Revista Argentina de Clínica Psicológica
2020, Vol. XXIX, N°3, 1249-1256
DOI: 10.24205/03276716.2020.954

Comprehensive analysis of *CD2* in the immune microenvironment of breast cancer

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Abstract

Background: Tumor microenvironment is essential for breast cancer progression and metastasis. Our study sets out to examine the genes affecting stromal and immune infiltration in breast cancer progression and prognosis.

Methods: This work provides an approach for quantifying stromal and immune scores by using the ESTIMATE algorithm based on the gene expression matrix of breast cancer patients in the TCGA database. We found differentially expressed genes (DEGs) through the limma R package. Functional enrichments were accessed through Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Besides, we constructed a protein-protein network, identified several key genes in Cytoscape. Through univariate COX analysis and online website Kaplan-Meier plotter analysis, genes significantly related to prognosis were determined. Their level of expression was determined through an online database (TIMER; HPA). Furthermore, their relationship with infiltrating immune cells was evaluated by Tumor IMmune Estimation Resource (TIMER) web tool. A prognosis analysis based on the expression levels of CD2 was further performed in related immune cells subgroup.

Result: To better understand the relationship between immune and stromal cell-related genes and prognosis, we screened patients with breast cancer in The Cancer Genome Atlas (TCGA) database and divided them into high and low groups based on immune/stromal scores. Immune score results showed that the high score subgroup was significantly associated (p = 0.008) with a better prognosis. Differential gene identification results show that there are 473 up-regulated genes and 49 down-regulated genes. Functional analysis of DEGs revealed their potential functions in immune response and extracellular interaction. Ten related prognostic genes were identified through univariate COX analysis and PPI network analysis. Multiple online databases have verified their expression levels, and the results show that *CD2* is significantly high in tumors. We found that in the BRCA, *CD2* was positively correlated with B cells (cor = 0.552), CD4 + T cells (cor = 0.679), CD8 + T cells (cor = 0.595), neutrophils (cor = 0.653) and dendritic cells (cor = 0.728). The expression level of *CD2* was also correlated with related immune cells subgroup. On the whole, further research on *CD2* can reveal a new understanding of the potential relationship between tumor microenvironment and breast cancer prognosis.

Keywords: breast cancer, tumor microenvironment, immune infiltration, The Cancer Genome Atlas

1. Introduction

Breast cancer is one of the most common malignant tumors in women (Hill et al., 2018; DeSantis et al., 2011; Liu et al., 2018; Takuwa et al., 2018; Munoz et al., 2018). The incidence rate of the World's breast cancer is increasing every year, and China is no exception. In 1990, the incidence of breast cancer in China is increasing much faster than that in other countries. According to authoritative analysis, the number of breast cancer patients in China will exceed 2 million in 2021, so breast cancer research is needed (Campbell, 2002; Li et al., 2016; Fan et al., 2014; Sitt et al., 2018; Pan et al., 2017). About 500000 It is imminent. With the development of medical technology, the direction of the tumor microenvironment (TME) has become one of the important directions to conquer breast cancer.

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According to incomplete data statistical analysis, TME has a certain impact on the gene expression of tumor cells and has a positive role in clinical diagnosis and treatment (Li et al., 2017; Lim et al., 2018; Houthuijzen and Jonkers, 2018; Dias et al., 2019). TME is the environment where tumor cells live. The expression of gene sequence is different when the environment changes. To estimate the stromal and immune infiltration level of the tumor and provide clues for researches on this field, Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data (ESTIMATE) is modeled and tested to calculate tumor purity, stromal and immune scores of patients via expression profile and give an overall view of the tumor microenvironment (Weng et al., 2019; Rasha et al., 2019; McCormack et al., 2018; Li et al., 2017; Leyrer et al., 2017; Giannakeas and Narod, 2018; Hill, 2014).

In this study, we obtained Breast cancer data from the TCGA database and verified it in the online database. Through protein-protein interaction (PPI) network analysis, univariate COX regression analysis, we finally identified 10 genes (*CD2*, *CD40LG*, *CCL19*, *CD3E*, *IL7R*, *CD48*, *CD5*, *CD52*, *ITK*, and *SPN*) related to prognosis. The online database was further verified, and the results showed that *CD2* was significantly high in breast cancer and was significantly related to a better prognosis. We also found that *CD2* has a significant correlation with clinical and a positive correlation with most immune infiltrating cells. The results of *CD2* may provide new insights into the treatment and prediction of Breast cancer.

2. Materials and Methods

2.1 Datasets and Preparation

The data provided by TCGA and ICGC are public and open, so the approval of ethics committee is not required. (https://tcga-data.nci.nih.gov/tcga/), containing 1109 BRCA tissues and 113 adjacent nontumorous tissue samples as of May 2020. Combined with the inherent law of gene expression, the influence mechanism of matrix and immune microenvironment was analyzed by using probability and statistics analysis.The analysis method is integrated in the “estimate” R package in R 3.6.3.

2.2 Identification of Valid Differentially Expressed Genes (DEGs)

We divided stromal cells and immune cells into two groups based on the scores, and then identified the differential genes of immune cell subgroups and stromal cell subgroups respectively. Differentially expressed genes (DEGs), defined as dysregulated genes with $|\log FC| > 1$ and $FDR < 0.05$ between high and low score groups in this study. The corresponding parameter data can be obtained by intervening r-packet "limma".

2.3 Functional enrichment analysis and protein-protein interaction (PPI) network construction

The Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analysis of all DEGs by R software with $P < 0.01$ as the threshold. Meanwhile, the DEGs were inputted into online website STRING (https://string-db.org/) to predict protein-protein interactions, with confidence >0.9 as the cut-off criterion. Then, we processed the PPI network using Cytoscape software (v3.7.2) and calculated the number of its nodes through R software, and visualized the top 30.

2.4 Screening and identification of prognostic genes

Univariate Cox proportional hazard regression was conducted to screen for the genes. $P < 0.05$ was considered statistically significant. Then, the obtained genes and the 30 genes identified by the PPI network are intersected, and the Venn diagram was visualized by R software. In the end, 10 prognostic genes were obtained. In order to analyze the expression levels of these 10 genes in BRCA, we analyzed them through the online database TIMER (https://cistrome.shinyapps.io/timer/). In addition, we also identified their protein level expression through the human protein atlas database (HPA, https://www.proteinatlas.org/).

2.5 Genes’ Correlation with Immune CellsInfiltration

In order to further analyze the internal relationship between key gene and infiltrating immune cells, the related parameters were analyzed with the help of a TIMER (tumor immune evaluation resource) web tool, and the mechanism between genes and immune cells were obtained. The number of different cells was obtained by the Kaplan Meier mapping instrument. (http://kmplot.com/).

2.6 Statistical analysis

In this study, we used R software (version 3.6.3) to explore the whole process. We used the R software packages "ggsignif", "ggpubr" and "ggplot2" to make box plots and quantitative statistical studies of differential expression. In R, we performed univariate Cox analysis. A $p < 0.05$ was considered significant.

3. Result

3.1 Association of immune and stromal scores with prognosis

1109 BRCA tissues and 113 adjacent non-tumor tissue samples were obtained from the TCGA database. We calculated the stromal score and immune score in each sample through the ESTIMATE algorithm, divided the patients into high and low groups based on the median score, and combined them with the survival time to obtain the survival curve. Immune score results showed that the high score subgroup was significantly associated (p = 0.008) with a better prognosis (Figure 1A). The stromal score results showed that there was no significant correlation between score subgroups and prognosis (Figure 1C). Then we used the R package "limma" to identify differential genes in the two subgroups. Due to too many differential genes, we visualized the first 50 genes (Figure 1B; Figure 1D).

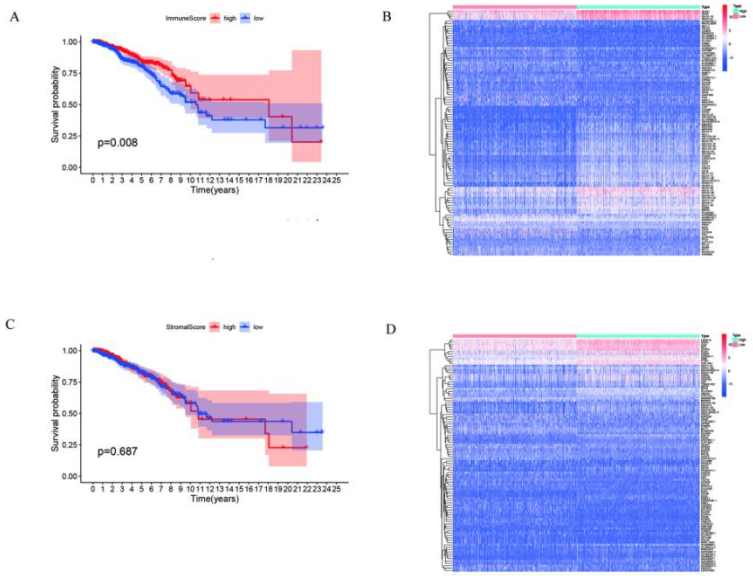

Figure 1**. Association of immune and stromal scores with prognosis**

(A) The results showed that there was a significant association between breast cancer and the level of immune scores. (B) DEGs between high and low immune score groups (top 50). (C) The results showed that there was no significant correlation between breast cancer and stromal score levels. (D) DEGs between high and low stromal score groups (top 50).

3.2 Public DEGs identification and functional enrichment analysis

In order to identify the same DEGs in the two groups, we crossed the DEGs of the immune subgroup with the DEGs of the stromal subgroup. The results showed that there were 473 up-regulated genes (Figure 2A) and 49 down-regulated genes (Figure 2B). In order to further analyze the functions of these genes in BRCA, we performed Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of them (Figure 2C, 2D).

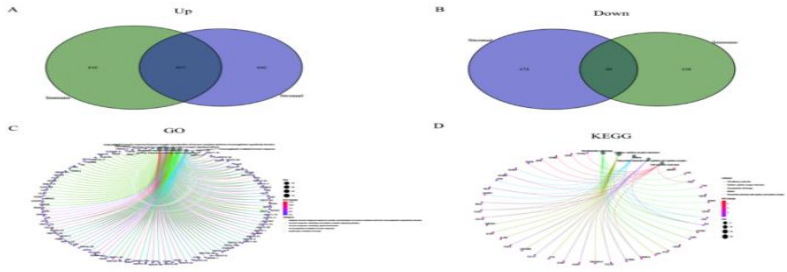

Figure 2. **Public DEGs identification and functional enrichment analysis**

(A) The results showed that there were 473 up-regulated genes. (B) The results showed that there were 49 down-regulated genes. (C) Gene Ontology (GO) analysis. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.

3.3 PPI Network Construction and Key Gene Screening

Through the online website STRING, we built a PPI network between DEGs, and then we visualized it through Cytoscape software. We can see from Figure 3A that this PPI network has a total of 1103 edges and 184 nodes. Red were genes that are up-regulated in breast cancer, and blue were genes that are down-regulated in breast cancer. In addition, we calculated the number of interactions between each node and visualized the first 30 nodes, as shown in Figure 3B. Because we hope to find genes related to the prognosis of breast cancer, we used univariate Cox regression analysis on the previously identified differential genes and found that 59 genes were critical to the prognosis of breast cancer (Figure 4A). Then we intersected the 30 genes in the PPI network with the genes of the univariate COX results (Figure 4B), and got 10 key genes (*CD2*, *CD40LG*, *CCL19*, *CD3E*, *IL7R*, *CD48*, *CD5*, *CD52*, *ITK*, and *SPN*). Then through Kaplan-Meier plotter databases analysis, the results show that these 10 genes were prognostic related (Figure 5)

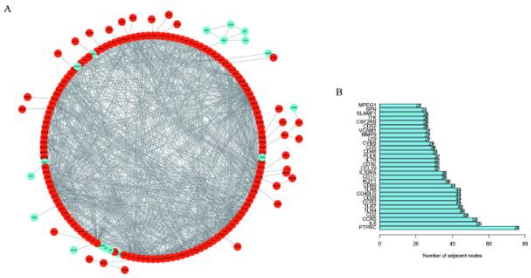

Figure 3**. PPI Network Construction**
(A) PPI Network analysis. (B) Top 30 genes with the most nodes.

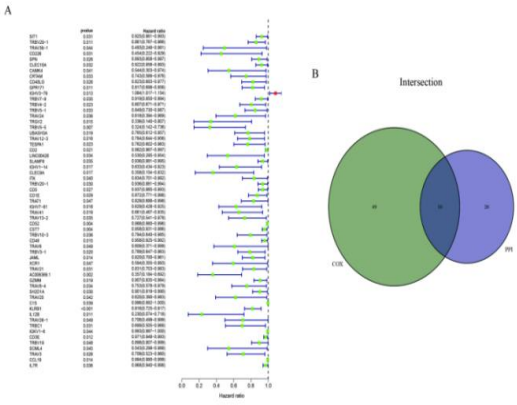

Figure 4. **Key Gene Screening**
(A) Univariate COX analysis showed that 59 genes were related to prognosis. (B) 10 key genes (*CD2*, *CD40LG*, *CCL19*, *CD3E*, *IL7R*, *CD48*, *CD5*, *CD52*, *ITK*, and *SPN*).

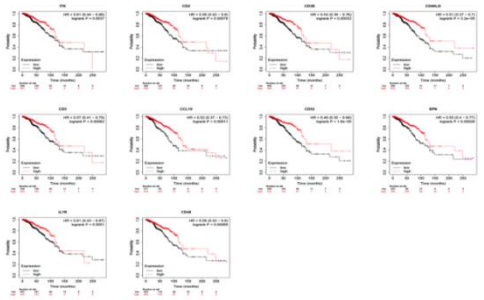

Figure 5. **Survival analysis of key genes in breast cancer (Kaplan-Meier plotter databases)**

3.4 Identification of key gene expression levels

The results of differential identification showed that these 10 genes are all genes that are significantly up-regulated in breast cancer in the TCGA-BRCA data. In order to further identify the expression levels of these genes in breast cancer, we conducted an analysis through online databases (TIMER), and the results showed that *CD2* ($p < 0.05$), *CD5* ($p < 0.001$), *CD40LG* ($p < 0.001$), *CD48* ($p < 0.05$), and *IL7R* ($p < 0.001$) were all significantly highly expressed in breast cancer (Figure 6). Then we used the HPA database to identify its protein level and the results showed that CD2 was highly expressed in tumors (Figure 7). There was no data for IL7R in HPA database.

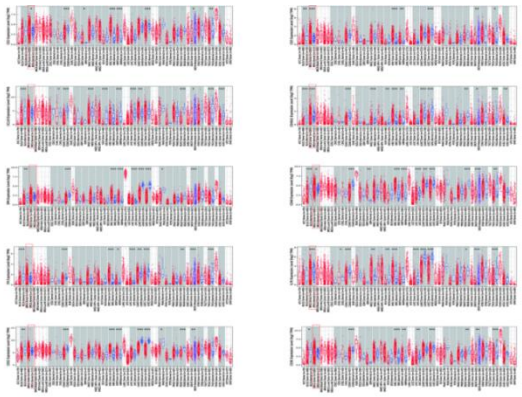

Figure 6. **Identification of mRNA expression levels of key genes (TIMER databases)**

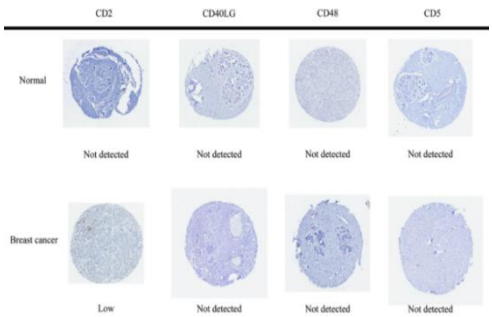

Figure 7**. Identification of the expression level of key gene proteins (HPA databases)**

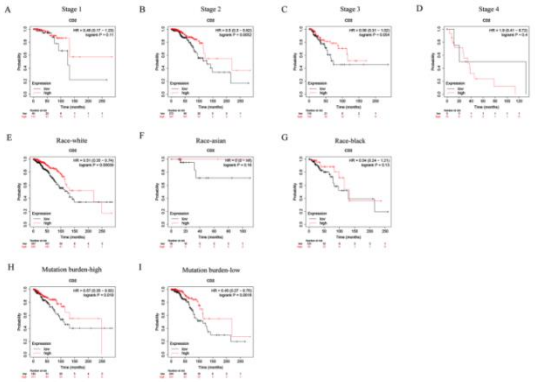

Figure 8**. Clinical correlation analysis**

3.5 Clinical correlation analysis

In order to further analyze the relationship between *CD2* and clinical, we analyzed it through Kaplan-Meier plotter databases, and the results showed that the high expression of *CD2* in the Stage 2 (p = 0.0052), Race-white (p = 0.00039), Mutation burden-high (p = 0.019) and Mutation burden-low (p = 0.0018) subgroups were all related to a better prognosis (Figure 8B, Figure 8E, Figure 8H, Figure 8I).

3.6 Identification of the Correlation between CD2 and Immune Infiltration

In order to predict the possible effect of *CD2* on immune cell infiltration (B cells, CD4 + T cells, CD8 + T cells, macrophages, neutrophils and dendritic cells), we analyzed it with the TIMER network tool. We found that in the BRCA group, *CD2* was positively correlated with B cells (cor = 0.552), CD4 + T cells (cor = 0.679), CD8 + T cells (cor = 0.595), neutrophils (cor = 0.653) and dendritic cells (cor = 0.728). In addition, in the BRCA subgroup, the results were also consistent (Figure 9).

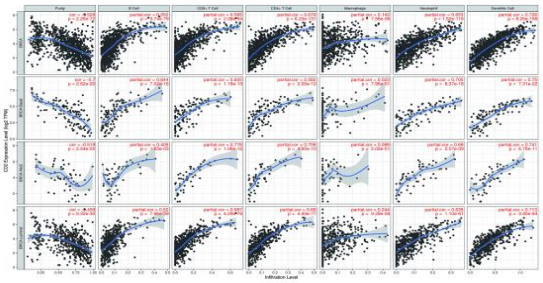

Figure 9. **Identification of the Correlation between *CD2* and Immune Infiltration**

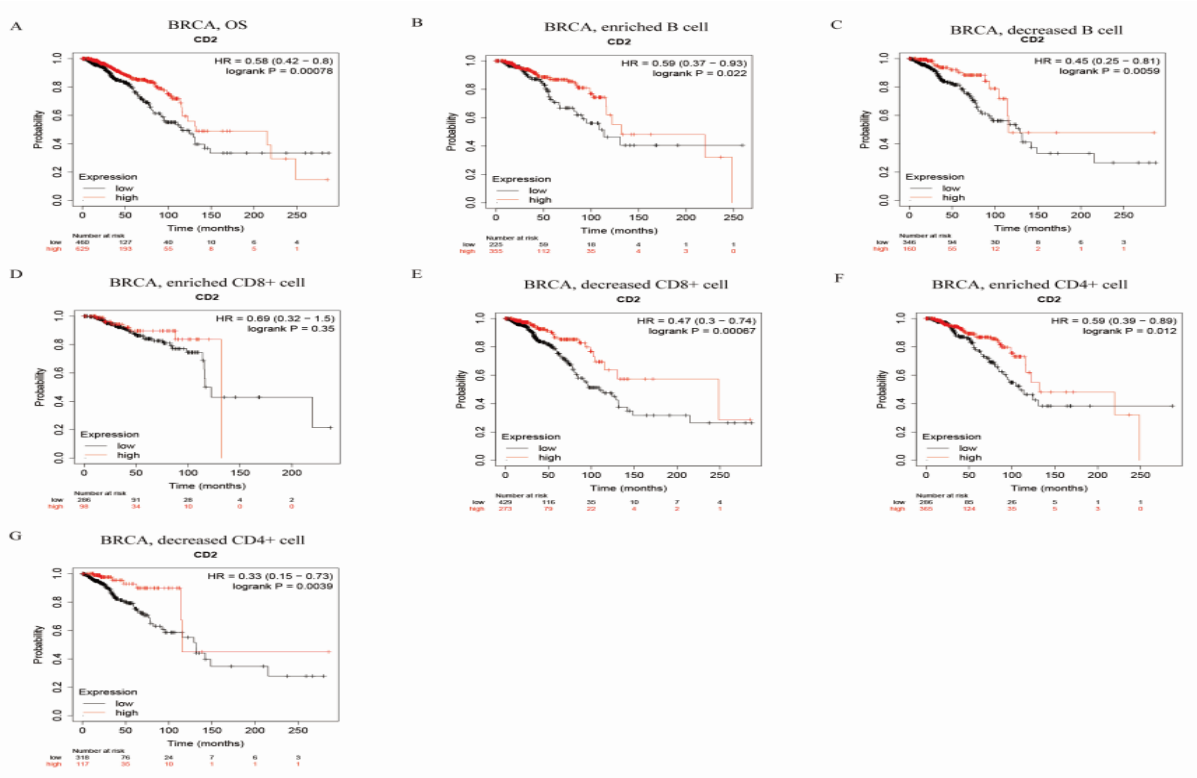

Figure 10. **Prognostic analysis of CD2 expressions in different tumors based on immune cells**

3.7 Prognostic analysis of *CD2* expressions in different tumors based on immune cells

Through a large number of data and research results, the expression of *CD2* has a direct relationship with BRCA immune infiltration and is also closely related to the prognosis mechanism. Therefore, it can be further obtained that tumor gene expression is also highly correlated with

immune invasion. Therefore, we can use the Kaplan Meier plotter to study the *CD2* expression sequence of BRCA, and then conduct targeted prognosis research. The results showed that the high expression of *CD2* of BRCA in enriched B cells (HR = 0.59), decreased B cells (HR = 0.45), enriched CD4+ memory T cells (HR = 0.59), decreased CD4+ memory T cells (HR = 0.33), and decreased CD8+ T cells (HR = 0.47) cohort had better prognosis respectively (Figure 10B, 10C, 10E, 10F, 10G). The above analysis suggested that high *CD2* expressions in BRCA may affect prognoses in part due to immune infiltration.

4. Discussion

Breast cancer is a common malignant tumor in the world and is one of the main causes of cancer death in modern women. Now a large number of data and information show that the immune system has irreplaceable value in the pathogenesis and prognosis of breast cancer (Dong et al., 2019; Sau et al., 2019; Allaire et al., 2017; Green, 2013; Emens, 2018; Nagini, 2017; Blackley and Loi, 2019). Through gene expression and construction of the corresponding host environment between tumor noumenon and host immunity, the corresponding strong immune system is also constructed; therefore, the research on immune escape and tumor progression is inseparable. The successful clinical application of immunotherapy at specific immune checkpoint PD-1 / PD-L1 and the generation of TNBC induced mechanisms all indicate that immune infiltration is a good prognostic marker, especially in TNBC, HER2 + and high proliferative ER-positive tumors (Bayraktar et al., 2019; Pondé et al., 2018; Fahad, 2019; Wein and Loi, 2017; Vikas et al., 2018; Waks and Winer, 2019; Howlader et al., 2014; Gandhi and Das, 2019).

In order to explore the role of the immune microenvironment in breast cancer, we first obtained breast cancer data from the TCGA database, and then calculated the stromal score and immune score of each sample through the ESTIMATE algorithm, and divided patients into high and low groups based on the median score. Immune score results showed that the high score subgroup was significantly associated (p = 0.008) with a better prognosis (Figure 1A). Then we identified the genes with significant differences and performed Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of them (Figure 2). As shown in Figure 2C, 10 biological processes (BPs), 10 molecular functions (MFs) and 10 cellular components (CCs) were enriched, such as immunoglobulin complex, T cell receptor complex, exogenous protein bindin, MHC protein binding, cytokine receptor activity, immune receptor activity, immunoglobulin receptor binding. Regarding the KEGG pathway analysis as shown in Figure 2D, immune-related pathways were enriched such as the T cell receptor signaling pathway, Cell adhesion molecules, Chemokine signaling pathway and

JAK−STAT signaling pathway, which revealed potential mechanisms and pathways activated during tumor progression. Through protein-protein interaction (PPI) network analysis, univariate COX regression analysis, we finally identified 10 genes (*CD2*, *CD40LG*, *CCL19*, *CD3E*, *IL7R*, *CD48*, *CD5*, *CD52*, *ITK*, and *SPN*) related to prognosis. The online database was further verified, and the results showed that *CD2* was significantly high in breast cancer and was significantly related to a better prognosis. In addition, we also found that CD2 has a certain clinical correlation with breast cancer. The results showed that the high expression of *CD2* in the Stage 2 (p = 0.0052), Race-white (p = 0.00039), Mutation burden-high (p = 0.019) and Mutation burden-low (p = 0.0018) subgroups were all related to a better prognosis. We also found that in the BRCA, CD2 was positively correlated with B cells (cor = 0.552), CD4 + T cells (cor = 0.679), CD8 + T cells (cor = 0.595), neutrophils (cor = 0.653) and dendritic cells (cor = 0.728). These strongly confirmed the positive correlation between *CD2* and immune infiltration in BRCA. Prognostic analysis of *CD2* expression levels in different tumors based on immune cells was performed, the high *CD2* expression level in BRCA had a favorable prognosis in the enriched B cells, and CD4 + memory T cells subgroups (Figure 10). The high CD2 expression level in BRCA had a favorable prognosis in the decreased B cells, CD4 + memory T cells, and CD8 + T cells subgroups (Figure 10). To sum up, *CD2* expression increased significantly in BRCA. Elevated *CD2* was positively correlated with immune infiltration and prognoses of BRCA.

Competing Interests

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

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