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Research article

Immune-related prognostic genes signatures in the tumor microenvironment of sarcoma

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Abstract: Sarcomas are a heterogeneous group of malignant mesenchymal neoplasms. This study aimed to investigate the immune-related prognostic gene signatures in the tumor microenvironment of sarcoma. The RNA-sequencing data and clinical phenotype data of 260 sarcoma samples and two normal samples were downloaded from The Cancer Genome Atla (TCGA) database. Tumor purity and immune cells infiltration were evaluated by Estimation of Stromal and Immune cells in Malignant Tumors using Expression data (ESTIMATE) deconvolution algorithm. Differentially expressed genes (DEGs) were screened in high vs. low immune score groups. Survival analysis was performed using Kaplan-Meier curve with log-rank test. Tumor infiltrating of immune cells was analyzed by Tumor Immune Estimation Resource (TIMER). High immune score and ESTIMATE score were associated with favorable prognosis. A total of 623 immune DEGs were screened. The majority of these genes (532 genes accounting for 85% of the DEGs) were up-regulated, and these genes were significantly enriched in various immune related biological processed and pathways, such as neutrophil activation, T cell activation, antigen processing and presentation. A total of 146 prognosis-related immune DEGs, and seven hub genes were identified, including B2M, HLA-DRB1, HLA-DRA, HLA-E, LCK, HLA-DPA1, and VAV1. Survival analysis showed that high expression of these genes was associated with a favorable prognosis. There were negative correlations between the expression of these hub genes and tumor purity, while positive correlations between expression of these hub genes and f infiltration levels of B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages and dendritic cells. These results help to stratify patients with different immune subtypes and help to design immunotherapy strategies for these patients in sarcoma.

Keywords: sarcoma; immune score; prognosis; immunotherapy; antigen presentation

1. Introduction

Sarcomas are a heterogeneous group of malignant neoplasms arising from mesenchymal mesenchymal stem cells [1]. There are over 70 histological sarcoma subtypes, and the occurrence of most subtypes are not confined to age and a particular part of the body. Sarcomas can be divided into soft tissue and bone sarcomas these two main groups [2]. Standard treatments for sarcoma contain surgery and chemoradiotherapy. Nevertheless, there are relatively low response rates to chemotherapy, and also current chemotherapy drugs usually have no effect on chondrosarcoma, chordoma, and other several sarcomas [3]. Besides, metastatic or relapsed sarcoma is arrantly difficult to treat, with a 5-year survival rate of less than 20% [4,5]. Hence, it is necessary to develop new efficacious therapeutic strategies for sarcoma.

Immune checkpoint-based immunotherapy can destroy the immune tolerance to cancer cells by the blockage of suppressor receptors expressed by antitumor T cells, which has been demonstrated to show significant benefit in clinical outcomes in many solid tumors [6,7]. These clinical successes have stimulated the development of immunotherapy in sarcomas, and emphasize the importance of understanding the tumors microenvironment of sarcoma [8,9]. Studies have shown that clinical manifestations often differ across the different sarcoma subtypes, and responses to immune checkpoint blockade vary greatly [10]. Oike et al. firstly reported the prognostic value of tumor immune microenvironment in synovial sarcoma, and they showed that elevated CD163+ macrophages infiltrating was correlated with poor outcomes and tumor progression [11]. Toulmonde et al. suggested that the effect of programmed cell death protein 1 (PD-1) inhibition in advanced soft-tissue sarcoma and gastrointestinal stromal tumor were limited due to the immunosuppressive in tumors microenvironment caused by enhanced macrophage infiltrating [12]. Another study revealed that the PD-L1 expression showed correlations with the tumor infiltrating of dendritic cells, T cells and natural killer cells in osteosarcoma, and tumor infiltrating dendritic cells and macrophages showed correlations with poor survival [13]. Additionally, immune-related genes have been demonstrated to be implicated in the carcinogenesis and tumors microenvironment, and showed prognostic value in soft tissue sarcoma [14]. Based on multi-omic study, Gu et al. identified five immune-related prognostic genes, which was useful to stratify soft tissue sarcoma patients with different risk and was useful to predict response to immunotherapy [15]. Moreover, incorporating these biomarkers in clinical had been found to contribute to optimize the therapeutic decision [16].

In this study, based on the RNA sequencing (RNA-seq) data of sarcoma, the immune score of sarcoma tumor was evaluated utilizing a deconvolution algorithm. Then, screening of immune-associated genes in high vs. low immune score was completed. Then their functions, prognosis value and the correlations between their expression and immune infiltrating level were investigated.

2. Materials and methods

2.1. Data acquisition

The RNA-seq data as well as matched clinical data of The Cancer Genome Atlas-Sarcoma (TCGA-SARC) were acquired from the University of California Santa Cruz database (https://xenabrowser.net/). In all, 262 samples having both RNA-seq and clinical data were analyzed in the current study, including 260 sarcoma samples and two normal samples. The procedures of

current study were shown in Figure S1.

2.2. Calculation of immune score

The GENCODE v22 [17] (https://www.gencodegenes.org/) was utilized to annotate the ENSEMBL ID to obtain Gene symbol in line accordance with the gene transfer format (GTF) annotation files. The final expression value was determined by the mean value if there were numerous probes matching to one gene. The Estimation of Stromal and Immune cells in Malignant Tumors using Expression data (ESTIMATE) algorithm [18] was utilized to evaluate the tumor purity and stromal/immune cells infiltration, and these were presented by the Estimate score, stromal score and immune score for all tumor samples. In line accordance with the median score of each score, samples were categorized as high- and low- score groups, and correlations of these three scores with prognosis were calculated utilizing Kaplan-Meier curves with log-rank test, respectively.

2.3. Differential expression analysis

In line accordance with the median value of immune score, samples were categorized as high- and low- immune score groups. Then genes having differential expression in high vs. low immune score were selected utilizing the classical Bayesian approach in limma package [19] (Version 3.10.3) in R, and genes having P value < 0.05 and $|\log FC| > 1$ were considered as immune-related differentially expressed genes (immune DEGs).

2.4. Functional enrichment analysis for immune DEGs

The biological processes in Gene Ontology annotations and kyoto encyclopedia of genes and genomes (KEGG) pathways were enriched for immune DEGs utilizing the clusterProfiler package [20] (Version 3.2.11) in R. We selected the terms with count ≥ 2 and P value < 0.05 to present the enrichment results. Utilizing clusterProfiler package in R, Gene Set Enrichment Analysis (GSEA) was carried out to evaluate the differences of pathways in high vs. low- immune score with reference gene sets c2.cp.kegg.v7.2.symbols.gmt from MSigDB v7.2 database [21]. Benjamini-Hochberg adjusted P value < 0.05 was utilized to screen the statistically significant results.

2.5. Protein-protein interaction (PPI) analysis

The interactions among proteins encoded by immune DEGs were retrieved from the Search Tool for the Retrieval of Interacting Genes (STRING) database [22] (Version: 11.0). Based on the PPI score of 0.9, the PPI network was constructed utilizing Cytoscape [23] (version:3.2.0). The topology properties of nodes were investigated utilizing CytoNCA plug-in (Version 2.1.6) with parameter setting as without weight. The functional clustered modules in the network were retrieved utilizing Molecular Complex Detection (MCODE) plug-in [24] (Version1.4.2) with default parameters (Degree Cutoff: 2, Node Score Cutoff: 0.2, K-Core: 2, Max. Depth: 100). The significant modules were screened on basis of score > 5. *Survival analysis*

The clinical data related to prognosis, containing overall survival and overall survival status, were utilized in the survival analysis. The samples were categorized as high- and low-expression groups on basis of the median expression value of genes, the correlations of these genes with

prognosis were evaluated by Kaplan-Meier curves with log-rank test. Genes with $P < 0.05$ were considered as the prognosis-related genes.

2.6. Prognosis-related immune DEGs

The reduplicated genes in prognosis-related genes and immune DEGs were selected utilizing Venn analysis, and these overlapped genes were regarded as the prognosis-related immune DEGs. Then, functional enrichment analysis, PPI network and significant modules analyses were performed for these prognosis-related immune DEGs using the methods described above.

2.7. Tumor Immune Estimation Resource (TIMER) analysis

The infiltrating abundance of prognosis-related immune DEGs in tumor tissues and immune cells were analyzed utilizing the TIMER algorithm [25] to further investigate the abundance of 6 infiltrating immune cells in tumor tissues, including B cells, CD4+ T cells, CD8+ T cells, neutrphils, macrophages and dendritic cells.

3. Results

3.1. Screening of immune DEGs

The Estimate score, stromal score and immune score for all tumor samples were evaluated to explore the tumor purity and stromal/immune cells infiltration. The correlations of these three scores with prognosis were assessed by Kaplan-Meier curves with log-rank test. We found that immune score ($P = 0.015$) and Estimate score ($P = 0.047$) were significant associated with prognosis, while stromal score ($P = 0.15$) showed no significant associations with prognosis (Figure 1A-C). It could be seen that patients with high immune scores and Estimate scores were related to a favorable prognosis. In line accordance with the immune score median value, samples were categorized as high- and low- immune score, and differential expression analyseswere performed to screen immune DEGs. A total of 623 immune DEGs were screened, of which 532 genes were up-regulated while 91 genes were down-regulated (Figure 1D–E).

3.2. The involved functions of the immune DEGs

Enrichment analysis was carried out for up-regulated and down-regulated genes to investigate the involved functions of these immune DEGs, respectively. The results showed that up-regulated immune DEGs were significantly enriched in 1070 biological processes and 66 KEGG pathways. Of which, most of the top 10 significant biological processes were immune-related terms, including neutrophil activation/degranulation, neutrophil mediated immunity, neutrophil activation involved in immune response, and T cell activation (Figure 2A). The significant KEGG pathways included cell adhesion molecules, viral protein interaction with cytokine and cytokine receptor, antigen processing and presentation and so on (Figure 2B). While the down-regulated immune DEGs were significantly enriched 14 biological processes and 8 KEGG pathways, such as muscle system process, muscle contraction, muscle tissue development, vascular smooth muscle contraction and extracellular matrix (ECM)−receptor interaction (Figure 2C–D).

3.3. GSEA for immune DEGs

GSEA analysis was performed for the immune DEGs ranked by logFC to evaluate the differences of KEGG pathways between high- and low- immune score groups. A total of 60 KEGG pathways were obtained. Of which 50 pathways were enriched for the high immune score group, while 10 pathways were enriched for the low immune score group (Table S1). The top 5 enriched pathways for the high immune score group contained cytokine-cytokine receptor interaction, chemokine signaling pathway, Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway, natural killer cell-mediated cytotoxicity, cell adhesion molecules cams (Figure 3A). While cell cycle, dilated cardiomyopathy, lysine degradation and other pathways were enriched for the low immune score group (Figure 3B).

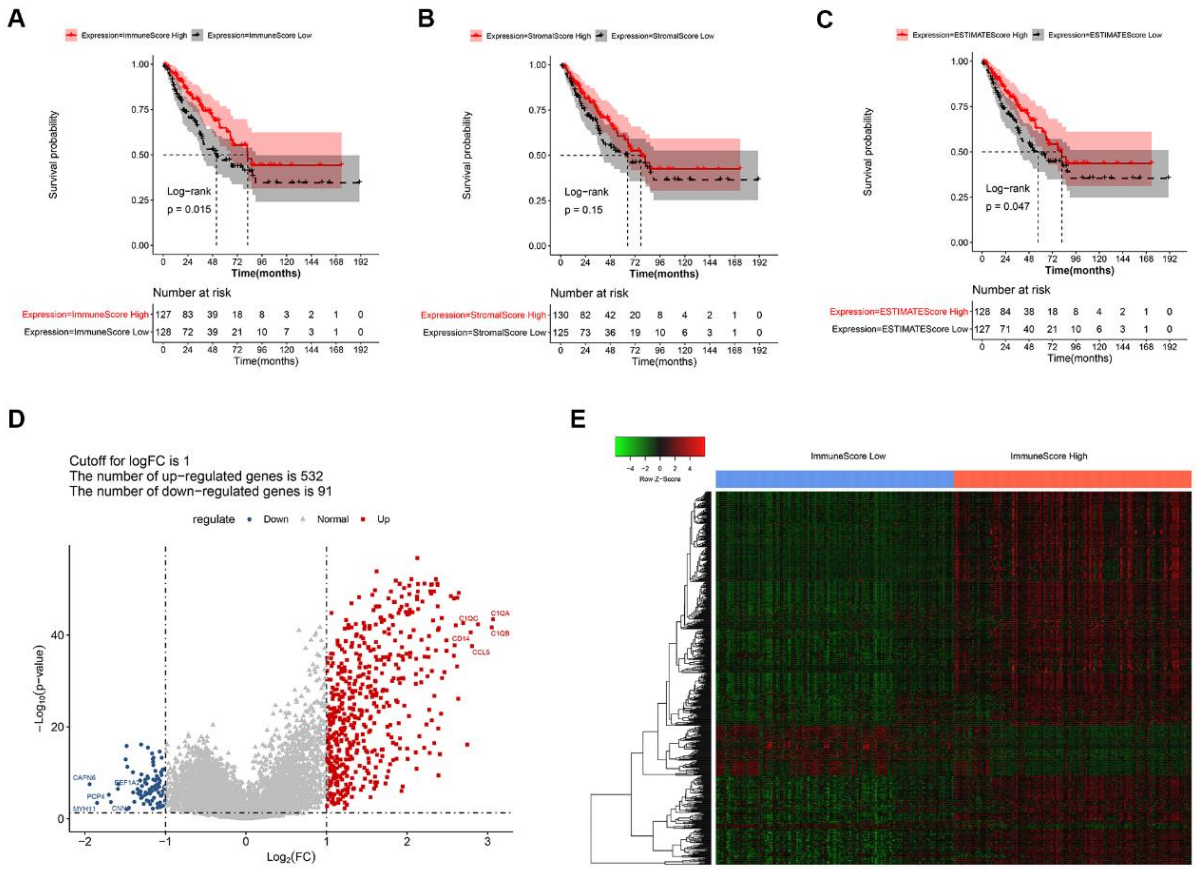

Figure 1. Results of differential expression analysis between high and low immune score. Kaplan-Meier curves of the immune score (A), stromal score (B) and ESTIMATE score (C) calculated by ESTIMATE algorithm; the volcano plot (C) and heatmap (D) of DEGs screened in high vs. low immune score.

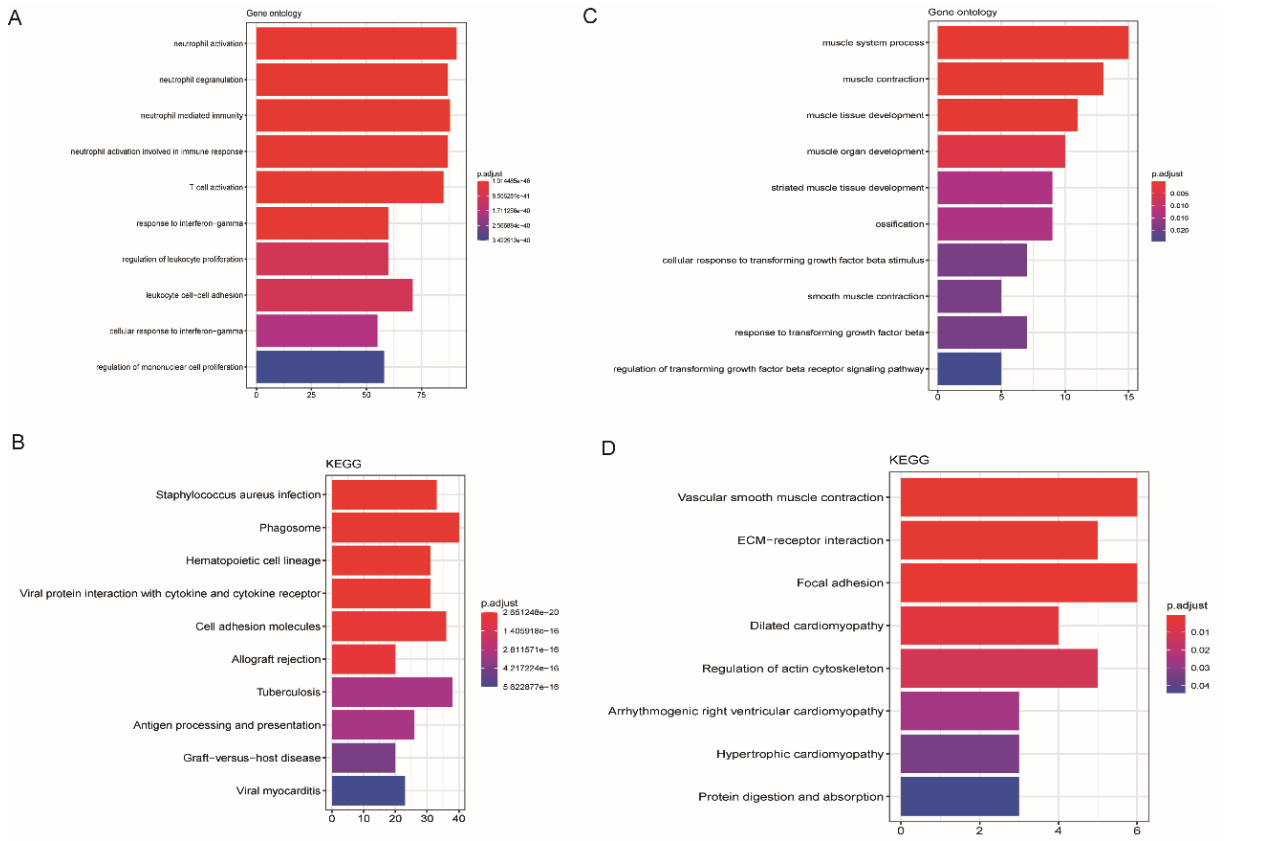

Figure 2. Functional enrichment analysis for immune DEGs. The histogram shows the top 10 enriched Gene ontology biological processes (A) and top 10 KEGG pathways (B) for up-regulated immune DEGs; the top 10 enriched Gene ontology biological processes (C) and top 10 KEGG pathways (D) for down-regulated immune DEGs.

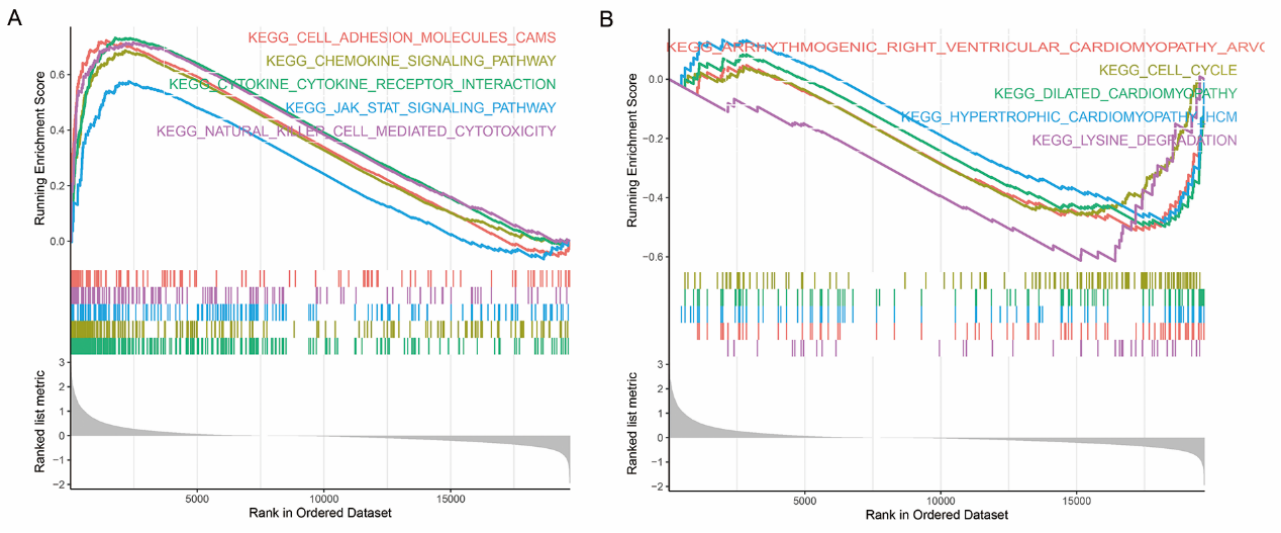

Figure 3. Gene set enrichment analysis. The top 5 enriched pathways for high immune score (A, positive correlation with NES > 0) and low score (B, negative correlation with NES > 0) tumors.

3.4. PPI network and modules for immune DEGs

PPI network was constructed to explore the interactions among proteins encoded by the immune DEGs (Figure 4). The PPI network contained 371 nodes and 2513 interactions. The topology properties of nodes were listed in Table S2. Among the top 20 hub nodes (ranked by degree), there were 14 human leukocyte antigens (HLA), such as HLA-A, HLA-E, HLA-DRA, HLA-B and so on. Besides, beta-2 microglobulin (B2M), complement C3 gene (C3), platelet-activating factor cell-surface receptor (PTAFR), Protein Tyrosine Phosphatase, Nonreceptor Type 6 (PTPN6) were also hub nodes. A total of 7 modules with score > 5 were selected. It could be seen that all the HLAs, B2M, C3 and PTAFR were clustered in one module (Figure S2).

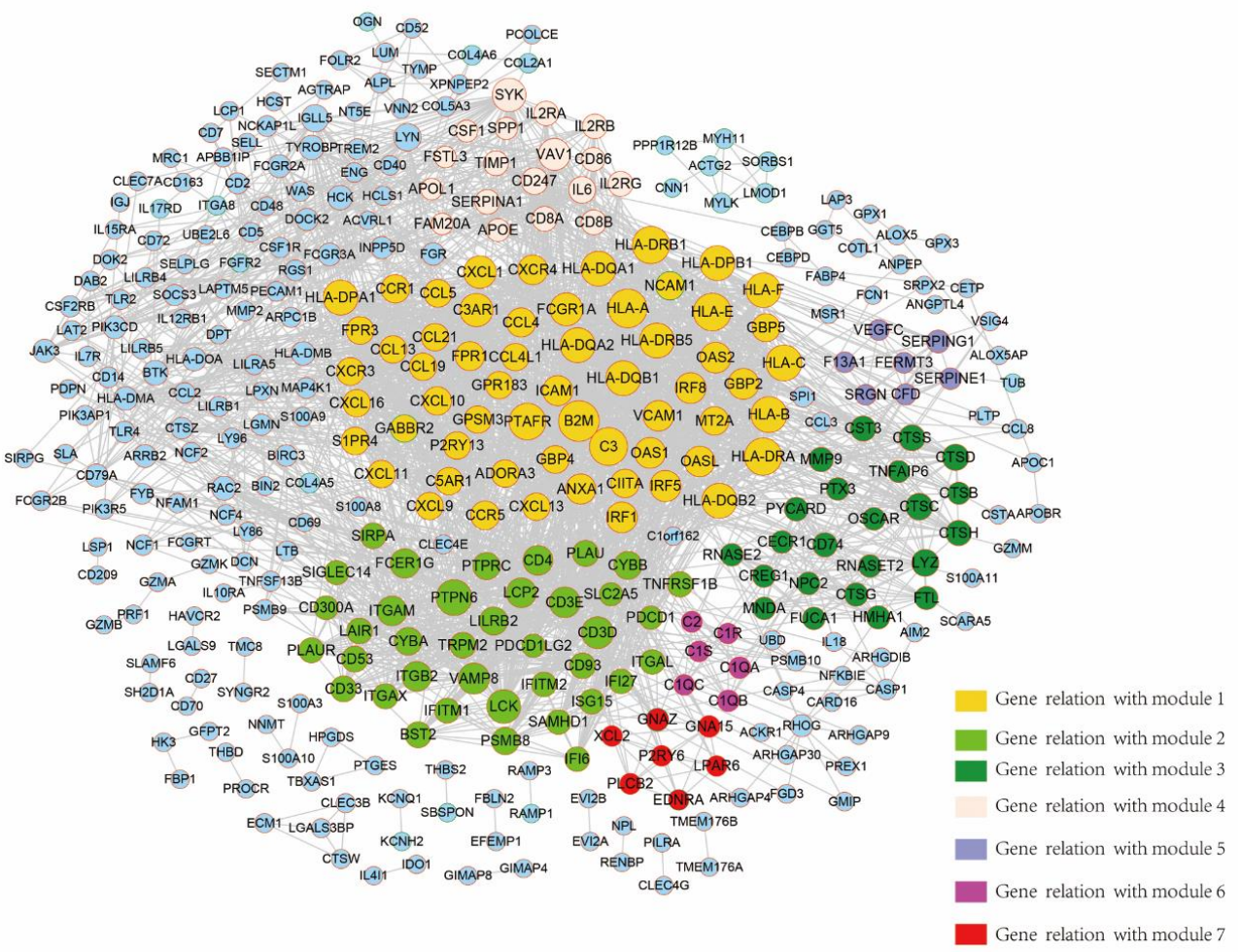

Figure 4. The PPI network for immune DEGs. Nodes with red edge represent up-regulated genes and nodes with green edge represent down-regulated genes; bigger node size represents larger degree value; blue nodes represent the genes that are not in significant modules.

3.5. Prognosis-related immune DEGs

Survival analysis revealed that 2393 genes were related to prognosis (Table S3). In all, 146 prognosis-related immune DEGs were selected by Venn analysis (Figure 5A, Table S4). Functional

enrichment analysis was carried out to investigate these genes involved biological processes and pathways. In all, 589 biological processes and 49 KEGG pathways were obtained. As shown in Figure 5B-C, these prognosis-related immune DEGs were significantly enriched in T cell activation, leukocyte cell−cell adhesion, lymphocyte proliferation, antigen processing and presentation, etc. We further investigated the interactions among these genes, and 80 genes involving 248 interactions were obtained from the STRING database (Figure 6, Table S5). There were seven nodes with degree > 15, and they were considered as hub nodes in the PPI network. B2M was a hub node with highest degree (degree = 25), followed by HLA-DRB1 (degree = 20), HLA-DRA (degree = 20), HLA-E (degree = 19), LCK (degree = 17), HLA-DPA1 (degree = 16) and VAV1 (degree = 16). Four significant modules were obtained (Figure S3). Module 1 contained 11 nodes, B2M, HLA-DRB1, HLA-DRA, HLA-E and HLA-DPA1 were also clustered in one module, indicating that these genes were implicated in the same biological function. Additionally, chemokines ligands, including C-C Motif chemokine ligand 5 (CCL5), CCL4, CCL19 and chemokine receptor, C-X-C motif chemokine receptor 3 (CXCR3) were clustered in one module.

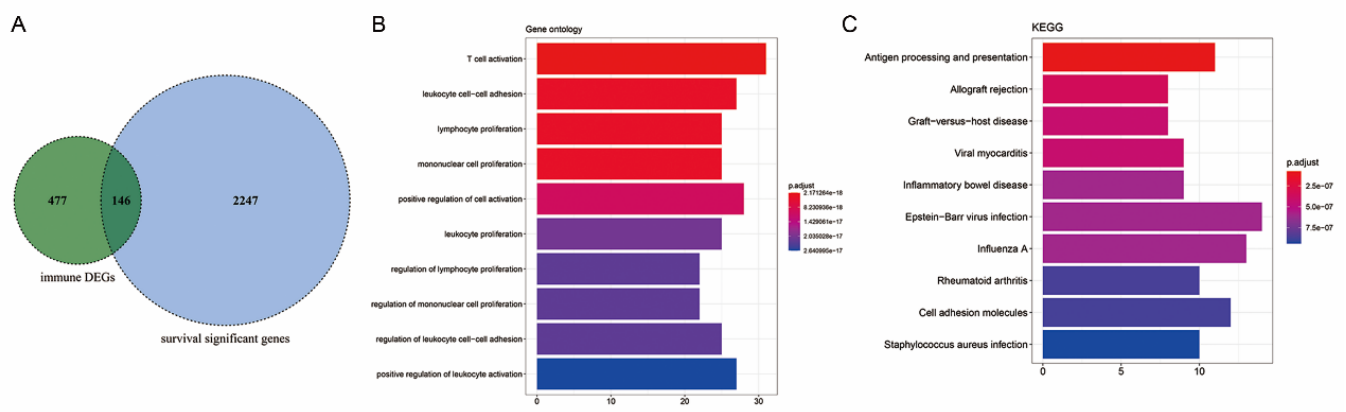

Figure 5. Functional enrichment analysis prognosis-related immune DEGs. (A), Venn plot shows the overlapped genes between immune DEGs and prognosis DEGs; histogram shows the top 10 enriched Gene ontology biological processes (B) and top 10 KEGG pathways (C) for prognosis-related immune DEGs.

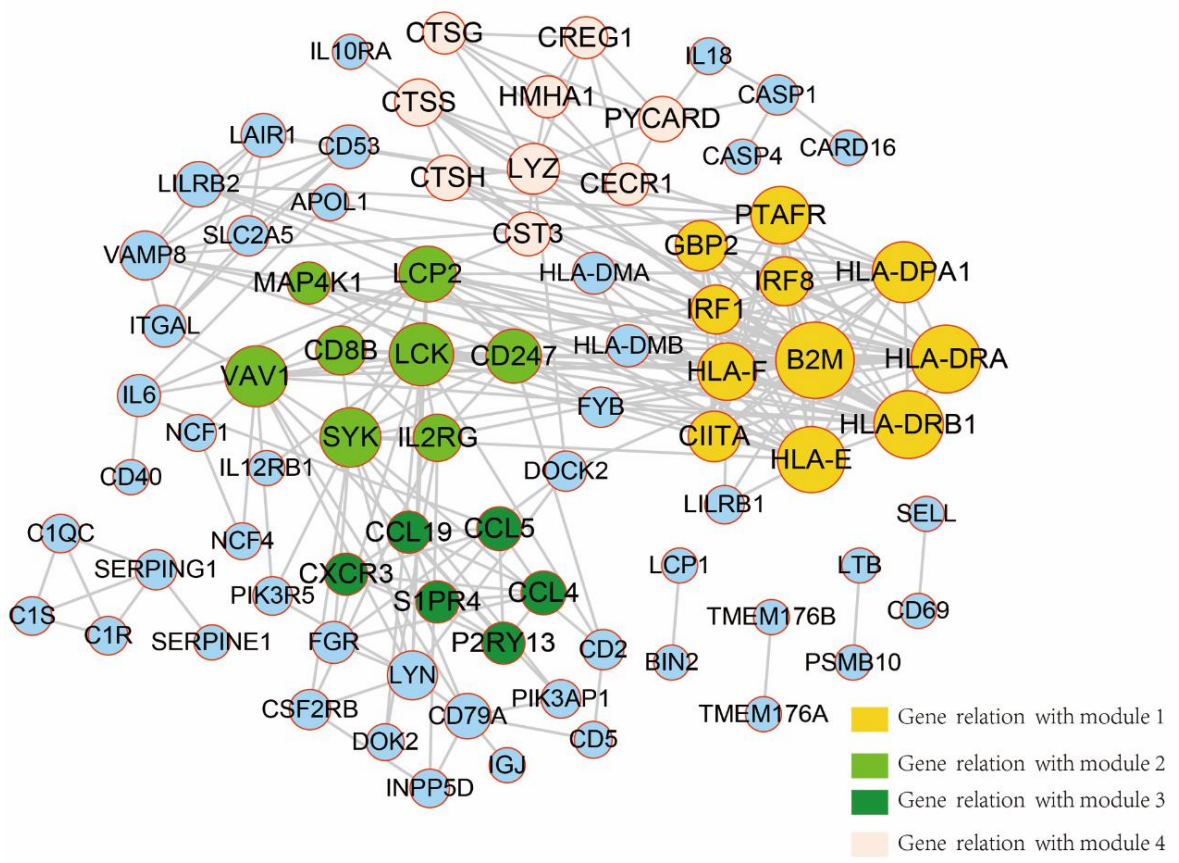

Figure 6. The PPI network for prognosis-related immune DEGs. Nodes with red edge represent up-regulated genes and nodes with green edge represent down-regulated genes; bigger node size represents larger degree value; blue nodes represent the genes that are not in significant modules.

3.6. Correlations of genes expression with immune infiltrating level

The correlations of the seven hub genes (B2M, HLA-DRB1, HLA-DRA, HLA-E, LCK, HLA-DPA1 and VAV1) expression with immune infiltration level in sarcoma were investigated utilizing TIMER. There were negative correlations between the expression of these hub genes and tumor purity, while there were positive correlations between the expression of these hub genes and the infiltration levels of B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages and dendritic cells (Figure 7). Consistently, KM curves of these seven genes revealed that the elevated expression of these genes showed correlations with a favorable prognosis (Figure 8).

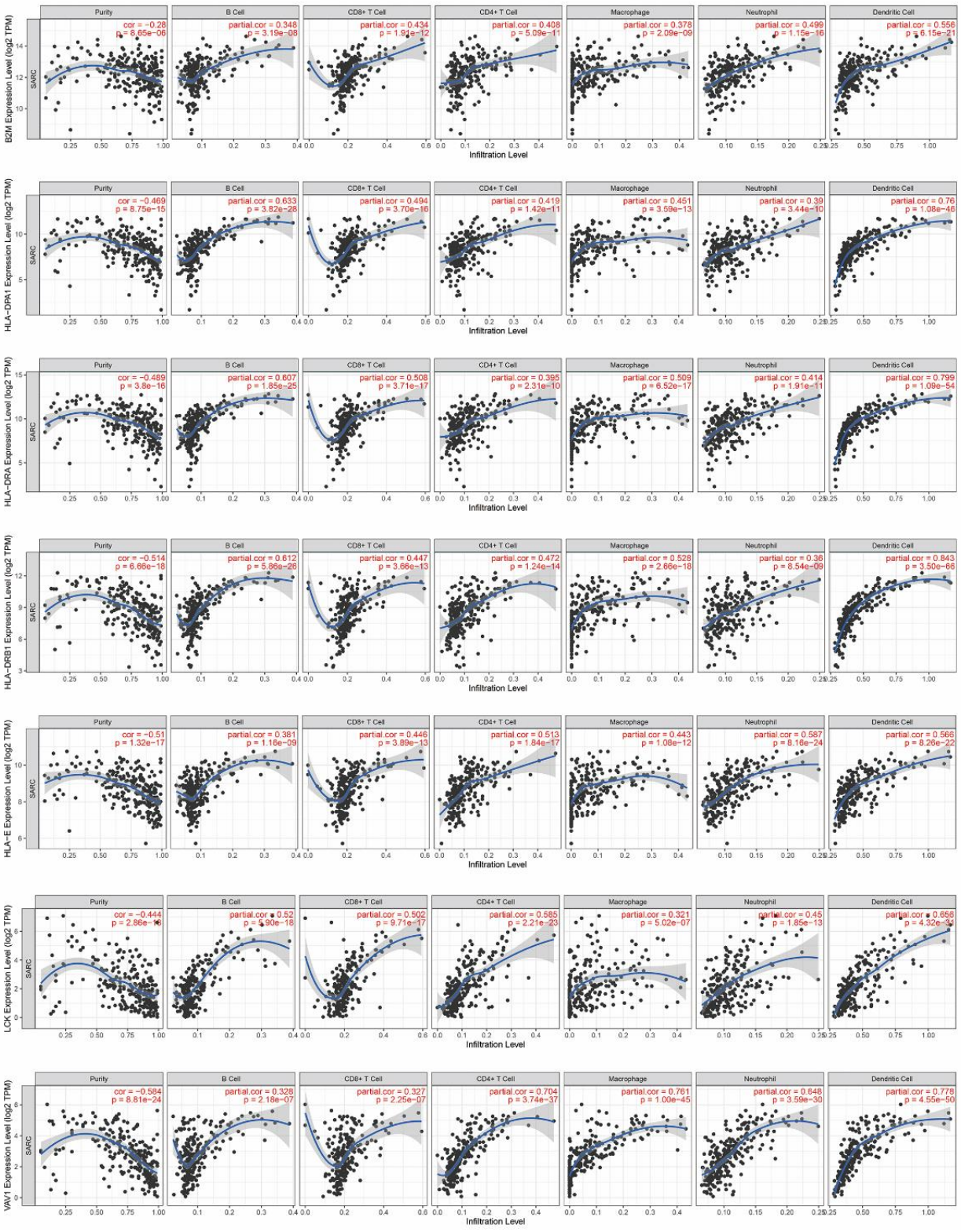

Figure 7. Genes expression with immune infiltration level and prognosis in sarcoma. Expression of B2M, HLA-DRB1, HLA-DRA, HLA-E, LCK, HLA-DPA1 and VAV1 showed negative correlations with tumor purity and positive correlations with infiltrating levels of B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages and dendritic cells.

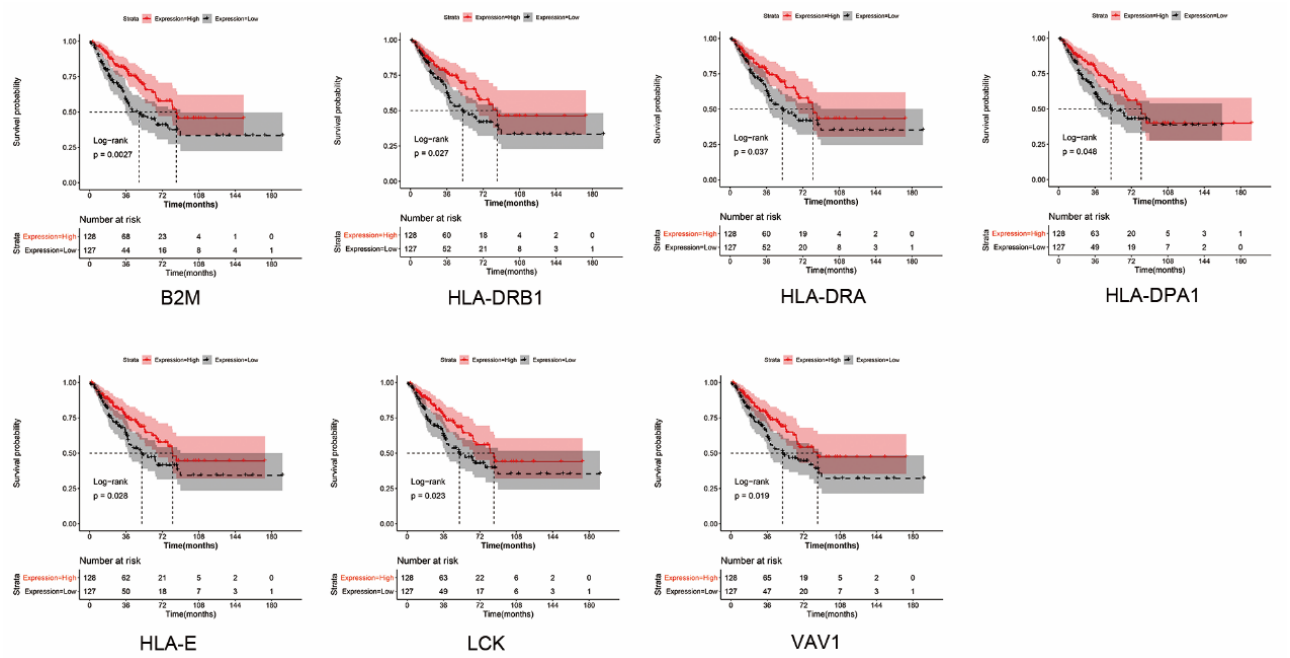

Figure 8. Survival analysis. Kaplan-Meier curves showed that the expression of B2M, HLA-DRB1, HLA-DRA, HLA-E, LCK, HLA-DPA1 and VAV1 was associated with prognosis in sarcoma.

4. Discussion

In recent years, the success and progress of tumor immunotherapy emphasizes the significance in clarifying the immune-regulatory mechanism in tumors microenvironment. Immunotherapy attacks cancer cells by the activation of the immune system of patients [26]. Increasing studies demonstrated that immune response had a significant impact on the progression of cancers [27]. In this study, we found that the immune score of sarcoma was significantly related to prognosis, and a high immune score was associated with a favorable prognosis. This suggested the potential benefits of immune activation in the treatment of sarcoma.

To investigate the underlying mechanism, we screened the genes having differential expressions in high immune score vs. low immune score. The majority of these genes (532 genes accounting for 85% of the DEGs) were up-regulated, and these genes were significantly enriched in various immune-related biological processed and pathways, such as neutrophil activation, T cell activation, antigen processing and presentation. Neutrophils are the most abundant leukocytes in the blood circulation. They play crucial roles in immune defense and are regarded as an instructor for the immune system [28]. Kuwabara et al. showed that neutrophil activation in tumors made tumors to be an immune target and contributed to the tumor regression [29]. The antigenicity and antigen presentation efficiency of the tumor are important decisive factors for the immunogenicity which is considered as the fundamental basis for the response to immune checkpoint inhibitors in immunotherapy [30]. Antigen presentation defects have been demonstrated to affect the effectiveness of immune checkpoint inhibitors [31]. Therefore, we concluded that the up-regulated immune genes activated these pathways, which might be responsible for the reason of favorable prognosis in patients with a high immune score.

Furthermore, the prognostic value of these immune genes was investigated, and 146 immune

genes were revealed to be related to prognosis, such as chemokines ligands CCL5, CCL4, CCL19 and chemokine receptor CXCR3. And these chemokines were clustered in one module. Chemokines in tumors microenvironment are considered as a transmitter for the communications among cells during the progression of cancers, which play roles in tumor proliferation, metastasis, immune cell recruitment and various processes [32]. Sun et al. had demonstrated that CCL5 expression could be an independent factor in predicting prognosis, and could be a potential therapeutic target in osteosarcoma [33]. Tang et al. suggested that the expression of CXCR3 showed a positive correlation with the abundance of infiltrating CD8 T cells, macrophages and the activation of NK cells, which could be an independent prognostic predictor in osteosarcoma [34].

Except for these chemokines, multiple HLAs (HLA-DRB1, HLA-DRA, HLA-E, HLA-DPA1, etc.) were also demonstrated to show correlations with prognosis. High expression of these genes was associated with favorable prognosis, and these HLAs and B2M were clustered in one module, and were significantly enriched in antigen processing and presentation pathway. Moreover, there were negative correlations between the expression of these hub genes and tumor purity, while positive correlations between the expression of these hub genes and the infiltration levels of immune cells. HLAs are antigen-presenting proteins that are expressed on the surface of some immune cells. Changes in their expression on cancer cells were found to regulate the ability of the immune system to kill cancer cells and to mediate the metastasis in several cancers [35]. The recognition of cellular immunity mainly relies on the expression of HLAs, which are crucial for immunotherapy. It had been proved that the decrease or absence of HLAs expression was observed in most advanced-stage Ewing sarcoma, suggesting there was immune escape, which helped to develop immunotherapy strategies for these patients [36]. A previous study had reported that the reduced level of HLA-DRA and HLA-DPA1 showed correlations with the tumor progression and worse survival in adult adrenocortical tumors, and HLA-DPA1 was an independent factor for prognosis prediction [37]. Luk et al. demonstrated that the increased expression of HLA-I in synovial sarcoma showed positive correlations with infiltrating T cells [38]. B2M, beta-2-microglobulin, is considered as a stabilizing scaffold, which together with HLAs encoded alpha chain to form the Major Histocompatibility Complex class I (MHC-I) molecule. MHC-I molecule contributes the immune system to identify and undermine tumor cells by presenting intracellular peptides to T cells. B2M is indispensable for MHC-I complex formation and peptide presentation [39].

5. Conclusions

From all above, we concluded that tumor with high immune score was correlated with favorable prognosis in sarcoma. The elevated expression of HLAs, B2M and chemokines might be responsible for the reason of favorable prognosis by mediating antigen processing and presentation, and intercellular communications in high immune score tumor. These findings help to stratify patients with different immune subtypes and help to design immunotherapy strategies for these patients in sarcoma.

Conflict of interest

The authors declare that they have no competing interests.

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