Article

Methylation-based reclassification of bladder cancer based on immune cell genes

Qizhan Luo [1], and Thomas-Alexander Vögeli [1,*]

[1] Department of Urology, RWTH Aachen University, Pauwelsstrasse 30, 52072 Aachen, Germany; qizhan.luo@rwth-aachen.de(Q.L.); tvoegeli@ukaachen.de(T-A.V.)

* Correspondence: tvoegeli@ukaachen.de

Simple Summary: Bladder cancer (BC) development is highly related to immune cell infiltration and inflammation. This study aimed to construct a new classification of bladder cancer (BC) molecular subtypes based on immune cells-associated CpG sites. The classification was accurate and stable. BC patients could be divided into three subtypes based on the immune cells-associated CpG sites. The distribution of immune cells, level expression of checkpoints, stromal score, immune score, ESTIMATEScore, tumor purity, APC_co_inhibition, APC_co_stimulation, HLA, MHC_class_I, Type_I_IFN_Reponse, and Type_II_IFN_Reponse were significant difference among three subgroups. The distribution of genomic alterations was different among them. High level immune infiltration was a correlation with high level methylation. The lower RNAss score was associated with higher immune infiltration and higher level expression of CD274.

Abstract: Background: Bladder cancer (BC) development is highly related to immune cell infiltration and inflammation. This study aimed to construct a new classification of bladder cancer (BC) molecular subtypes based on immune cells-associated CpG sites. Methods: The genes of 28 types of immune cells were obtained from previous studies. Then methylation sites corresponding to immune cells-associated genes were acquired. Differentially methylation sites (DMSs) were identified between normal samples and bladder cancer samples. Unsupervised clustering analysis of differentially methylation sites was performed to divide into several subtypes. Then the potential mechanism of different subtypes was exploded. Result: Bladder cancer patients were divided into three groups. Cluster 3 (methylation-L) subtype had the best prognosis. Cluster 1 (methylation-M) had the worst prognosis. The distribution of immune cells, level expression of checkpoints, stromal score, immune score, ESTIMATEScore, tumor purity, APC_co_inhibition, APC_co_stimulation, HLA, MHC_class_I, Type_I_IFN_Reponse, and Type_II_IFN_Reponse were significant difference among three subgroups. The distribution of genomic alterations was different among them. Conclusion: The classification was accurate and stable. BC patients could be divided into three subtypes based on the immune cells-associated CpG sites. Specific biological signaling pathways, immune mechanisms, and genomic alterations were various among three subgroups. High level immune infiltration was a correlation with high level methylation. The lower RNAss score was associated with higher immune infiltration and higher level expression of CD274.

Keywords: Immune cell; DNA CpGs; Bladder cancer; Subtype; mutation; CNV; Immune score; Immune Checkpoints

1. Introduction

Recently, diverse immunotherapy has been proven to successfully treat numerous lethal cancers[1]. These included cytokine treatment, cellular therapy, immune checkpoint blockade, and

therapeutic vaccines[2]. Immune checkpoint inhibitors have shown remarkable anti-tumor function in several human cancers, including programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), and PD-1 ligand (PD-L1) antibodies[3-5]. FDA approved two cytokines as anti-tumor agents against kidney malignancy and metastatic melanoma[6]. Preventive and therapeutic vaccines have a significant anti-tumor function in several cancers, such as hepatitis B virus vaccines[7], Sipuleucel-T, human papillomavirus[8], and GVAX vaccine against prostate cancer[9, 10]. However, there is heterogeneity in response rates, and not all immunotherapy is successful in treating patients[11].

BC development is highly related to immune cell infiltration and inflammation. A previous study also revealed the interaction of various types of immune cells and signaling pathways between the tumor and immune cells[12]. There are several kinds of immunotherapy to treat BC, such as intravesical administration of the Bacillus Calmette-Guerin vaccine for treating high-risk no-muscle invasive bladder cancer (NMIBC) [13]. Patient prognosis and treatment response were predicted by immune cells with current molecular stratification in patients with BC[14].

In this study, we divided BC into three distinct subtypes based on immune cell-related methylation sites profiles. The three methylation sites subtypes are related to different molecular features, cellular characteristics, and clinical results. The classification of immune-related methylation sites subtypes may promote the optimal scheme of BC patients responsive to immunotherapy.

2. Results

2.1 Three subgroups based on Differentially methylation sites (DMSs)

Seven hundred and eighty-two immune cell biomarker-associated genes were selected from previous studies, and corresponding 8703 immune cell biomarker-associated methylation sites were acquired. By Parameter of infiltration was adjusted P-value < 0.05 and |deltabeta| > 0.2, 715 Differentially methylation sites (DMSs) between normal samples and tumor samples were identified (Fig. 1A).

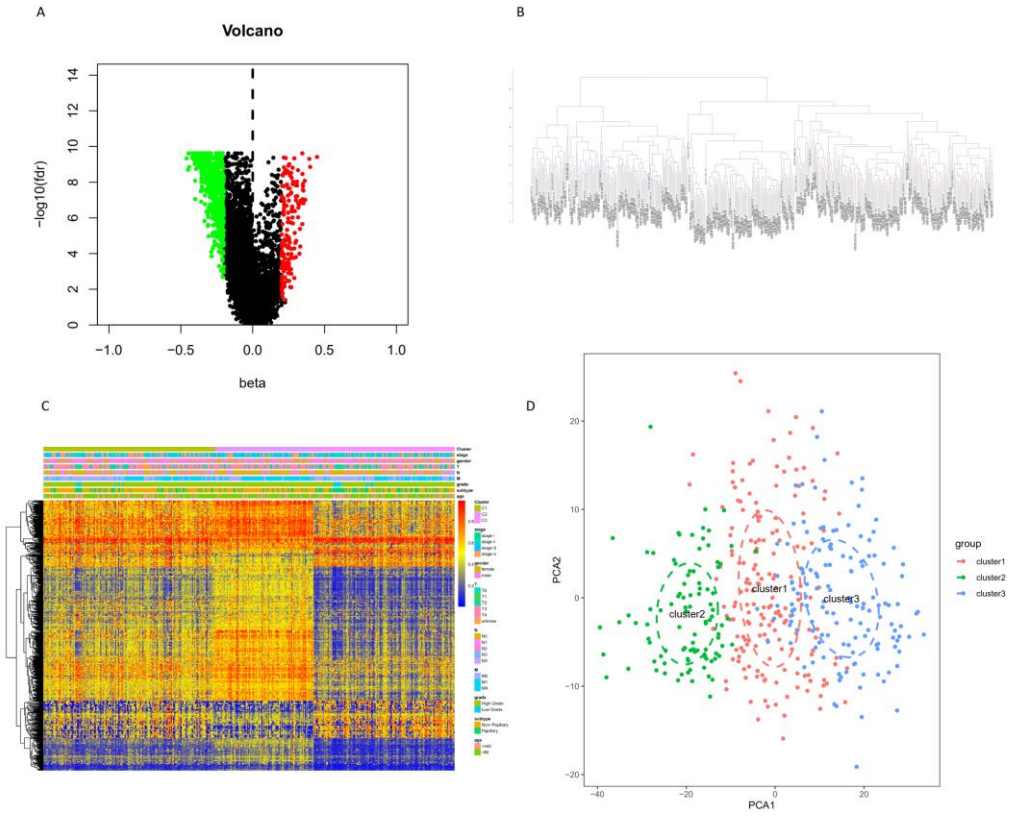

Figure 1. A. 715 Differentially methylation sites (DMSs) between normal samples and bladder cancer samples. 1B.The consensus clustering of 715 Differentially methylation sites (DMSs) was divided into three CpGs subgroups. B.The heatmap of methylation based on 715 Differentially methylation sites (DMSs). C.Principal component analysis (PCA) validated the stability of classification.

2.2. Classification of methylation subtypes of BC

The consensus clustering of 715 DMSs was classified into three subtypes(Fig. 1B). Cluster 1 showed middle-methylation. Cluster 2 showed hyper-methylation. And cluster 3 showed hypo-methylation (Fig. 1C). Principal component analysis (PCA) was utilized to check the stability of the consensus classification(Fig. 1D) .

The overall survival (OS) curve of BC subsets was gotten with Kaplan–Meier method (Fig. 2A). Cluster 1 had the worst prognosis. Cluster 3 had the best prognosis. A barplot showed the relationship between clinical traits and the biological characteristics of subtypes.(Fig. 2.B-H). Based on our results, Cluster 3 had more stage I, more low grade, and less T3.

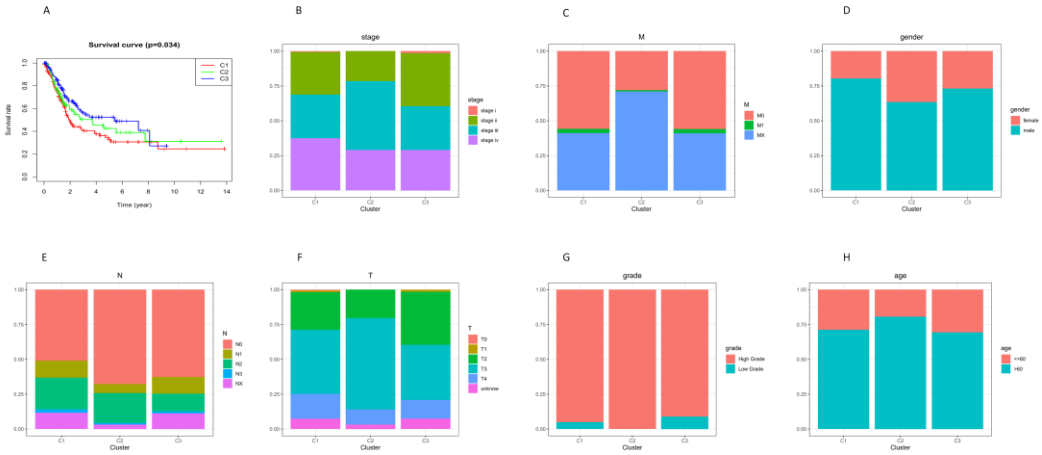

Figure 2. A.Overall survival (OS) curve. B-H. Clinicopathologic features among the three subgroups.

2.3. Identifying different methylation level and distinct gene expression level of different subgroups

Differentially immune cell biomarker-associated methylation levels was shown in Fig. 3B. Cluster 1 revealed middle-methylation. Cluster 2 revealed hyper-methylation. And cluster 3 revealed hypo-methylation. It was consistent with Fig 1C.

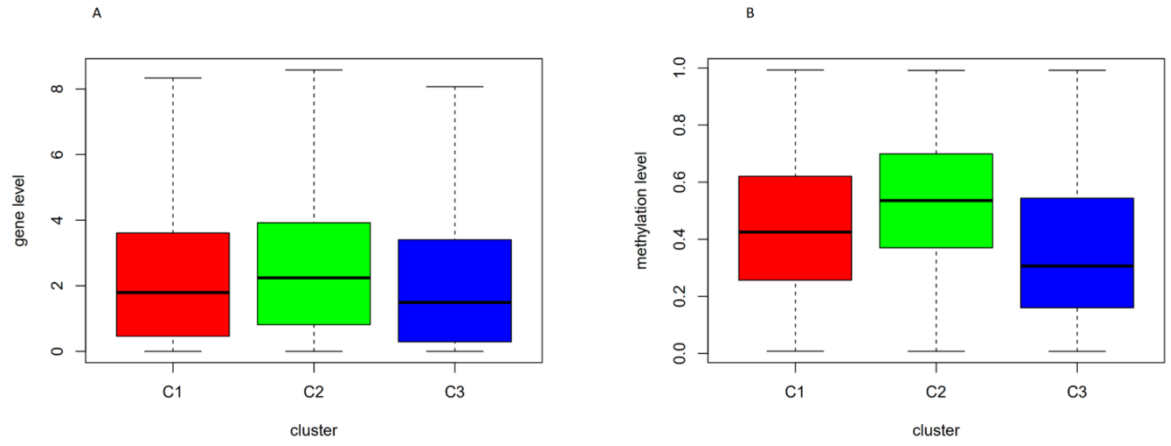

Figure 3. A. Gene level among the three subgroups. 3B. Methylation level among the three subgroups.

A previous study reported that the correlation between DNA methylation and gene expression in lung cancer was identified for about 750 genes. They found one-third of these correlations were positive, indicating the challenges in finding widespread and strong negative correlations between gene expression and genome-wide CpG methylation[30]. In fig 3A, immune cell genes expression is a high expression in cluster 2, low expression in cluster 3, and middle expression in cluster1. In the present study, high methylation level had high gene expression level.

2.4. Immune in different subgroups

In Fig. 4A, cluster 1 had middle immune infiltration. Cluster 2 was a correlation with high immune infiltration, and cluster 3 had low immune infiltration. Immune infiltration was compared among the three subtypes, and there were remarkable differences among these subtypes (Fig. 4B). Immune checkpoints were also significant differences among these subtypes (Figure 4E). Three

asterisks are P-value < 0.001. Two asterisks are P-value < 0.01. One asterisk is P-value < 0.05. Ns means that there is no significance.

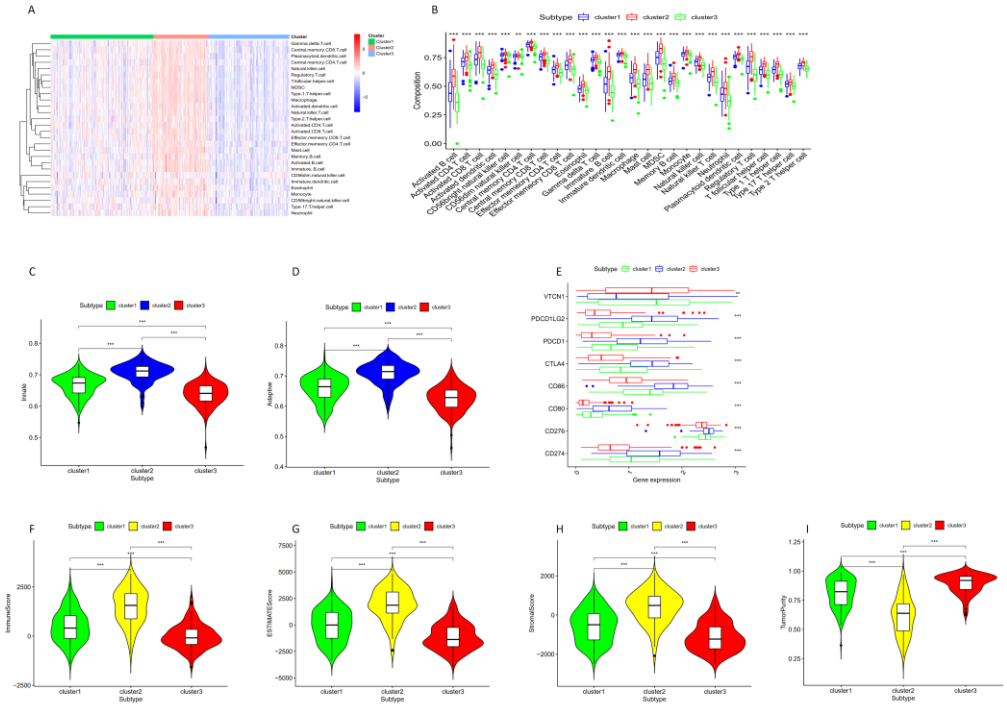

Figure 4. A. Immune cells infiltration among the three subtype. B.Immune cells the among three subgroups. C-D. Innate immune cells and adaptive immune cells among the three subgroups. E.Immune checkpoints among the three subsets. F-I.Immune microenvironment among the three groups.

Three asterisks are P-value less than 0.001. Two asterisks are P-value less than 0.01. One asterisk is P-value less than 0.05. Ns means that there is no significance

2.5. Tumor microenvironment (TME)

The tumor microenvironment contains stromal cells, tumor cells, and immune cells. The higher stromal score and immune score, the lower purity of tumor. In Fig4.F-I, cluster 2 had the highest stromal score, immune score, ESTIMATEScore, and the lowest purity of tumor. Cluster 3 had the lowest stromal score, immune score, ESTIMATEScore, and the highest tumor purity.

2.6. Single sample gene set enrichment analysis (ssGSEA)

The bio-marker of APC_co_inhibition, APC_co_stimulation, Endothelial cells, Fibroblasts, HLA, Inflammation-promoting, MHC_class_I, Type_I_IFN_Reponse, and Type_II_IFN_Reponse were significantly different among these subtypes (Figure 5).

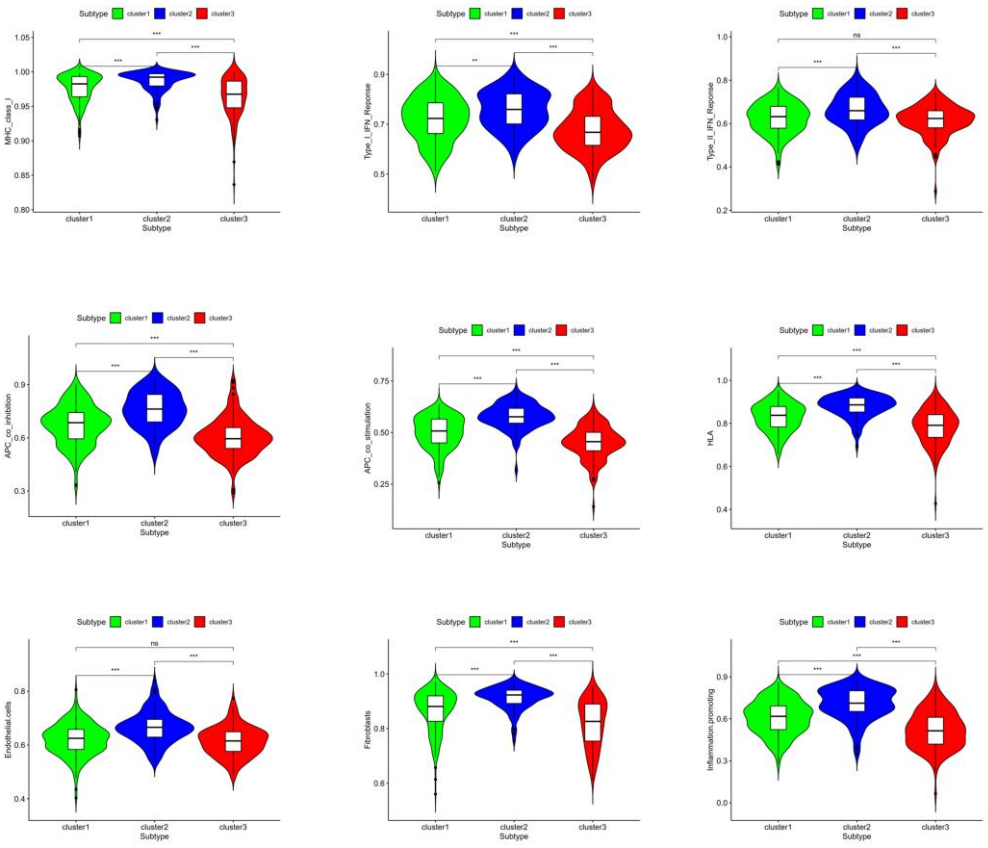

Figure 5. APC_co_inhibition, APC_co_stimulation, Endothelial cells, Fibroblasts, HLA, Inflammation-promoting, MHC_class_I, Type_I_IFN_Reponse and Type_II_IFN_Reponse among the three subgroups.

2.7. DNA-methylation (DNAss) and mRNA (RNAss) among subgroups

The DNA hypermethylation of those promoter genes suppressed gene expression, which in turn benefited cancer cells. Therefore, down-regulation of those genes may lead to cancer stem and progenitor cells' occurrence by DNA hypermethylation[24, 25]. RNA stemness score and DNA stemness score were the lowest in cluster 2 in Fig.6.

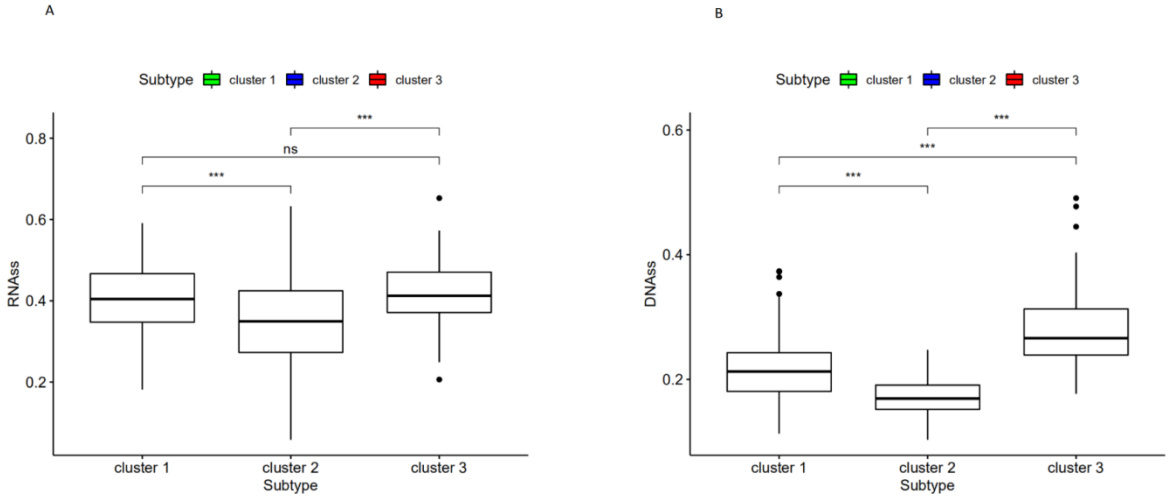

Figure 6. RNAss and DNAss among the three subgroups.

2.8. Analysis of mutations and CNVs among three subgroups

Thirty immune-cell-associated- genes with the highest mutation proportion in each subtype were shown in Fig. 7.A-C. And 58 immune-cell-associated- genes were identified from the above 30 genes in each subgroup. It meant that there was less overlap among the three subtypes (Fig. 7 A-C). The mutations of ITGA9, ENG, EVI5，ATIC，and FZD2 in cluster 1 were significantly higher than those in other subtypes. The mutations of CTSZ, HOXA1, and KLRF1 in cluster 2 were significantly higher than those in other subtypes. The mutations of DLC1, OSBPL1A, RRP12, C3AR1, MPZL1, and ITK in cluster 3 were significantly higher than those in other subtypes. TMB was a significant difference only between cluster 1 and cluster 2. TMB was a remarkable difference only between cluster 1 and cluster 2. (Fig. 7 D).

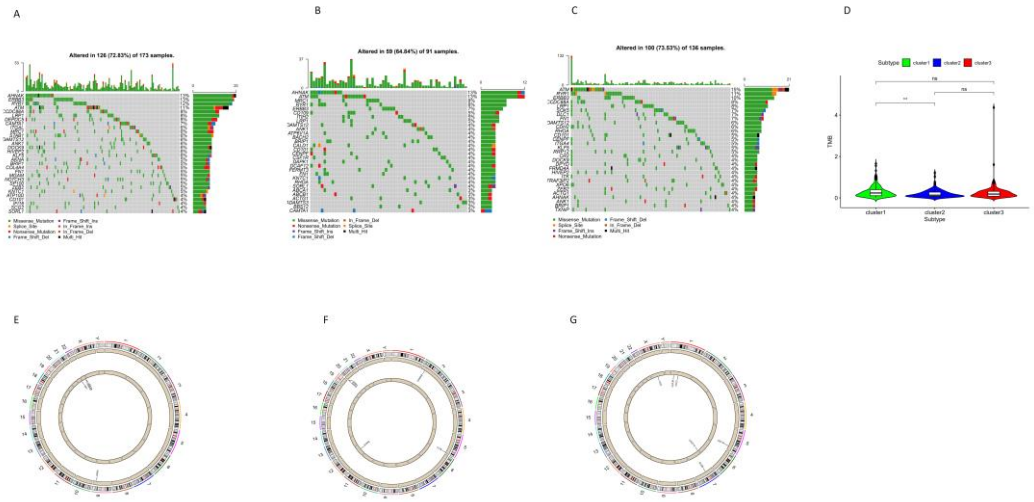

Figure 7. A. Immune cell genes-associated mutation in cluster1. B. Immune cell genes-associated mutation in cluster2. C.Immune cell genes-associated mutation mutation in cluster3. D. TMB among the three subtypes. E-G. Immune cell genes-associated CNVs in three subgroups, respectively.

Next, the CNV data was analyzed, 391 normal tissue and 410 tumor tissue were extracted. In figure 7E, CNV data in one subgroup was compared with the rest two subgroups. One gene with significant copy number gains was in cluster 1, and three genes with significant copy number losses were in cluster 1. Figure 7F showed four genes with significant copy number gains and one gene with significant copy number losses in cluster 2. In figure 7G, there were two genes with significant copy number gains and four genes with significant copy number losses in cluster 3.

3. Discussion

Altered DNA methylation patterns are hallmarks of tumors. Usually unmethylated promoters may alter into densely methylated, which will lead to the silencing of critical genes such as tumor suppressor genes[31]. Other sequences may alter into hypomethylated in tumors, which result in the abnormal activation of genes that are usually suppressed by DNA methylation[32]. Hypermethylation events have also been reported to be biomarkers of human tumors, for an early examination of blood, urine and other body fluids, for prognosis or prediction of response to treatment, and for monitoring cancer recurrence[33].

BC development is highly related to immune cell infiltration and inflammation. A previous study also revealed the interaction of various types of immune cells and signaling pathways between

the tumor and immune cells[12]. There are several kinds of immunotherapy to treat BC, such as intravesical administration of the Bacillus Calmette-Guerin vaccine for treating high-risk NMIBC [13]. Patient prognosis and treatment response were predicted by immune cells with current molecular stratification in patients with BC[14]. To understand the mechanism of cancer, guide therapy, and improve prognosis, it is vital for us to identify accurate subtypes. Several studies were reported to identify subtype based on DNA methylation, including colon adenocarcinoma[34], cervical cancer[35], glioblastoma[36], and bladder cancer[37]. This study divided BC into three distinct subtypes based on immune cells - related methylation profiles (Fig. 1B-C). To check for stability of the classification, PCA was utilized to validate the stability of the classification(Fig. 1D), and PCA proved the classification was stable and accurate. The three immune subtypes are related to significantly different clinical results(Fig. 2). The methylation levels among the three subtypes were different. Differentially immune cell biomarker-associated methylation level was shown in Fig. 1C. Cluster 1 revealed middle-methylation. Cluster 2 revealed hyper-methylation. And cluster 3 revealed hypo-methylation. This was consistent with Fig3B. The distribution of immune cells, level expression of checkpoints, stromal score, immune score, ESTIMATEScore, APC_co_inhibition, APC_co_stimulation, HLA, MHC_class_I, Type_I_IFN_Reponse, and Type_II_IFN_Reponse were significant difference among the three subgroups. All of those verified the stability and accuracy of the classification(Fig 4 and Fig 5).

In the present study, different subtypes had different survival. This may be caused by several reasons as followings. 1. Abnormal DNA methylation may lead to poor prognosis in cancer patients[38]. The progression and prognosis of cancer may be affected by Hyper-methylation of DNA[39]. 2. Tumor cells in the microenvironment can express high levels of immunosuppressive cytokines to forbid T cell proliferation and activity while facilitating tumor development and progression[40, 41]. Tumor-expressing specific molecular can be enough to induce immunosuppressive and facilitate immune evasion[42]. Subtle changes in the compositions of immune cells can have different influences on tumor progression[43]. Previous studies reported that a high density of macrophage in the microenvironment was correlated with poor prognosis of bladder cancer patients[44]. In our study, cluster 3 had good survival. In Fig 1C and Fig 4A, cluster 3 showed the hypo-methylation and low immune cell infiltration. Hypo-methylation and low immune cell infiltration might be the reason that cluster 3 had good survival.

However, middle-methylation and middle immune infiltration were in cluster 1 that had the worst survival. Hyper-methylation and high immune infiltration were in cluster 2 that had intermediate survival. We may find the reason from checkpoints. VTCN1 expression up-regulation in bladder cancer led to worse survival[45, 46]. B7x (VTCN1) was remarkably overexpressed in many human cancers, and it repressed the antitumor immune effect and regulated to escape immunosurveillance[47] High-level expression of CD80 and CD86 may result in a good survival in patients with nasopharyngeal carcinoma[48]. The absence or low-level expression of CD80 and CD86 in cancers may be one mechanism by which cancers escape immunosurveillance[48]. In fig.4E, these checkpoints were significant differences among the three subgroups. Among them, VTCN1 (B7-H4) was the higher expression in cluster 1. CD80 and CD86 were the lower expressions in cluster 1. So these may cause cluster 1 with worse survival than cluster 2.

The tumor microenvironment contains stromal cells, tumor cells, and immune cells. The higher stromal score and immune score, the lower purity of tumor. In Fig, cluster 2 had the highest stromal score, immune score, and the lowest tumor purity. Cluster 3 had the lowest stromal score, immune score, and the highest purity of the tumor. The distribution of immune score among three subgroups was consistent with the distribution of immune cells(Fig 4F-I).

Endothelial cells can remodel the local immune microenvironment and help tumor cells escape immunosurveillance in many ways[49]. Endothelial cells release chemokines to promote leukocyte migration into tumor tissues and express adhesion protein to facilitate peripheral leukocyte capture[50]. Endothelial cells can also forbid the activation and chemotaxis of immune cells and

mediate inhibitory molecules to facilitate immune tolerance[51, 52]. Endothelial cells also show increased expression of PD-L1 to repress T cell activation[53-55]. Besides, FasL expression in endothelial cells promotes their ability to suppress activation CD8+ T cells, causing endothelial cells-associated immune cell death and promote tumor escape[56, 57]. In the present study, the density of endothelial cells in cluster 2 was the highest, and immune infiltration was the highest in cluster2(Fig 5). It inferred that the endothelial cells might help tumor cells escape immunity.

Cancer cells had interaction with cancer-associated macrophages and tumor-associated fibroblasts, which promotes tumor progression in bladder cancer[58]. In the present study, the distribution of fibroblasts among three subgroups was consistent with macrophages' distribution (Fig. 5). It suggested there was a correlation between fibroblasts and macrophages.

The patients in the therapy of tumors can benefit from the many inflammatory molecules that also have an important role in cancer progression and development. The dual role of inflammatory molecular is far from being fully understood[59]. In the present study, the distribution of inflammation-promoting among three subgroups was consistent with the distribution of immune cells(Fig.5). It indicated the inflammation-promoting and immune cells might affect each other. But the detailed role of inflammation-promoting may need more studies to be explored.

A previous study reported that the correlation between DNA methylation and gene expression in lung cancer was identified for about 750 genes. They found one-third of these the correlation was positive, indicating the challenges in finding widespread and strong negative correlations between genes expression and genome-wide CpG methylation[30]. In fig 3A, immune cell genes expression is a high expression in cluster 2, low expression in cluster 3, and middle expression in cluster1. It also challenged the finding widespread and strong negative correlations between genes expression and genome-wide CpG methylation.

The DNA hypermethylation of those promoter genes suppressed gene expression, which in turn benefited cancer cells. Therefore, down-regulation of those genes may lead to the occurrence of cancer stem and progenitor cells by DNA hypermethylation[24, 25]. The range of scores was from 0 to 1. Zero means high differentiation, and one means undifferentiation[26]. However, in the present study, RNA stemness score and DNA stemness score were the lowest in cluster 2 in Fig 6. It challenged the above findings which down-regulation of those genes may lead to the occurrence of cancer stem and progenitor cells by DNA hypermethylation. A previous study found that for several tumor types, such as BLCA, LUSC, HNSC, and GBM, there was a negative correlation between DNAss score with leukocyte fraction and/or lower PD-L1 expression[26]. In the present study, cluster 2 had the highest immune infiltration and high-level expression of CD274 (Fig.4E), but cluster 3 had the lowest DNAss score. This result was the same as the previous work. In present work, we also found the lower RNAss score was associated with higher immune infiltration and higher-level expression of CD274(Fig 6 and Fig. 4E).

TMB was a remarkable difference only between cluster 1 and cluster 2. (Fig. 7 D). It suggested the TMB might be not correlated with methylation level. But the composition of genes of mutations was different among the three subtypes. In Fig. 7. and 58 immune-cell-associated-genes were identified from the highest mutant 30 genes in each subgroup. It meant that there was less overlap among the three subtypes (Fig. 7 A-C). The mutations of ITGA9, ENG, EVI5, ATIC，and FZD2 in cluster 1 were significantly higher than those in other subtypes. These genes are the bio-marker of Mast cell, Plasmacytoid dendritic cell, Type 2 T helper cell, Immature dendritic cell, and Macrophage[15, 16]. The mutations of CTSZ, HOXA1, and KLRF1 in cluster 2 were significantly higher than those in rest subtypes. These genes are the bio-marker of Natural killer cell, CD56 bright natural killer cell, and Gamma delta T cell[15, 16]. The mutations of DLC1, OSBPL1A, RRP12, C3AR1, MPZL1, and ITK in cluster 3 were significantly higher than those in rest subtypes. These genes are the bio-marker of Type 2 T helper cell, Eosinophil, Effector memeory CD8 T cell, Activated CD8 T cell, and Activated CD4 T cell[15, 16]. These immune cells with mutant genes among the three

subgroups were different. It is possible to become promising drug targets based on these mutant genes.

In figure 7E, CNV data in one subgroup was compared with the rest two subgroups. AKNA with significant copy number gains was in cluster 1 and the gene is the biomarker of Activated B cell[15, 16]. PARVG, SIK1 and UPK3A with significant copy number losses were in cluster 1 and these genes are the biomarker of MDSC, Effector memeory CD8 T cell, and Monocyte[15, 16]. Figure 7F showed CLTB, GEMIN6, SIRPA and SIRPG with significant copy number gains. These genes are the biomarker of Immature dendritic cell, Activated CD8 T cell, Plasmacytoid dendritic cell, and Central memory CD4 T cell[15, 16]. DYRK2 with significant copy number losses was in cluster 2 and the gene is the biomarker of CD56 dim natural killer cell[15, 16]. In figure 7G, CSF1R and GUSB with significant copy number gains were in cluster 3 and these genes are the biomarker of T follicular helper cell and Central memory CD8 T cell[15, 16]. CDC7, CHST12, CSF3R and OGT with significant copy number losses were in cluster 3. These genes are the biomarker of Type 2 T helper cell, T follicular helper cell, Immature dendritic cell and Plasmacytoid dendritic cell[15, 16]. These immune cells with mutant genes among the three subgroups were totally different. It also is possible to become promising drug targets based on these CNV genes.

In conclusion: The classification was accurate and stable. BC patients could be divided into three subtypes based on the immune cells-associated CpG sites. Specific biological signaling pathways, immune mechanisms, and genomic alterations were various among three subgroups. High-level immune infiltration was a correlation with high-level methylation. The lower RNAss score was associated with higher immune infiltration and higher-level expression of CD274.

4. Materials and Methods

4.1. Data pre-processing

Methylation data from Illumina Human Methylation 450 arrays was obtained from UCSC Xena and had 437 samples (https://xenabrowser.net/datapages/, 2020-07-15). DNA-methylation (DNAss), mRNA stemness (RNAss), RNA-sequencing data from 430 BC samples and clinical data also were downloaded from UCSC Xena website. The Masked Somatic Mutation data (MuTect2. Variant0. Maf) and the CNV data set (Masked Copy Number Segment, affymetrix snp 6.0) were obtained from TCGA website (https://portal.gdc.cancer.gov/repository). The CNV data was comprised of 814 samples. Because the databases were the public databases, and our data was obtained directly from these databases. There was no requirement for ethical approval.

4.2. Immune cells-associated genes selection

Immune cells-associated bio-markers were obtained from previous studies[15, 16]. And their corresponding methylation sites were obtained. The criteria for exclusion probes from the analysis was as followings: 1. If the CpG site data missed more than 70% in the samples, the CpG sites were excluded from the analysis[17]. 2. Cross-reactive genome CpG sites were deleted. 3. Probes on the X and Y chromosomes were moved excluded from the analysis. The remaining sites were imputed with the k-nearest neighbors (KNN) imputation procedure[18].

4.3. Unsupervised hierarchical cluster analysis

The methylation sites corresponding to immune cells-associated genes were acquired. Differentially methylation sites (DMSs) were identified between normal samples and bladder cancer samples with adjusted P-value < 0.05 and |deltabeta| > 0.2. Unsupervised hierarchical clustering was performed based on immune cell-associated methylation data to identify subtypes of BC with "sparcl" R software package. The overall survival (OS) curve of BC subsets was gotten with Kaplan–Meier method and with "survival" package in R software. Principal component analysis (PCA) was

performed to validate the classification. A barplot showed the relationship between clinical traits and the biological characteristics of subtypes.

4.4. Single sample gene set enrichment analysis (ssGSEA) based on immune cells bio-marker

Single sample gene set enrichment analysis was performed to quantify the infiltration of immune cells. The ssGSEA ranked the genes based on their absolute expression in a sample with "GSEABase" and "GSVA" R package. The enrichment score is calculated by integrating the differences between the empirical cumulative distribution functions of the gene ranks[19, 20]. Activated B cell, activated CD8 T cell, effector memeory CD8 T cell, central memory CD8 T cell, activated CD4 T cell, effector memeory CD4 T cell, central memory CD4 T cell, regulatory T cell, gamma delta T cell, immature B cell, memory B cell, type 17 T helper cell, T follicular helper cell, type 1 T helper cell, and type 2 T helper cell are adaptive immune cells. CD56 dim natural killer cell, CD56 bright natural killer cell, Eosinophil, Activated dendritic cell, Immature dendritic cell, MDSC, Macrophage, Monocyte, Mast cell, Plasmacytoid dendritic cell, Natural killer cell, Natural killer T cell, and Neutrophil are innate immune cells.

Immune checkpoints were a selected from previous studies[21, 22] to be compared among the subtypes. Kruskal-Wallis. Test was performed.

4.5. Tumor microenvironment (TME)

ESTIMATE algorithm was obtained from the public source website (https://sourceforge.net/projects/estimateproject/) to estimate the scores of stromal and immune cells based on gene expression signature in tumor samples. Then, we calculated stromal scores, immune scores, tumor purity, and ESTIMATE scores for each sample. Stromal scores, immune scores, tumor purity, and ESTIMATE scores were compared among subtypes.

4.6. Single sample gene set enrichment analysis (ssGSEA)

The bio-marker of APC_co_inhibition, APC_co_stimulation, Endothelial cells, Fibroblasts, HLA, Inflammation-promoting, MHC_class_I, Type_I_IFN_Reponse and Type_II_IFN_Reponse were selected from studies[19, 20]. Single sample gene set enrichment analysis was performed to rank the genes based on their absolute expression in a sample.

4.7. DNA-methylation (DNAss) and mRNA stemness (RNAss) among subgroups

During cancer progression, a differentiated phenotype was lost, and progenitor and stem-cell-like characteristics were acquired[23]. The DNA hypermethylation of those genes suppressed gene expression, which in turn benefited cancer cells. Therefore, down-regulation of those genes may lead to the occurrence of cancer stem and progenitor cells by DNA hypermethylation[24, 25]. RNA stemness score based on mRNA expression (RNAss) and DNA stemness score based on DNA methylation pattern (DNAss) were utilized to measure tumor stemness[26]. The rang of scores was from 0 to 1. Zero means high differentiation, and 1 means undifferentiation[26]. DNA-methylation (DNAss) and mRNA (RNAss) among three subgroups were analyzed.

4.8. Analysis of mutations and CNVs among subgroups.

The 'maftools' software package was utilized to analyze and visualize immune cell biomarker-associated mutation data[27]. Immune cell biomarker-associated mutation data was compared between one group with the rest groups with Chi-square Test. The P-value is less than 0.05. TMB is called the density of tumor genes mutation[27]. TMB (tumor mutation burden) was compared among subtypes based on immune cell biomarker-associated mutation data.

Then, immune cell biomarker-associated CNV data was analyzed. Genomic identification of significant targets in cancer (GISTIC) algorithm was utilized to classify the copy number variant

genes with remarkable gains and losses[28, 29]. Parameter thresholds were set to 0.2 and -0.2 for genomic gains and losses, respectively[28, 29]. Immune cell biomarker-associated copy number variant data was compared between one group with the rest groups with a Chi-square Test and P-value < 0.01.

5. Conclusions

The classification was accurate and stable. BC patients could be divided into three subtypes based on the immune cells-associated CpG sites. Specific biological signaling pathways, immune mechanisms, and genomic alterations were various among three subgroups. High-level immune infiltration was a correlation with high-level methylation. The lower RNAss score was associated with higher immune infiltration and higher-level expression of CD274.

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Conflicts of Interest: The authors declare that they have no competing interests.

Data available: Our data was gotten directly from public databases and the publishing policies of these databases were strictly obeyed by us, and there was no requirement for ethical approvals.

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