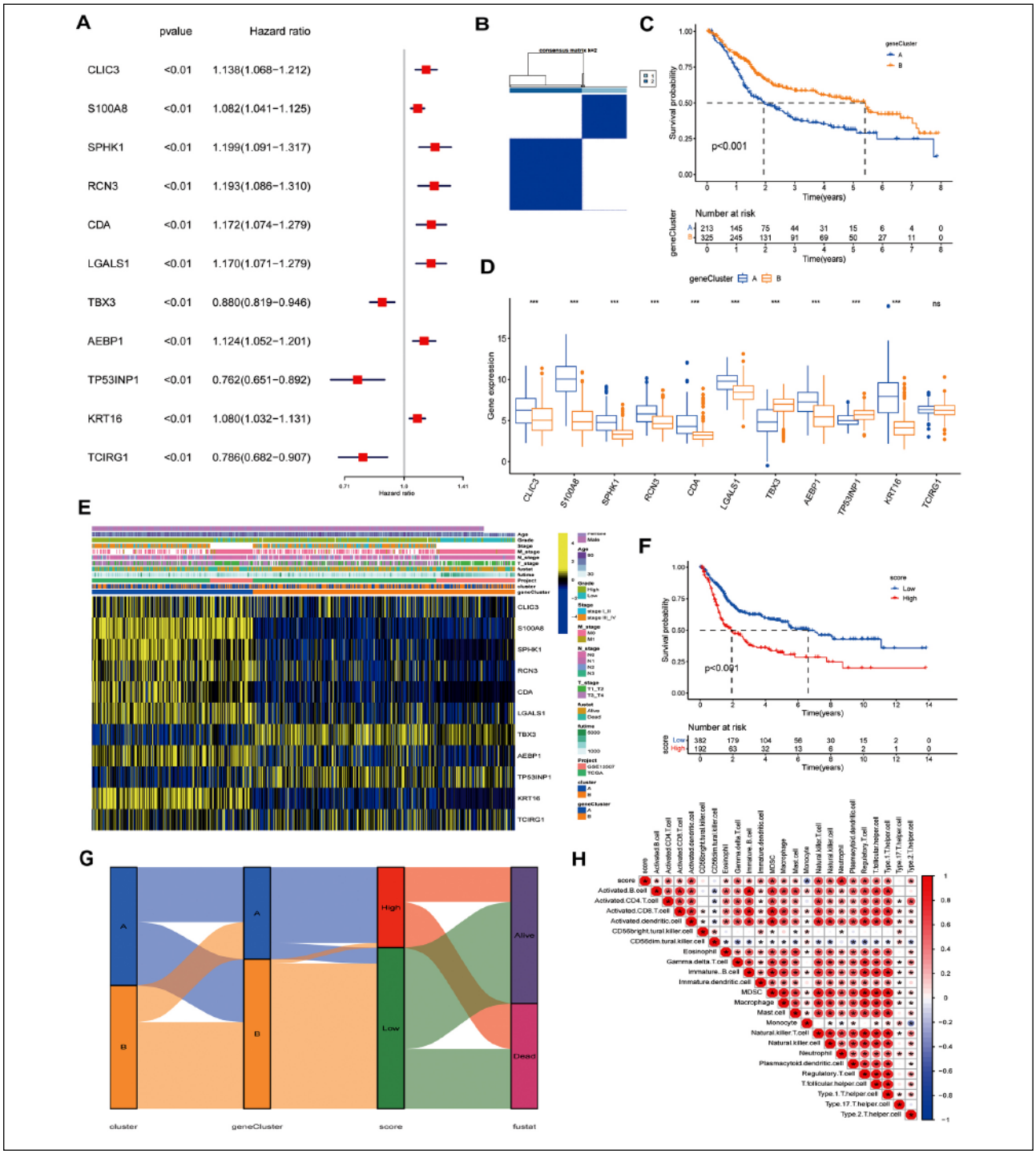


Figure 5.
Construction of lactylation score in BLCA:
A. Forest plot presenting results of univariate Cox regression analysis. B. Consensus matrix heatmap defining various clusters and their correlation area. C. Kaplan-Meier analysis of BLCA patients in two geneClusters. D. The expression of indicated genes in two geneClusters. E. Distributions of clinical features and expression levels of 11 DEGs between the two geneClusters. F. Kaplan-Meier analysis of lactylation score in BLCA. G. The sankey diagram visualized the correlation between cluster, geneCluster, lactylation score, and survival status of BLCA patients. H. The correlation between lactylation score and immune cell infiltration. Red color represents positive correlation, blue color represents negative correlation.



Genetic alteration of prognostic genes

We further explored the expression and genetic alterations of 11 prognostic genes. The SNV frequency of these genes was generally low, with TBX3 having the highest SNV frequency (***Supplementary Figure S2A-B***). Supplementary Figure S3A displays the percentage of *copy number variation* (CNV) for each gene. TP53INP1 had the highest amplification CNV frequency, while LGALS1 showed a significant

CNV deletion. We also represented the percentage of heterozygous and homozygous CNV of each gene in BLCA, including heterozygous amplification, heterozygous deletion, homozygous amplification, and homozygous deletion (***Supplementary Figure S3B***). The linear CNV levels of TCIRG1, TP53INP1, and TBX3 were positively correlated with their mRNA expression (***Supplementary Figure S3C***). The expression of these genes was generally negatively correlated with their DNA methylation level (***Supplementary Figure S3D***).

The association of lactylation score with clinical features

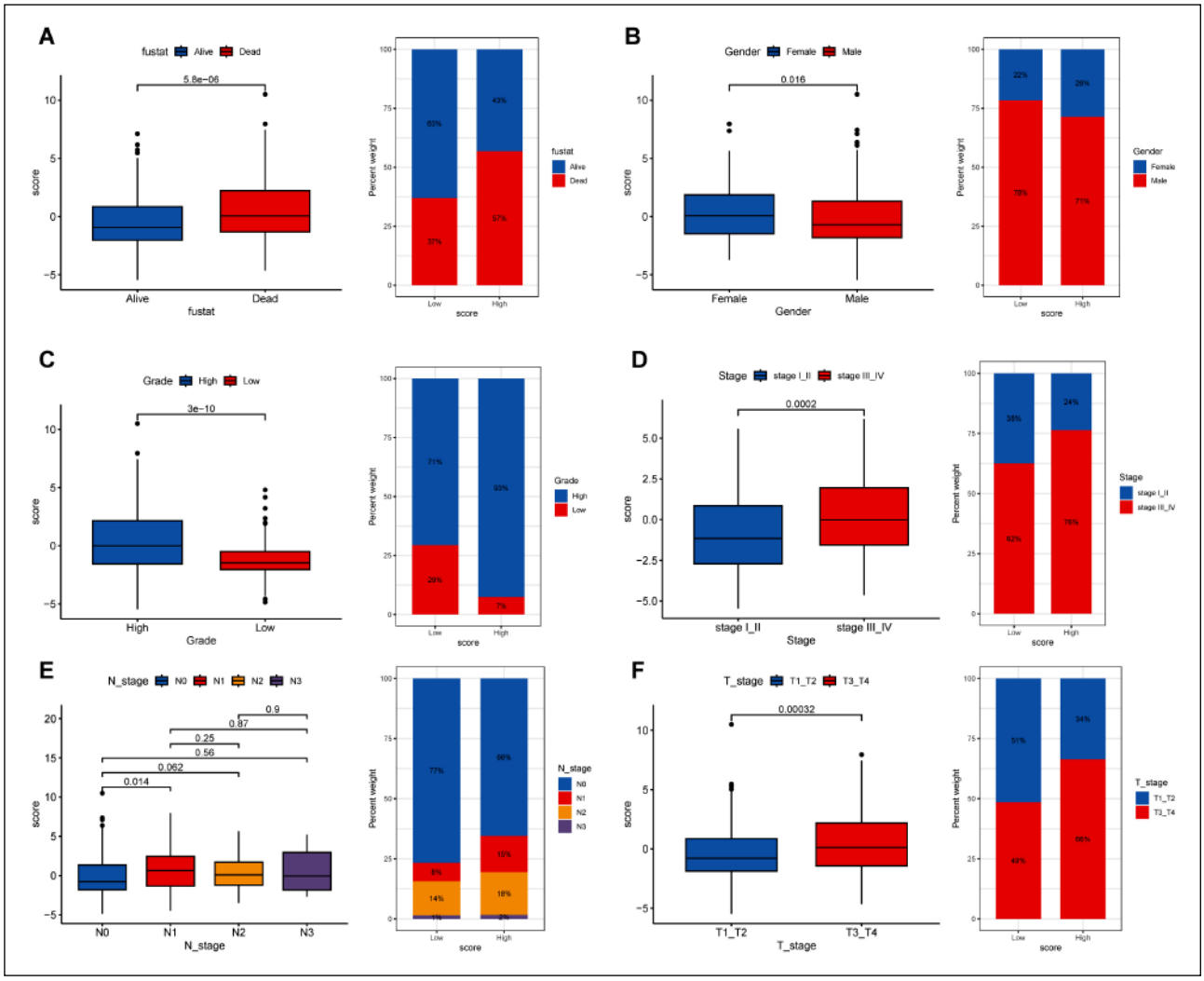
Additionally, we discovered that patients who are alive have lower lactylation scores compared to those who have passed away (Figure 6A). The proportion of patients who are alive in the high lactylation score group (43%) was lower than that in the low lactylation score group (63%). Furthermore, a higher lactylation score was strongly associated with other clinical features, including gender, grade, WHO stage, N stage, and T stage in BLCA (Figure 6B-F).

The correlation of lactylation score with immune microenvironment and efficacy of immunotherapy

The correlation between the immune microenvironment and lactylation score was evaluated. As illustrated in Figure 7A, chemokine and receptor expression were notably elevated in the high lactylation score category. The lactylation score exhibited a favorable correlation with immune-related pathways such as inflammatory response, IL6-JAK-STAT3 signaling pathway, IL2-STAT5 signaling pathway, and interferon GAMMA response (Figure 7B). Additionally, the high lactylation score group demonstrated an elevated immune checkpoint expression, including

Figure 6.

The association of lactylation score with clinical features: A. Left: The lactylation score in alive and dead groups. Right: The percentage of alive and dead patients in high and low lactylation score groups. B. Left: The lactylation score in various gender. Right: The percentage of various gender patients in high and low lactylation score groups. C. Left: The lactylation scores in high- and low-grade groups. Right: The percentages of high- and low-grade patients in high and low lactylation score groups. D. Left: The lactylation score in high- and low-stage groups. Right: The percentage of high and low stage patients in high and low lactylation score groups. E. Left: The lactylation score in high and low N stage groups. Right: The percentage of high and low N stage patients in high and low lactylation score groups. F. Left: The lactylation score in high and low T stage groups. Right: The percentage of high and low T stage patients in high and low lactylation score groups.

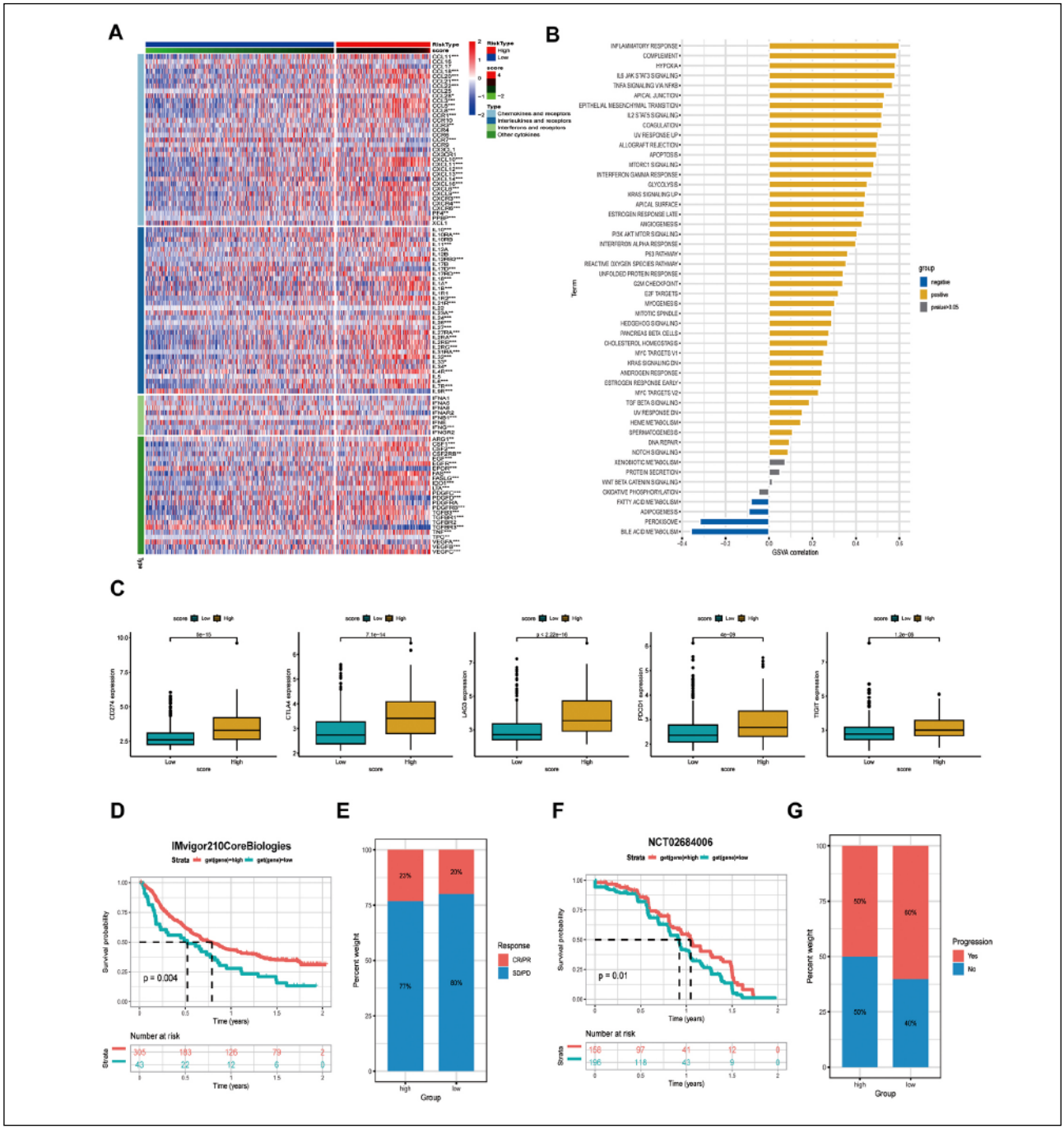


CD274, CTLA4, LAG3, PDCD1, and TIGIT (Figure 7C). Moreover, the high lactylation score group presented a higher frequency of gene mutations (***Supplementary Figure S4A-C***). These findings further support the theory that cancer patients with a high lactylation score may respond well to immunotherapy.

To verify our hypothesis, further analysis was performed using immunotherapy datasets. We confirmed that patients with a high lactylation score were responsive to ICI treatment in the IMvigor210 cohort (20) (Figure 7D-E; Urothelial carcinoma) and NCT02684006 cohort (21) (Figure 7F-G; Renal clear cell carcinoma).

Figure 7.

The correlation of lactylation score with immune microenvironment and efficacy of immunotherapy: A. The expression of chemokines and chemokine receptors in high and low lactylation score group in BLCA. B. The correlation of lactylation score with HALLMARK pathway scores. C. The expression of immune checkpoints in high and low lactylation score group. D. Kaplan-Meier analysis of patients in high and low lactylation score groups in IMvigor210 cohort. E. The percentage of patients with different progress status after ICI treatment in high and low lactylation score groups in IMvigor210 cohort. F. Kaplan-Meier analysis of patients in high and low lactylation score groups in NCT02684006 cohort. G. The percentage of patients with different progress status after ICI treatment in high and low lactylation score groups in NCT02684006 cohort.



Apart from immunotherapy, we also analyzed other antitumor drugs. Based on the predicted results using the R package "*pRRophetic*", we displayed 6 commonly used chemotherapeutic agents for BLCA, including gemcitabine, cisplatin, vinblastine, doxorubicin, docetaxel, and paclitaxel, may be resistant to patients with a high lactylation score. Furthermore, 5 targeted agents, including sunitinib, pazopanib, gefitinib, erlotinib, and tipifarnib, were also revealed to be resistance in high lactylation score group (***Supplementary Figure S5***).

Discussion

Lactylation modification seems to have an important place in cancer therapeutics research. It has been well shown that tumor cells undergo metabolic reprogramming, resulting in increased lactate production and alterations in the microenvironment (22-24). Related with this issue, lactate has been shown to play a critical role in tumor growth and progression by promoting angiogenesis, immune evasion, and metastasis (25). Moreover, lactylation of proteins has been found to modulate cellular processes involved in carcinogenesis regarding the survival, proliferation, and invasion status (26, 27). These findings suggest that lactylation modification may offer novel therapeutic targets in the management of cancer. However, further studies are needed to identify specific lactylated targets and explore the possible underlying mechanisms of this process in different types of tumors.

In recent years, increasing attention has been focused on the possible role of lactylation modification in carcinogenesis of different tumor types such as bladder cancer (28), lung cancer (29), liver cancer (27, 30), and gastric cancer (31). A recent study indentified a unique cluster of cisplatin-resistant epithelial cells by constructing a BLCA single-cell atlas. The elevated lactylation was founded in the cisplatin-resistant cluster and verified in BLCA cell lines with cisplatin resistance. Furthermore, H3K18la was up-regulated in cisplatin-resistant BLCA cell lines, and heightened transcription factor expression such as YBX1 and YY1, ultimately driving cisplatin resistance in BLCA (28). More and more studies demonstrated the emerging evidence suggesting that lactylation modification plays a critical role in the pathogenesis and progression of cancer. However, further research is certainly needed to explore the exact underlying mechanisms of lactylation in different types of tumors and to develop novel lactylation-based therapies for the treatment of cancer.

In this study, firstly we evaluated 11different lactylation genes. By examining the expression of these lactylation genes, we identified two subtypes of BLCA (cluster A and B). Additionally, we observed the difference in malignant cancer-promoting pathways and immune-related pathways between cluster A and B, such as TGF BETA SIGNALING, MTORC1 SIGNALING, and WNT SIGNALING PATHWAY. Based on these findings, in the second stage we investigated the immune infiltration in BLCA samples. The stromal, immune, and ESTIMATE scores were all higher in cluster A, and most immune cell levels were found to be highly infiltrated in cluster A than in cluster B.

Based on this information in our study we aimed to develop a lactylation score for quantifying patient risk. Initially, we screened 275 DEGs across two clusters and later we conducted univariate Cox regression analysis to determine the prognostic value of these 275 DEGs. 11 genes being identified at the last stage have been found to be correlated with overall survival period ($p < 0.001$). Following this critical evaluation based on the PCA algorithm used , we established a lactylation score depending on these 11 prognostic genes. In addition to the poorer survival rates observed in patients with high lactylation scores; the correlation between the lactylation score and immune cell infiltration suggested a positive association between these two variables. Among the 11 genes included in the established lactylation score, CLIC3、TCIRG1 、S100A8 and LGALS1 were found to be related to glycolysis and lactate regulation in tumor cells. CLIC3 and TCIRG1, as a member of the chloride intracellular channel protein family and a subunit of a large protein complex known as a vacuolar H+-ATPase (V-ATPase) respectively, play important roles in regulating the pH of cells and their surrounding environment (32, 33). The acidification of the *tumor microenvironment* (TME) can regulate glycolysis in cells and mediate the production of lactate, thereby regulating the lactylation modification of proteins (34). S100A8 is a member of the S100 family of proteins containing 2 EF-hand calcium-binding motifs. It is involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. According to a recent study, S100A8 also play a critical role in promoting macrophage perturbation and glycolysis through the TLR4/MyD88/NF-κB signaling pathway (35). LGALS1, also known as Gal-1, is a member of the family of beta-galactoside-binding proteins implicated in modulating cell-cell and cell-matrix interactions. Previous study had revealed that the TLR4/Gal-1 signaling pathway could regulate lactate-mediated EMT processes in CRC cells (36). Although no studies have revealed that the other seven genes are directly or indirectly related to lactylation modification, many studies have reported that they are closely related to the occurrence and development of bladder cancer. Among them, SPHK1, RCN3, CDA, TBX3, AEBP1 and KRT16 are widely reported oncogenes. It can regulate cell apoptosis, promote cell proliferation, migration, or resist immune killing by activating STAT3 and NF-κB signaling pathways, participating in collagen biosynthesis, inducing specific gene mutations, or acting as transcriptional promoters (37-43). TP53INP1 has been reported to be a tumor suppressor gene in bladder cancer, which can inhibit the migration and invasion of bladder cancer cells by inducing autophagy and inhibiting the activation of *extracellular regulated kinase* (ERK) (44).

The effectiveness of an ICI treatment can be predicted by chemokines, chemokine receptors, and immune checkpoints (45, 46). Chemokines are critical in directing immune cell migration necessary to mount and deliver an effective antitumor immune response. Chemokine secretion is often altered in the TME, and an aberrant chemokine profile can facilitate the differentiation and infiltration of immunosuppressive pro-tumorigenic cells into the tumor, namely Treg cells, MDSCs and TAMs. Due to their multifaceted role in the tumor immune response and tumor biology, the chemokine network has emerged as a potential

immunotherapy target (47). In addition, immune checkpoints are another key regulator of the tumour immune response. Normally, the presence of immune checkpoints avoids the damage caused by excessive immune response, but tumour cells can escape from the immune system by expressing corresponding ligands that bind to them, activating inhibitory pathways or inhibitory immune checkpoints within the immune cells, inhibiting immune cell activity or mediating apoptosis of immune cells. Immune checkpoint inhibitors can enhance the immune attack against tumours by blocking the interaction of these immune checkpoint proteins with their ligands and preventing the inactivation of T-cell function (48). Our results reveal that high expression levels of chemokines, chemokine receptors, and immune checkpoints were observed in the group with high lactylation scores, which indicated although high lactylation score is a poor prognostic factor for BLCA, patients with high lactylation score may respond better to an ICI treatment. This finding is highly valuable in an attempt to predict the efficacy of ICI treatment in a reliable manner in cases with BLCA. The analysis of immunotherapy datasets confirmed that patients with high lactylation scores had positive responses to ICI treatment in the IMvigor210 and NCT02684006 cohorts. These findings again further supported our findings.

However, as a bioinformatics-based analysis, the findings of this study are subjected to several limitations. First and foremost, as research in the protein lactylation field is still in its preliminary stages, there are relatively few genes directly related to lactylation modification reported. Therefore, the lactylation gene subtypes and lactylation score identified based on these genes may not be able to fully describe the characteristics of lactylation-related gene signature. Furthermore, the experimental validation of all signature genes currently faces practical difficulty due to the lack of corresponding lactylation antibodies. Another potential limitation of this study is that the findings of this study rely mainly on retrospective data collected from public databases. The accuracy of the lactylation score in predicting the response to ICI treatment by BCa still needs to be verified by large-scale clinical trials.

Conclusions

In the light of our findings and the limited data published so far in the literature, we may state that lactylation genes have a significant control over the immune microenvironment of tumors, clinical traits and treatment outcomes in cases with BLCA. Moreover, a lactylation score has been well established by our group and this scoring has proven its reliability in the prediction of the prognosis and efficacy of ICI treatment. All these observations indicated well the vital role of lactylation in clinical practice which could enable the physicians to potential customization of the applied treatments in BLCA patients.

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Declarations

Ethical approval: *The study was approved by the Institutional Review Board of Shantou Central Hospital and carried out following the Declaration of Helsinki.*

Availability of data and material: *The data that support the findings of this study are available with the article and its supplementary material, or are available from the corresponding authors upon reasonable request.*

Competing interests: *The authors declare that there is no conflict of interest.*

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