


Research Article

Mining TCGA database for tumor mutation burden and their clinical significance in bladder cancer

Jia Lv^{1,*}, Yongze Zhu^{2,*}, Alin Ji¹, Qi Zhang¹ and  Guodong Liao¹

¹Department of Urology, Zhejiang Provincial Peoples' Hospital, Peoples' Hospital of Hangzhou Medical College, Hangzhou 310014, China; ²Department of Laboratory, Zhejiang Provincial Peoples' Hospital, Peoples' Hospital of Hangzhou Medical College, Hangzhou 310014, China

Correspondence: Guodong Liao (liaoguodong2019@outlook.com)



Background: Bladder cancer is the ninth most-common cancer worldwide and it is associated with high morbidity and mortality. Tumor mutational burden (TMB) is an emerging biomarker in cancer characterized by microsatellite instability. TMB has been described as a powerful predictor of tumor behavior and response to immunotherapy.

Methods: A total of 443 bladder cancer samples obtained from The Cancer Genome Atlas (TCGA) were analyzed for mutation types, TMB values, and prognostic value of TMB. Differentially expressed genes (DEGs) were identified from the TMB groupings. Functional analysis was performed to assess the prognostic value of the first 30 core genes. CIBERSORT algorithm was used to determine the correlation between the immune cells and TMB subtypes.

Results: Single nucleotide polymorphism (SNP) and C>T were reported as the most common missense mutations and we also identified a high rate of mutations in TP53, TTN, KMT2D. Bladder cancer patients with high TMB showed a better prognosis. Enrichment analysis of the DEGs revealed that they were involved in the regulation of the P13K-Akt signaling pathway, cytokine–cytokine receptor interaction, and Ras signaling pathway. The high expression of hub genes *ADRA2A*, *CXCL12*, *S1PR1*, *ADAMTS9*, *F13A1*, and *SPON1* was correlated with poor overall survival. Besides, significant differences in the composition of the immune cells of T cells CD8, T cells CD4 memory activated, NK cells resting and Mast cells resting were observed.

Conclusions: The present study provides a comprehensive and systematic analysis of the prediction of TMB in bladder cancer and its clinical significance. Also, the study provides additional prognostic information and opportunities for immunotherapy in bladder cancer.

Introduction

Bladder cancer, the ninth most-common malignancy worldwide with an estimated 356000 new cases and 145000 deaths annually, has a propensity to relapse, requiring lifelong monitoring after diagnosis [1,2]. Until very recently, bladder cancer treatment had seen little progress since, over the last three decades, a limited range of treatment options with an overall 5-year survival rate was being used by clinicians to treat patients [3,4]. Approximately 25% of bladder cancer is muscle-invasive bladder cancer. Poor prognosis in muscle-invasive bladder cancer is reported with 85% of the patients dying within 2 years without treatment [5]. In recent years, the use of immunotherapy in the treatment of muscle-invasive and metastatic bladder cancer has shown great potential in clinical application [6]. However, there are no biomarkers for assessing the effectiveness of immunotherapy in bladder cancer.

Tumor mutational burden (TMB) refers to the number of somatic mutations per 1 million bases, excluding single nucleotide polymorphism (SNP), germline, copy number variation, and structural variation [7,8]. TMB is an emerging characteristic of cancer and is associated with microsatellite instability [9,10]. TMB increase in the human cancer genome is attributed to endogenous factors and environmental

*These authors contributed equally to this work and should be considered co-first authors.

Received: 15 December 2019

Revised: 31 March 2020

Accepted: 01 April 2020

Accepted Manuscript online:

02 April 2020

Version of Record published:

21 April 2020

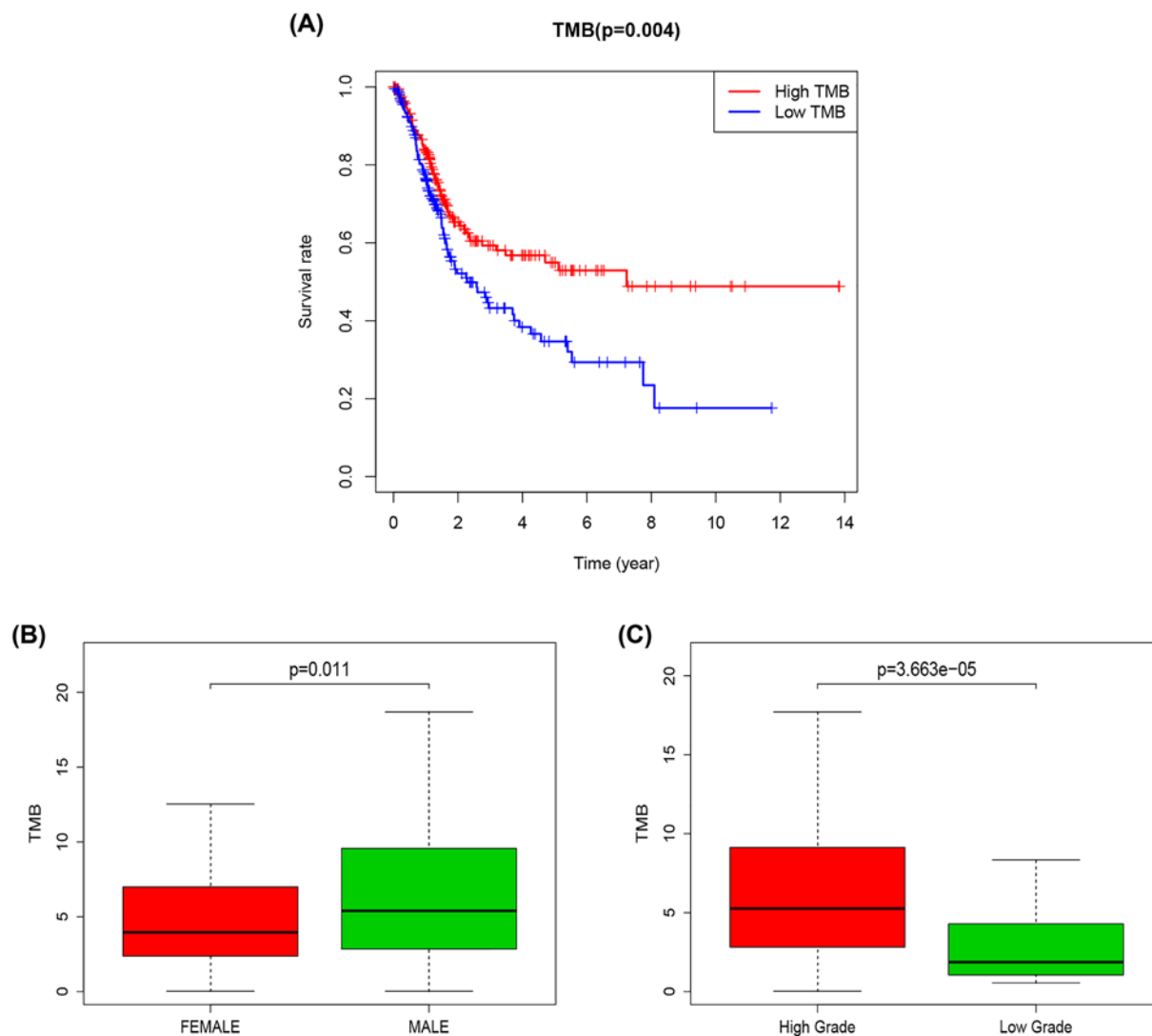


Figure 2. TMB correlation analysis

(A) Kaplan–Meier curves of overall survival of the high- and low-TMB groups. (B) Wilcox test for patients stratified by gender. (C) Wilcox test for patients stratified by grade.

Identification of differentially expressed genes

Based on the median TMB value (5.132), the TMB group was divided into a high-TMB group and a low-TMB group. The ‘limma’ R package was utilized to identify TMB-related differentially expressed genes (DEGs) [16], and all DEGs with $FDR < 0.05$ and $|\log_2 FC| > 0.5$ were exported, and the ‘pheatmap’ R package used to perform hierarchical clustering.

Functional analysis of DEGs

The gene ontology (GO) pathway enrichment analysis and KOBAS-Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis of DEGs were performed by ‘clusterProfiler, org.Hs.eg.db, plot, ggplot2’ in R package [17]. The protein–protein interaction (PPI) network of DEGs were constructed in the STRING database [18], and the number of core gene nodes in the PPI network were visualized using Cytoscape software in R package [19].

Core gene survival analysis

The survival package in R was used to assess the prognostic value for the top 30 core genes in bladder cancer.

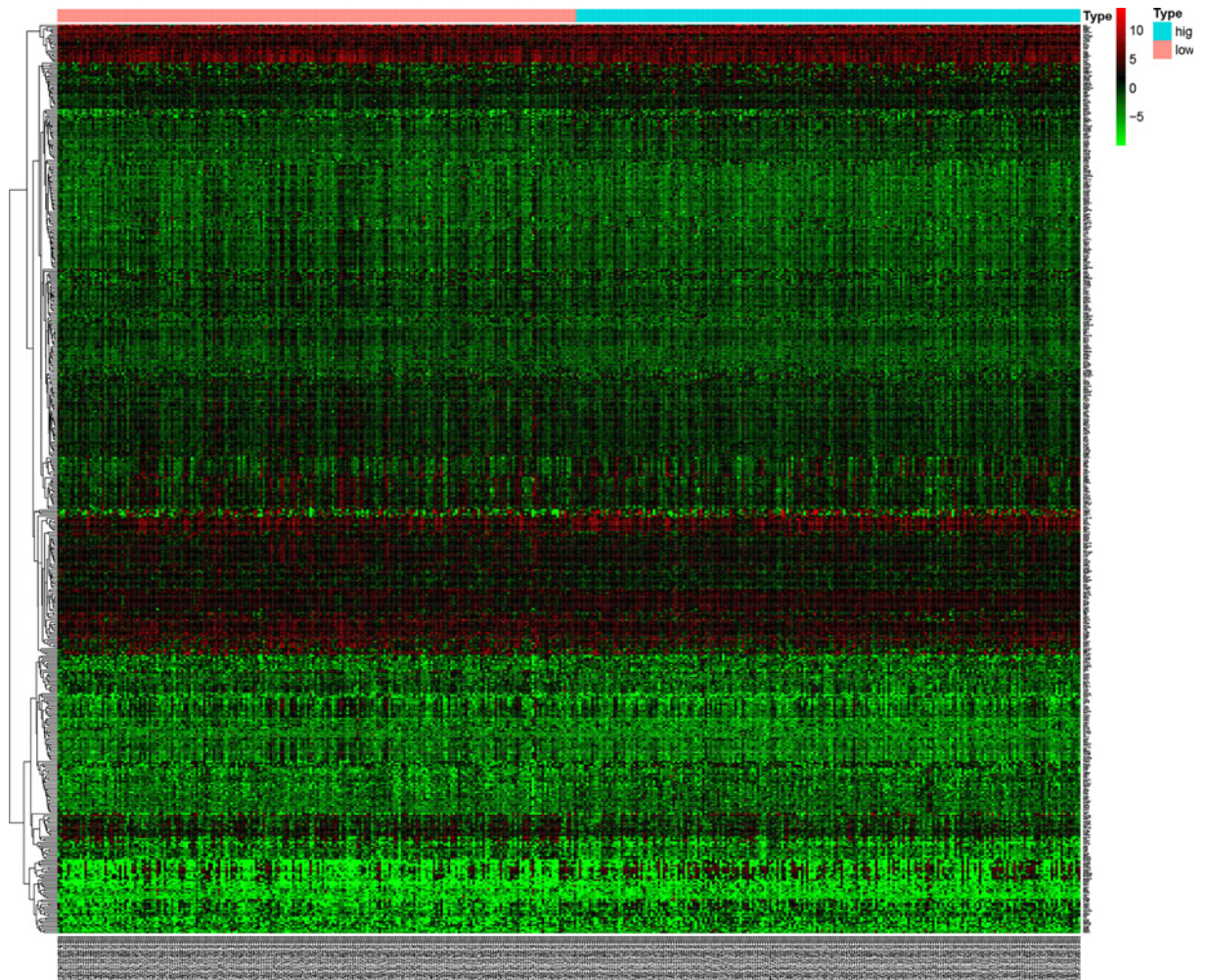


Figure 3. Hierarchical clustering heatmap of DEGs between high- and low-TMB groups

The higher and lower expressed genes were shown in red and green, respectively, and genes with the same expression level in black.

CIBERSORT analysis

CIBERSORT is a deconvolution tool that uses linear support vector regression to determine the expression matrix of human leukocyte subtypes [20]. The abundance of 22 leukocyte subtypes in bladder cancer was obtained using the ‘CIBERSORT’ R package, with a cut-off P -value <0.05 . Wilcoxon test was performed to analyze the differences not only in the immune cell abundance in the patients but also in the high-TMB and low-TMB groups which were visualized using the ‘beeswarm’ R package.

Statistical analysis

R Studio v 1.1.463 and Bioconductor (<https://www.bioconductor.org/>) were used for statistical analysis [21]. Overall survival was assessed by Kaplan–Meier and log-rank test methods, and subgroup differences were analyzed by the Wilcoxon test or Kruskal test, with P -values <0.05 considered to be statistically significant.

Results

Mutations in bladder cancer

We first evaluated the variation in each TCGA bladder cancer sample to provide insights into the factors associated with bladder cancer mutagenesis. The findings revealed that missense mutations, SNP, and C>T mutation were more

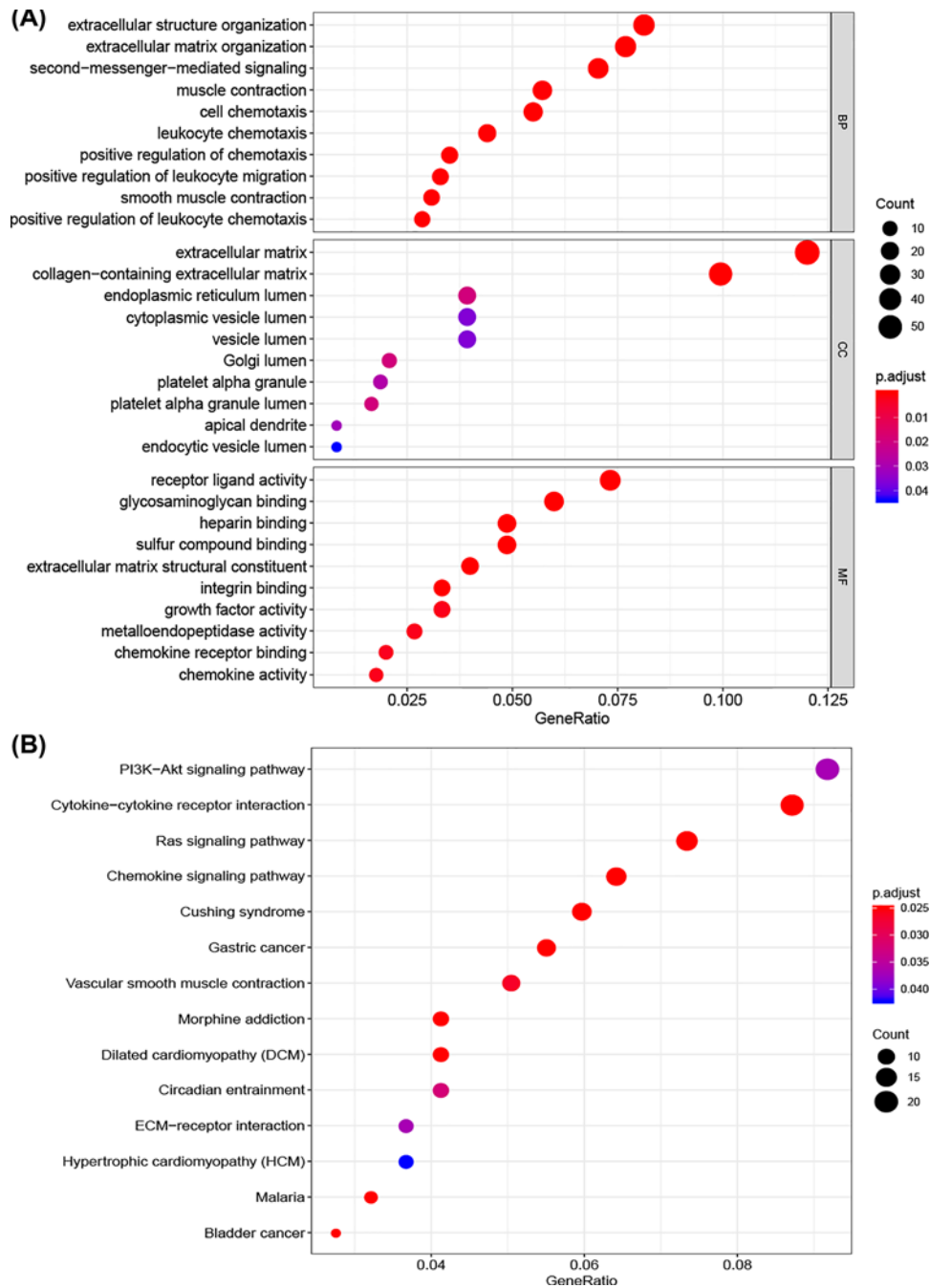


Figure 4. Functional enrichment analysis of DEGs

(A) Functional analysis of the top ten enriched biological processes (BPs), cell composition (CC), and molecular function (MF) of GO analysis. (B) KEGG enrichment diseases analysis.

common, with the highest mutation frequency being 3398 (Figure 1A). Based on the MutSigCV algorithm, the waterfall diagram revealed the integration status of somatic mutations in TCGA bladder cancer, and the results showed that the somatic mutations of TP53, TTN, KMT2D, ARID1A, MUC16, PIK3CA, and RB1 ($P < 0.001$) were higher (Figure 1B).

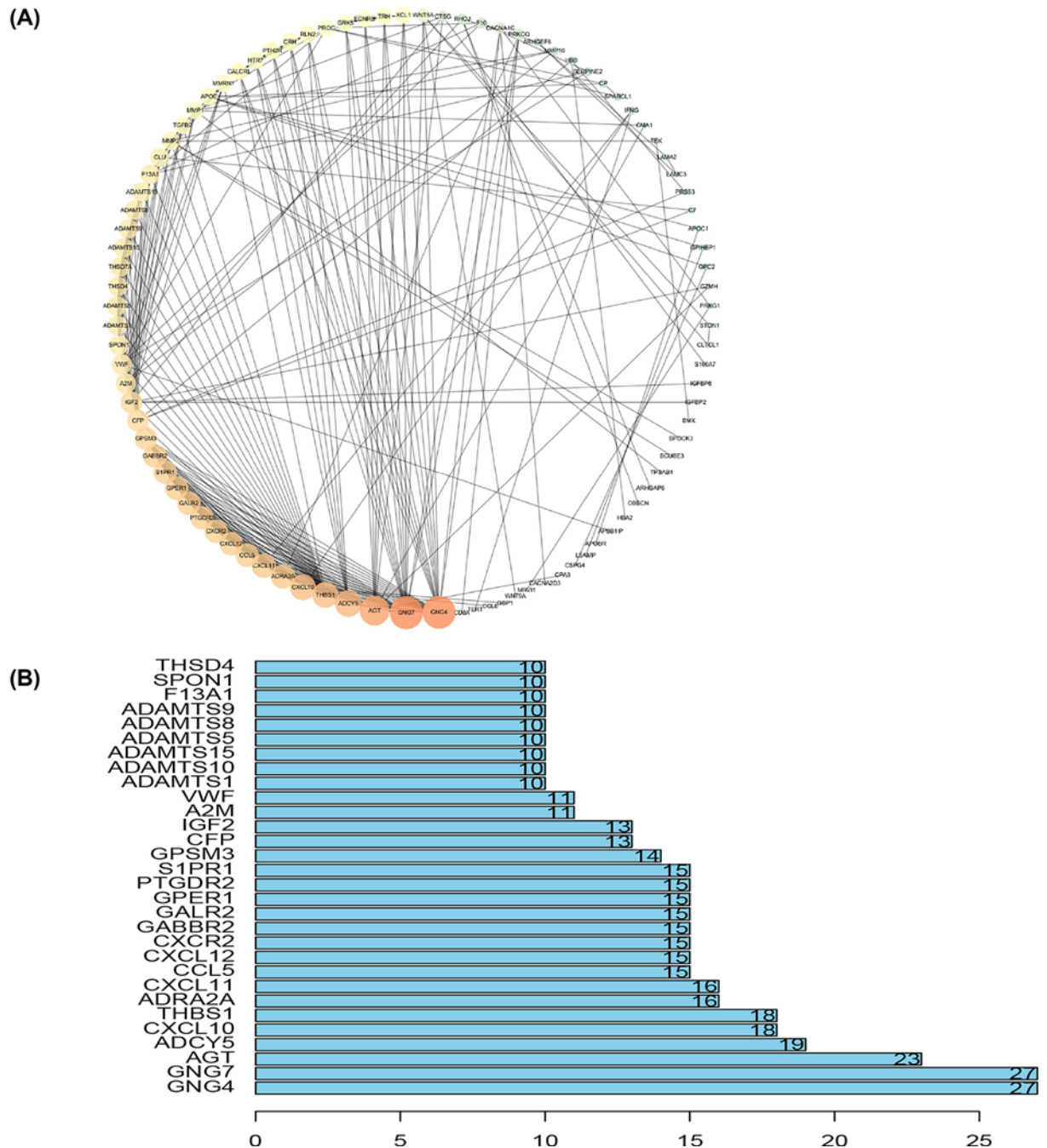


Figure 5. PPI network analysis

(A) PPI network. The color and size of the map node was determined by the degree value, which was a gradual process. Green and small circles represent low values, and orange and large circles represent high values. (B) Histogram of core genes.

TMB and clinical relevance

Kaplan–Meier analysis was used to assess the potential correlation of TMB in bladder cancer with prognosis. The results showed that TMB (Figure 2A, $P=0.004$) was associated with prognosis. Patients with high-TMB had a better prognosis, suggesting that the patients experienced better effects on the immune response. The correlation between TMB and clinicopathological features including patients' gender, tumor grade (tumor cell differentiation), and TNM staging revealed that TMB was associated with gender (Figure 2B, $P=0.011$) and tumor grade (tumor cell differentiation) (Figure 2C, $P=3.663e-05$) in patients with bladder cancer. The TMB of male patients was reported to be higher

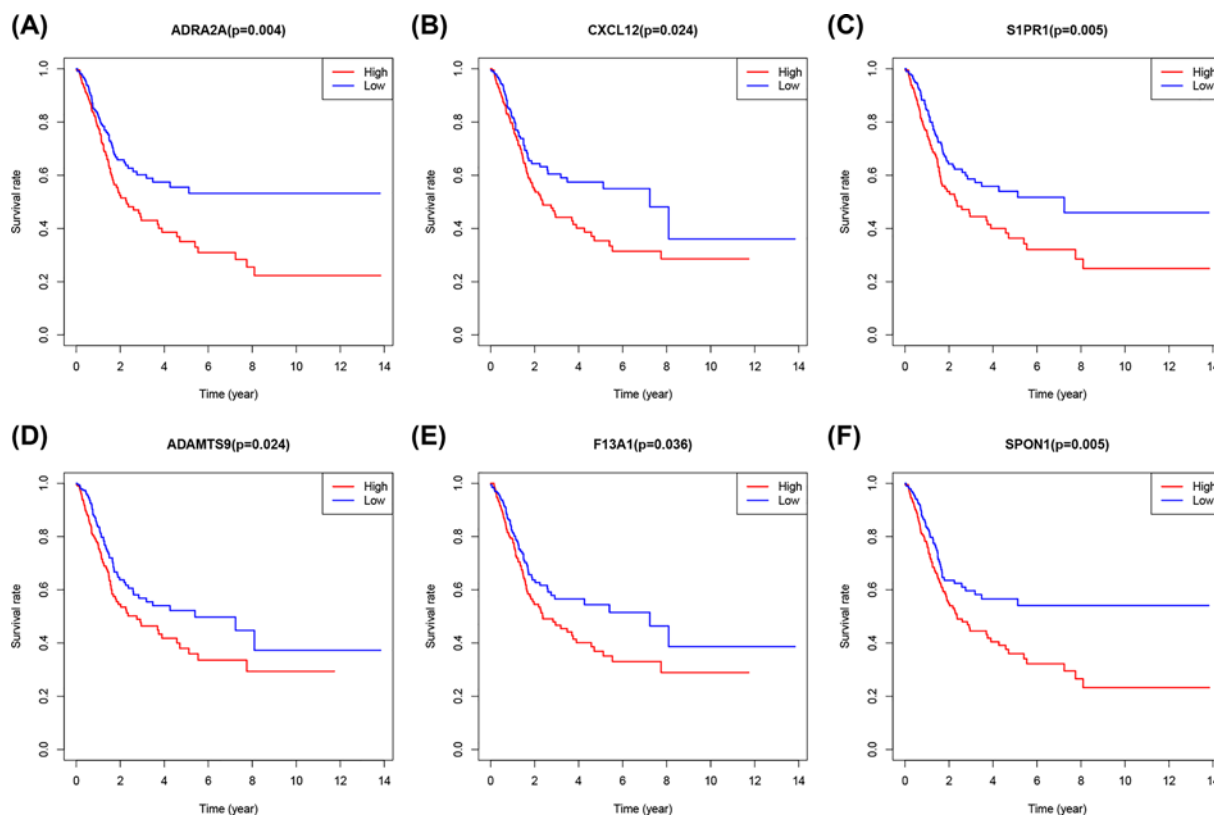


Figure 6. The overall survival of bladder cancer patients with high or low expression of ADRA2D (A) CXCL12 (B) S1PR1 (C) ADAMTS9 (D) F13A1 (E) and SPON1(F).

than that of female patients. Besides, bladder cancer patients with high tumor grade (well differentiation) had a higher TMB value.

Enrichment analysis for the DEGs

TMB-associated DEGs in bladder cancer were analyzed using the ‘limma’ package. A total of 506 DEGs were identified, including 181 up-regulated and 325 down-regulated genes. Figure 3 shows the hierarchical clustering heatmap. GO enrichment analysis was performed to elucidate the biological functions of the DEGs. Figure 4A shows the top 30 enriched GO terms which were associated with tumor immune cell response and composition of extracellular matrix (ECM). As shown in Figure 4B, KEGG pathway analysis showed that the DEGs were mainly enriched in the PI3K-Akt signaling pathway, cytokine–cytokine receptor interaction, Ras signaling pathway, chemokine signaling pathway, ECM–receptor interaction, and bladder cancer.

PPI network of DEGs

The PPI network of the DEGs was constructed using the STRING online database to determine interactions among DEGs and discover important genes involved in tumorigenesis. The networks were visualized using the Cytoscape software (Figure 5A). Among the top 30 core genes with the highest clustering included *GNG4*, *GNG7*, *AGT*, *ADCY5*, *CXCL10*, *THBS1*, *ADRA2A* and *CXCL11* etc. (Figure 5B). Kaplan–Meier analysis was used to investigate the prognostic values of the 30 potential core genes (Figure 5B). In conclusion, high expression of *ADRA2A* (Figure 6A), *CXCL12* (Figure 6B), *S1PR1* (Figure 6C), *ADAMTS9* (Figure 6D), *F13A1* (Figure 6E), and *SPON1* (Figure 6F) was associated with poor overall survival in bladder cancer patients, with a P -value <0.05 considered to be statistically significant.

Association of TMB and tumor immune microenvironment

After the previous calculation, the proportion of 22 immune cells in all samples was obtained. The findings revealed that the first three sites in the low TMB group were macrophages M0 (0.155), macrophages M2 (0.1443), and T

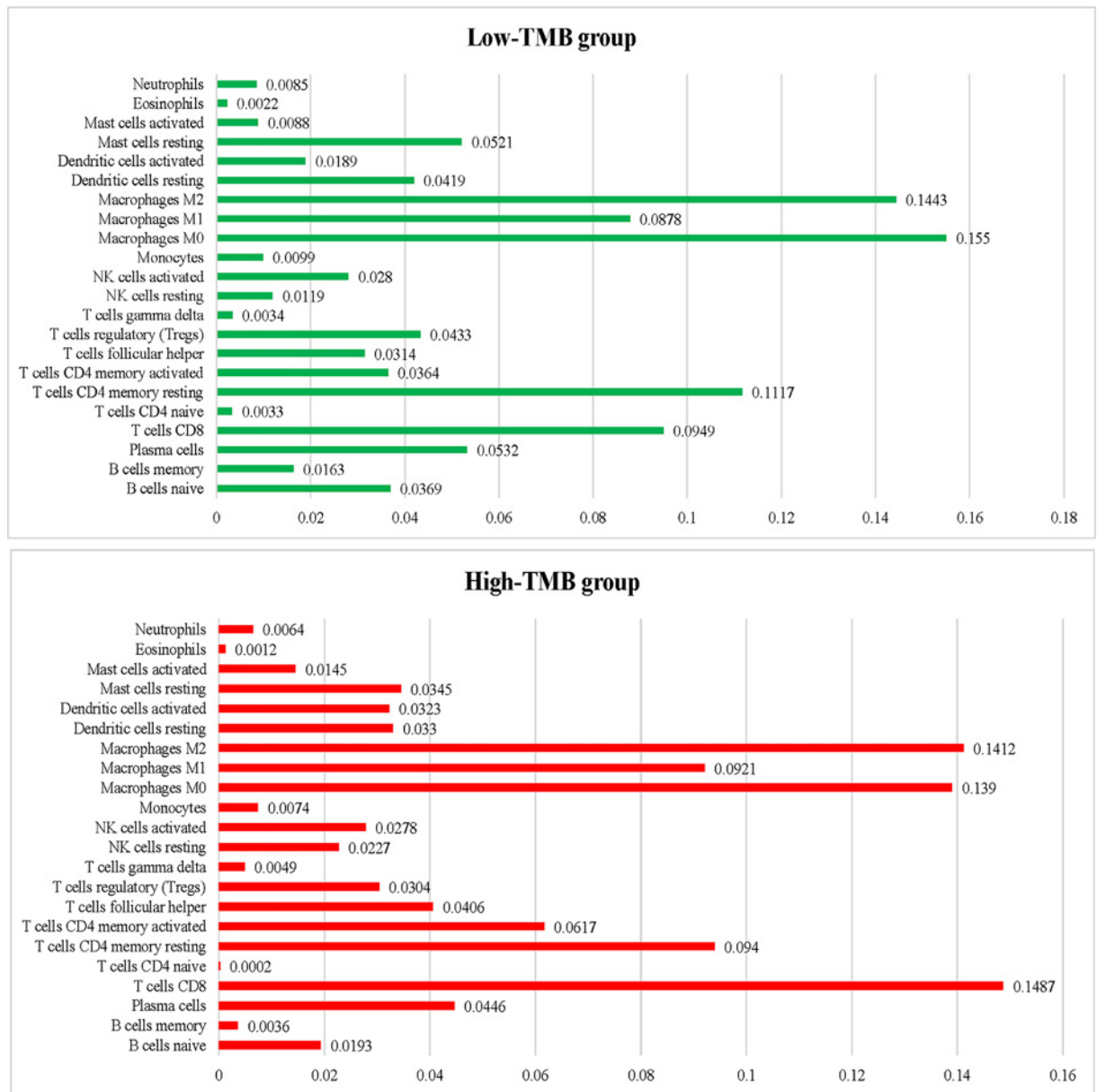


Figure 7. The average proportion of each type of tumor-infiltrating immune cells in in the low- and high-TMB groups

cells CD4 resting (0.1117), while in the high TMB group were T cells CD8 (0.1487), Macrophages M2 (0.1412), and Macrophages M0 (0.139), respectively (Figure 7). The differences in the abundance of each leukocyte subtype between the high- and low-TMB groups showed that samples with high-TMB had a significant increase in the abundance of T cells CD8 ($P < 0.001$), T cells CD4 memory activated ($P = 0.002$), and NK cells resting ($P = 0.023$) and a significant decrease in the abundance of mast cells resting ($P = 0.011$) (Figure 8). In conclusion, the difference in TIICs between the two groups suggests that they may have important clinical implications.

Discussion

The tumorigenesis is a complex multistep process, involving genetic alterations interacting with immune cells in tumor microenvironment [22,23]. Somatic missense mutations strongly contribute to the generation of novel tumor epitopes [13]. A better understanding of the relationship between TMB with highly immunogenic tumors may help to evaluate the effect of immunotherapy and provide a mechanistic explanation for the observed clinical survival patterns. Immune checkpoint molecule inhibitors have opened the possibility of immunotherapy for bladder cancer,

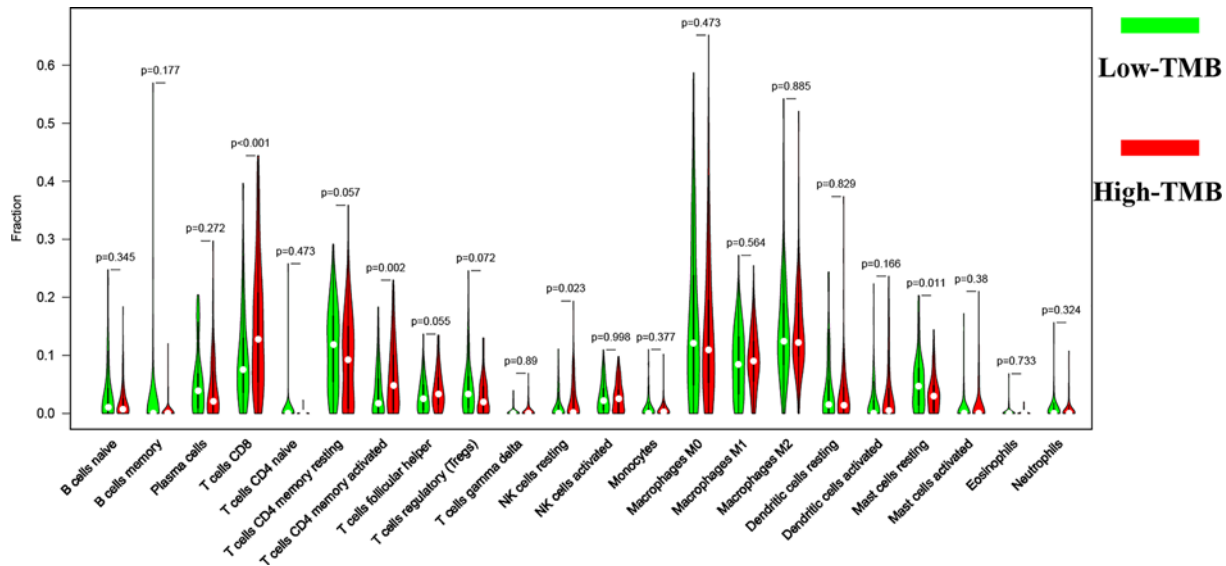


Figure 8. Differential analysis of tumor-infiltrating immune cells (TIICs) between high- and low-TMB groups

especially for muscle-invasive and metastatic bladder cancer [24,25]. Recent research has correlated bladder cancer and the immune environment [26]. However, there are no available biomarkers to assess the effectiveness of immunotherapy in bladder cancer.

In the present study, we analyzed mutations in bladder cancer samples. The findings revealed that missense mutations, SNP, and C>T mutations were the most common mutation forms in bladder cancer. Previous studies have demonstrated the significance of missense mutation and SNP in tumorigenesis, progression, and prognosis in various cancer types, including bladder cancer [27–30]. The three most frequently mutated genes were *TP53*, *TTN*, and *KMT2D*. *TP53* is one of the famous tumor suppressor genes reported to regulate the cell cycle thus inhibits the development of cancerous cells [31]. *P53* protein maintains genome stability and prevents the occurrence of genomic mutation [32]. *KMT2D* is a known cancer-related protein that regulates tumor growth and metastasis, thus influences prognosis [33,34]. In bladder cancer, *KMT2D* functions as a tumor suppressor and supports tumor cell viability, migration, and invasion [35].

The clinical significance of TMB in bladder cancer was analyzed. The results showed that TMB was higher in bladder cancer patients with high tumor grade. Bladder cancer patients with low TMB had a poor prognosis compared with those with high TMB. These results demonstrated that high-TMB often has a relatively favorable living condition. In breast cancer, TMB is a determinant of immune-mediated survival of patients and identify candidate immune-regulatory mechanisms associated with immunologically cold tumors [36]. Therefore, TMB is suggested to be an independent predictor of immunotherapy response in various types of cancers including bladder cancer [37–39].

The potential biological functions of TMB-associated DEGs were analyzed. The functions of TMB-associated DEGs were mainly associated with tumor immune cell response, PI3K-Akt signaling pathway, cytokine–cytokine receptor interaction, Ras signaling pathway, chemokine signaling pathway, ECM–receptor interaction, and bladder cancer. The PI3K/AKT signaling pathway shows frequent molecular alterations and increased activity in cancers. Previous studies have revealed the significant role of the PI3K/AKT pathway in bladder cancer. Leupaxin promotes bladder cancer proliferation, metastasis, and angiogenesis through the PI3K/AKT pathway [40]. Another study revealed that activation of the PI3K/AKT pathway plays a critical role in the initiation and progression of bladder cancer [41,42]. Ras signaling pathway is considered to exert an important role in tumorigenesis and progression of human cancers, including RCC and bladder cancer [43,44]. The immense diversity of ECM proteins confers distinct biochemical and biophysical properties that influence cell phenotype. The composition and organization of the ECM are spatiotemporally regulated to control cell biological processes (BPs), but an aberrant expression of ECM dynamics results in the occurrence of diseases such as cancer [45].

Kaplan–Meier analysis demonstrated the TMB-associated DEGs PPI network. *ADRA2A*, *CXCL12*, *SIP1*, *ADAMTS9*, *F13A1*, and *SPON1* were selected as the hub genes which were reported to be mainly involved in DNA

replication, cell cycle control, genomic stability, and mitosis [46–49]. Besides, the hub genes regulate tumor cell proliferation, invasion, apoptosis, and metastasis [50,51]. Therefore, the present study demonstrated that the identified hub genes played a significant role in bladder cancer.

The correlation between TMB and tumor-infiltrating immune cells was analyzed to reflect on the status of the immune microenvironment. In the current study, there was a significant increase in the abundance of T cells CD8, T cells CD4 memory activated, and NK cells resting and a significant decrease in the abundance of Mast cells resting in the high-TMB group compared with the low-TMB group. These results demonstrate that patients with higher infiltration levels of CD8⁺ T cell, CD4 T cell, NK resting cells, and lower Mast cells are more likely to present with better immunotherapeutic effect and prognosis. These findings confirm that CD4, CD8 T cells, and NK cells, may be major players in antitumor immunity in bladder cancers in patients with high TMB.

The present study was not without limitations that should be considered when interpreting our results. For instance, the results of the current study were not validated using an independent patient cohort. Thus, further *in vitro* or *in vivo* experiments are needed to validate our findings.

Conclusion

In conclusion, the present study provides a comprehensive and systematic analysis of the prediction of TMB in bladder cancer and its clinical significance in the recognition, surveillance, and prognosis of bladder cancer. In addition, the present study provides additional prognostic information and opportunities for immunotherapy in bladder cancer.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

The authors declare that there are no sources of funding to be acknowledged.

Author Contribution

Jia Lv and Yongze Zhu performed data analysis work and aided in writing the manuscript. Guodong Liao designed the study, assisted in writing the manuscript. Alin Ji and Qi Zhang edited the manuscript. All authors read and approved the final manuscript.

Ethics Approval

The study was approved by the Ethics Committee of Zhejiang Provincial Peoples' Hospital, Peoples' Hospital of Hangzhou Medical College.

Abbreviations

DEG, differentially expressed gene; ECM, extracellular matrix; FDR, false discovery rate; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; KOBAS, KEGG Orthology Based Annotation System; NK, Natural killer; PPI, protein–protein interaction; RCC, Renal cell carcinoma; SNP, single nucleotide polymorphism; TCGA, The Cancer Genome Atlas; TMB, tumor mutational burden; TMN, Tumor, Node, Metastasis.

References

- Li, Z. et al. (2018) Oncogene miR-187-5p is associated with cellular proliferation, migration, invasion, apoptosis and an increased risk of recurrence in bladder cancer. *Biomed. Pharmacother.* **105**, 461–469, <https://doi.org/10.1016/j.biopha.2018.05.122>
- Martinez Rodriguez, R.H., Buisan Rueda, O. and Ibarz, L. (2017) Bladder cancer: present and future. *Med. Clin. (Barc.)* **149**, 449–455, <https://doi.org/10.1016/j.medcli.2017.06.009>
- Grayson, M. (2017) Bladder cancer. *Nature* **551**, S33, <https://doi.org/10.1038/551S33a>
- Li, Z. et al. (2018) MiR-302b regulates cell functions and acts as a potential biomarker to predict recurrence in bladder cancer. *Life Sci.* **209**, 15–23, <https://doi.org/10.1016/j.lfs.2018.07.057>
- Farling, K.B. (2017) Bladder cancer: risk factors, diagnosis, and management. *Nurse Pract.* **42**, 26–33, <https://doi.org/10.1097/01.NPR.0000512251.61454.5c>
- Massari, F. et al. (2018) Immune checkpoint inhibitors for metastatic bladder cancer. *Cancer Treat. Rev.* **64**, 11–20, <https://doi.org/10.1016/j.ctrv.2017.12.007>
- Samstein, R.M. et al. (2019) Tumor mutational load predicts survival after immunotherapy across multiple cancer types. *Nat. Genet.* **51**, 202–206, <https://doi.org/10.1038/s41588-018-0312-8>
- Zehir, A. et al. (2017) Erratum: Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. *Nat. Med.* **23**, 1004, <https://doi.org/10.1038/nm0817-1004c>

- 9 Hatakeyama, K. et al. (2018) Tumor mutational burden analysis of 2,000 Japanese cancer genomes using whole exome and targeted gene panel sequencing. *Biomed. Res.* **39**, 159–167, <https://doi.org/10.2220/biomedres.39.159>
- 10 Steuer, C.E. and Ramalingam, S.S. (2018) Tumor mutation burden: leading immunotherapy to the era of precision medicine? *J. Clin. Oncol.* **36**, 631–632, <https://doi.org/10.1200/JCO.2017.76.8770>
- 11 Roberts, S.A. and Gordenin, D.A. (2014) Hypermutation in human cancer genomes: footprints and mechanisms. *Nat. Rev. Cancer* **14**, 786–800, <https://doi.org/10.1038/nrc3816>
- 12 Carbone, D.P. et al. (2017) First-line nivolumab in stage IV or recurrent non-small-cell lung cancer. *N. Engl. J. Med.* **376**, 2415–2426, <https://doi.org/10.1056/NEJMoa1613493>
- 13 Klebanov, N. et al. (2019) Burden of unique and low prevalence somatic mutations correlates with cancer survival. *Sci. Rep.* **9**, 4848, <https://doi.org/10.1038/s41598-019-41015-5>
- 14 Mayakonda, A. and Koeffler, H.P. (2016) MafTools: efficient analysis, visualization and summarization of MAF files from large-scale cohort based cancer studies. *BioRxiv* 052662, <https://doi.org/10.1101/052662>
- 15 Schumacher, T.N., Kesmir, C. and van Buuren, M.M. (2015) Biomarkers in cancer immunotherapy. *Cancer Cell* **27**, 12–14, <https://doi.org/10.1016/j.ccell.2014.12.004>
- 16 Ritchie, M.E. et al. (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* **43**, e47, <https://doi.org/10.1093/nar/gkv007>
- 17 Yu, G. (2018) clusterProfiler: universal enrichment tool for functional and comparative study. *bioRxiv* 256784, <https://doi.org/10.1101/256784>
- 18 Szklarczyk, D. et al. (2015) STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* **43**, D447–D452, <https://doi.org/10.1093/nar/gku1003>
- 19 Shannon, P. et al. (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* **13**, 2498–2504, <https://doi.org/10.1101/gr.1239303>
- 20 Newman, A.M. et al. (2015) Robust enumeration of cell subsets from tissue expression profiles. *Nat. Methods* **12**, 453–457, <https://doi.org/10.1038/nmeth.3337>
- 21 Huber, W. et al. (2015) Orchestrating high-throughput genomic analysis with Bioconductor. *Nat. Methods* **12**, 115–121, <https://doi.org/10.1038/nmeth.3252>
- 22 Hsu, Y.-C. et al. (2019) Tumor mutation burden and recurrent tumors in hereditary lung cancer. *Cancer Med.* **8**, 2179–2187, <https://doi.org/10.1002/cam4.2120>
- 23 Zeng, Q. et al. (2020) Bioinformatic identification of renal cell carcinoma microenvironment-associated biomarkers with therapeutic and prognostic value. *Life Sci.* **243**, 117273–117273, <https://doi.org/10.1016/j.lfs.2020.117273>
- 24 Lenfant, L. and Roupert, M. (2018) Immunotherapy and bladder cancer. *Biol. Aujourd'hui.* **212**, 81–84, <https://doi.org/10.1051/jbio/2018028>
- 25 Loidl, W. and Luger, F. (2018) Immunotherapy for locally advanced and metastasized bladder cancer. *Urol. A* **57**, 1334–1341, <https://doi.org/10.1007/s00120-018-0785-1>
- 26 Ma, Y. et al. (2019) Exploring the pathological mechanism of bladder cancer based on tumor mutational burden analysis. *Biomed. Res. Int.* **2019**, 1093815–1093815, <https://doi.org/10.1155/2019/1093815>
- 27 Korphaisarn, K. et al. (2017) FBXW7 missense mutation: a novel negative prognostic factor in metastatic colorectal adenocarcinoma. *Oncotarget* **8**, 39268–39279, <https://doi.org/10.18632/oncotarget.16848>
- 28 McIntyre, J.B. et al. (2014) PIK3CA missense mutation is associated with unfavorable outcome in grade 3 endometrioid carcinoma but not in serous endometrial carcinoma. *Gynecol. Oncol.* **132**, 188–193, <https://doi.org/10.1016/j.ygyno.2013.11.015>
- 29 Yuan, Y. et al. (2015) Nonsense and missense mutation of mitochondrial ND6 gene promotes cell migration and invasion in human lung adenocarcinoma. *BMC Cancer* **15**, 346, <https://doi.org/10.1186/s12885-015-1349-z>
- 30 Guo, G. et al. (2013) Whole-genome and whole-exome sequencing of bladder cancer identifies frequent alterations in genes involved in sister chromatid cohesion and segregation. *Nat. Genet.* **45**, 1459–1463, <https://doi.org/10.1038/ng.2798>
- 31 Fischer, M. et al. (2016) Integration of TP53, DREAM, MMB-FOXM1 and RB-E2F target gene analyses identifies cell cycle gene regulatory networks. *Nucleic Acids Res.* **44**, 6070–6086, <https://doi.org/10.1093/nar/gkw523>
- 32 Negrini, S., Gorgoulis, V.G. and Halazonetis, T.D. (2010) Genomic instability—an evolving hallmark of cancer. *Nat. Rev. Mol. Cell Biol.* **11**, 220–228, <https://doi.org/10.1038/nrm2858>
- 33 Dawkins, J.B. et al. (2016) Reduced expression of histone methyltransferases KMT2C and KMT2D correlates with improved outcome in pancreatic ductal adenocarcinoma. *Cancer Res.* **76**, 4861–4871, <https://doi.org/10.1158/0008-5472.CAN-16-0481>
- 34 Xiong, W. et al. (2018) Downregulation of KMT2D suppresses proliferation and induces apoptosis of gastric cancer. *Biochem. Biophys. Res. Commun.* **504**, 129–136, <https://doi.org/10.1016/j.bbrc.2018.08.143>
- 35 Sun, P. et al. (2019) KMT2D inhibits the growth and metastasis of bladder cancer cells by maintaining the tumor suppressor genes. *Biomed. Pharmacother.* **115**, 108924, <https://doi.org/10.1016/j.biopha.2019.108924>
- 36 Thomas, A. et al. (2018) Tumor mutational burden is a determinant of immune-mediated survival in breast cancer. *Oncoimmunology* **7**, e1490854, <https://doi.org/10.1080/2162402X.2018.1490854>
- 37 Devarakonda, S. et al. (2018) Tumor mutation burden as a biomarker in resected non-small-cell lung cancer. *J. Clin. Oncol.* **36**, 2995–3006, <https://doi.org/10.1200/JCO.2018.78.1963>
- 38 Goodman, A.M. et al. (2017) Tumor mutational burden as an independent predictor of response to immunotherapy in diverse cancers. *Mol. Cancer Ther.* **16**, 2598–2608, <https://doi.org/10.1158/1535-7163.MCT-17-0386>
- 39 Lee, D.W. et al. (2019) Tumor mutation burden and prognosis in patients with colorectal cancer treated with adjuvant fluoropyrimidine and oxaliplatin. *Clin. Cancer Res.* **25**, 6141–6147, <https://doi.org/10.1158/1078-0432.CCR-19-1105>

- 40 Hou, T. et al. (2018) Leupaxin promotes bladder cancer proliferation, metastasis, and angiogenesis through the PI3K/AKT pathway. *Cell. Physiol. Biochem.* **47**, 2250–2260, <https://doi.org/10.1159/000491536>
- 41 Kachrilas, S. et al. (2019) PI3K/AKT pathway genetic alterations and dysregulation of expression in bladder cancer. *J. BUON* **24**, 329–337
- 42 Neoh, C.A. et al. (2017) Flaccidoxide-13-acetate extracted from the soft coral *Cladiella kashmani* reduces human bladder cancer cell migration and invasion through reducing activation of the FAK/PI3K/AKT/mTOR signaling pathway. *Molecules* **23**, <https://doi.org/10.3390/molecules23010058>
- 43 Zeng, Q. et al. (2020) Identification of therapeutic targets and prognostic biomarkers among CXCL chemokines in the renal cell carcinoma microenvironment. *Front. Oncol.* **9**, 1555, <https://doi.org/10.3389/fonc.2019.01555>
- 44 Shinohara, N. and Koyanagi, T. (2002) Ras signal transduction in carcinogenesis and progression of bladder cancer: molecular target for treatment? *Urol. Res.* **30**, 273–281, <https://doi.org/10.1007/s00240-002-0275-0>
- 45 Walker, C., Mojares, E. and Del Rio Hernandez, A. (2018) Role of extracellular matrix in development and cancer progression. *Int. J. Mol. Sci.* **19**, 3028, <https://doi.org/10.3390/ijms19103028>
- 46 Karkoulas, G. et al. (2006) Alpha 2-adrenergic receptors decrease DNA replication and cell proliferation and induce neurite outgrowth in transfected rat pheochromocytoma cells. *Ann. N.Y. Acad. Sci.* **1088**, 335–345, <https://doi.org/10.1196/annals.1366.017>
- 47 Wei, L. et al. (2015) Inhibition of CXCL12/CXCR4 suppresses pulmonary arterial smooth muscle cell proliferation and cell cycle progression via PI3K/Akt pathway under hypoxia. *J. Recept. Signal Transduct. Res.* **35**, 329–339, <https://doi.org/10.3109/10799893.2014.984308>
- 48 Swan, D.J., Kirby, J.A. and Ali, S. (2012) Post-transplant immunosuppression: regulation of the efflux of allospecific effector T cells from lymphoid tissues. *PLoS ONE* **7**, e45548, <https://doi.org/10.1371/journal.pone.0045548>
- 49 Zhao, X. et al. (2019) A natural “GA” insertion mutation in the sequence encoding the 3’UTR of CXCL12/SDF-1alpha: Identification, characterization, and functional impact on mRNA splicing. *Gene* **681**, 36–44, <https://doi.org/10.1016/j.gene.2018.09.045>
- 50 Chen, L. et al. (2017) ADAMTS9 is silenced by epigenetic disruption in colorectal cancer and inhibits cell growth and metastasis by regulating Akt/p53 signaling. *Cell. Physiol. Biochem.* **44**, 1370–1380, <https://doi.org/10.1159/000485534>
- 51 Dai, W. et al. (2015) microRNA-506 regulates proliferation, migration and invasion in hepatocellular carcinoma by targeting F-spondin 1 (SPON1). *Am. J. Cancer Res.* **5**, 2697–2707