Xia D et al. (2023) SPP1 AS A PROGNOSTIC BIOMARKER IN COLORECTAL CANCER: IMPLICATIONS FOR IMMUNE INFILTRATION IN ATHLETES AND FITNESS ENTHUSIASTS. Revista Internacional de Medicina y Ciencias de la Actividad Física y el Deporte vol. 23 (93) pp. 310-342. **DOI:** https://doi.org/10.15366/rimcafd2023.93.021

ORIGINAL

SPP1 AS A PROGNOSTIC BIOMARKER IN COLORECTAL CANCER: IMPLICATIONS FOR IMMUNE INFILTRATION IN ATHLETES AND FITNESS ENTHUSIASTS

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Recibido 22 de agosto de 2022 **Received** August 22, 2022
Aceptado 25 de octubre de 2023 **Accepted** October 25, 2023

ABSTRACT

Background: Secretory phosphoprotein 1 (SPP1), also known as Osteopontin, is part of the small integrin-binding ligand N-linked glycoprotein family. Its overexpression in various cancers, including colorectal cancer (CRC), has been linked to prognosis. However, its role in the survival and immune infiltration in CRC, particularly in athletes and fitness enthusiasts, requires further investigation. **Methods:** Utilizing data from The Cancer Genome Atlas (TCGA) and the Genotype Tissue Expression Project (GTEx), we analyzed the expression profiles and clinical data of CRC patients, focusing on the relationship between SPP1 expression and cancer development. Wilcoxon rank tests compared SPP1 expression in normal and cancerous colorectal tissues. The predictive power of SPP1 in CRC was assessed using the receiver operating characteristic (ROC) curve. Logistic regression and Wilcoxon rank sum tests were used to correlate SPP1 expression with clinical features. Kaplan-Meier survival analysis, Progno Scan database, and Cox multivariate regression analysis evaluated SPP1's prognostic significance. R-package Cluster profiler enrichment analysis identified SPP1's biological functions and signaling pathways. Correlations between SPP1 and immune-infiltrating cells were appraised using single-sample gene set enrichment analysis (ssGSEA) and TIMER database, with further exploration of SPP1 and immune cell gene

markers through TIMER and GEPIA databases. **Results:** SPP1 was found to be highly expressed in CRC, significantly more so than in normal colorectal tissues, with an AUC of 0.846 indicating high accuracy in CRC prognosis prediction. SPP1 expression correlated with pathological stage and disease-specific survival (DSS), with overexpression leading to reduced overall survival (OS), DSS, and disease-free survival (DFS). Multivariable Cox regression analysis confirmed SPP1 as an independent prognostic biomarker for CRC (HR = 1.459; 95% CI: 1.030-2.067; P = 0.033). SPP1 was associated with various biological functions and signaling pathways. It also showed a positive correlation with the infiltration levels of macrophages, dendritic cells (DCs), CD8+ T cells, and neutrophils in CRC. SPP1 was strongly correlated with immune gene markers, including TAM, DC, T cell exhaustion, and Tregs. **Conclusion:** SPP1's elevated expression in CRC is closely associated with poor immune infiltration and prognosis. In athletes and fitness enthusiasts, who might experience unique immune system challenges due to their intense physical regimes, SPP1's role becomes particularly crucial. Understanding SPP1's impact on immune infiltration in this group could lead to tailored therapeutic approaches in CRC, positioning SPP1 as a promising prognostic biomarker in this demographic.

KEYWORDS: SPP1; Biomarkers; colorectal cancer; prognosis; immune infiltration

1. INTRODUCTION

Colorectal cancer (CRC) remains a major public health challenge worldwide, with increasing incidence and mortality rates. Among the diverse factors influencing CRC progression, immune infiltration within the tumor microenvironment plays a critical role. Secretory phosphoprotein 1 (SPP1), also known as Osteopontin, has emerged as a significant biomarker in various cancers, including CRC. Its role in modulating immune responses and influencing cancer prognosis is gaining attention, especially in specific population subsets like athletes and fitness enthusiasts(Sung et al., 2021).

Athletes and individuals engaged in regular, intense physical activity represent a unique cohort. Their heightened physical stress and distinct physiological adaptations can influence immune function and potentially alter cancer progression dynamics. Understanding the specific implications of biomarkers like SPP1 in such populations is crucial for developing tailored therapeutic strategies and improving prognostic assessments(W. Chen et al., 2016; D.-S. Wang et al., 2021). SPP1, part of the small integrin-binding ligand N-linked glycoprotein family, is known for its role in cell-matrix interactions and signaling. In the context of CRC, SPP1's overexpression has been linked to various pathological processes, including tumor growth, metastasis, and particularly, angiogenesis and immune infiltration. The latter is pivotal as it

shapes the tumor microenvironment, influencing the body's immune response to cancer cells(Ganesh et al., 2019; IJsselsteijn, Sanz-Pamplona, Hermitte, & de Miranda, 2019). The study of SPP1 in CRC has primarily focused on general populations, with less emphasis on how physical conditioning and fitness-related immune adaptations might impact its role. Athletes, due to their rigorous training routines, experience unique immune system responses. These could potentially interact with SPP1-related pathways, affecting the progression and prognosis of CRC in this group differently than in the general population(Barbee, Ogunniyi, Horvat, & Dang, 2015; Garon et al., 2015).

To investigate this, we utilize data from comprehensive genomic databases like The Cancer Genome Atlas (TCGA) and the Genotype Tissue Expression Project (GTEx), assessing SPP1 expression profiles in CRC patients. The study employs a range of analytical techniques, including ROC curve analysis, logistic regression, and Cox multivariate regression, to elucidate the prognostic significance of SPP1. Additionally, the study delves into the correlation between SPP1 expression and immune infiltration, particularly focusing on how this relationship manifests in athletes and fitness enthusiasts (Chung et al., 2010; Le et al., 2015; Overman et al., 2017; Waniczek et al., 2017). SPP1, a member of the SIPING family, is not only known as osteopontin or the other,but also can specifically trigger matrix metalloproteinase at tumors (Su et al., 2020), which involved in cell evolution at complete period. It has been proved that SPP1 became overexpressed as a biomarker for predicting adverse outcomes, including hepatocellular carcinoma (J. Wang, Hao, Fei, & Chen, 2019), gastric cancer (Song et al., 2019), ovarian cancer (Zeng, Zhou, Wu, & Xiong, 2018) and Glioblastoma multiforme (Kijewska et al., 2017). In this project, we conducted bioinformatics analysis using R language, analytical tools and online websites to investigate SPP1 expression in CRC and potential function and mechanism at tumor-immunity, and even furtherly to clear and definite whether attributes to an independent prognosis biomarker.

2. Materials and methods

2.1 Gene Express Analysis

The TCGA database (Tomczak, Czerwińska, & Wiznerowicz, 2015) and the GTEX database (Lonsdale et al., 2013) provided Gene expression profiling and clinical data on colorectal cancer patients for our study. Grade 3 HTSeq-FPKM data keep convert to transcripts reads permillion for investigating differentiate of SPP1 between colorectal cancer and matched samples, which included unpaired and paired tissues. We also obtained TPM-formatted RNAseq data for TCGA and GTEX by uniform processing of the UCSC Xena database (Vivian et al., 2017) and analyze SPP1 differences at pan-cancer. The CRC cohort provided by the TCGA data includes the colon adenocarcinoma (COAD) and rectal adenocarcinoma (READ) datasets.

Through analysis of the clinical data in CRC cohort, the relationship between SPP1 and CRC sufferers' clinic opathological features was deeply investigated and moreover the diagnostic value of SPP1 by ROC curve keep appraised. The HPA database provides us with differential data on SPP1 protein levels in two opposite samples including colorectal cancer and the normal after immuno histochemical staining (IHC) (Uhlén et al., 2015; Uhlen et al., 2010). ROC curves remained performed utilizing the PROC package [version 1.17.0.1] to assess prediction accuracy. Expression data after transformation with log2[TPM (per million transcripts) + 1] show expression levels of SPP1.

2.2 Survival Prognosis Analysis

The optimal segmentation point for SPP1 TPM separation of continuous variables was determined by R package survminer's Surv cutpoint function. Then, the Kaplan-meier of log-rank test was adopt to appraise SPP1 prognostic in colorectal cancer patients as well as in different clinical subgroups of colorectal cancer patients. R packet survminer [version 0.4.9] was used to visualize the survival curve. The association between SPP1 and the sufferer survival of CRC was also discovered through the Progno Scan. The multivariable Cox proportional hazards model in TIMER database was picked to assess SPP1 outcome significance, which was completed through the function coxph (). It was considered that the baseline variables were relevant to the outcome whether univariate or clinically, included in the multivariable Cox proportional hazards model (Stone et al., 2011).

2.3 Different Express Gene and Functional Enrichment Analysis

Taking SPP1 median as the dividing point, the colorectal cancer patients in TCGA were put into two different groups including SPP1 high expression and the low, which was completed by R-package DESeq2 (Love, Huber, & Anders, 2014), adjusted $p<0.05$,|log2 fold change (FC)|>1.5 as the threshold for differentially genes. Volcano and heat maps were drawn by R language Ggplot2 package (https://ggplot2.tidyverse.org). The Genome Ontology term and Kyoto Encyclopedia of Genes and Genomes（KEGG）were studied to differential gene pathways through using cluster Profiler (Subramanian et al., 2005; Yu, Wang, Han, & He, 2012) of R and Ggplot2 packages. Similarly, such analysis also keeps implemented through Cluster Profiler, with adjusted $p< 0.05$ and false discovery rate (FDR) < 0.25 determined to be statistically enriched functional.

2.4 Immune Infiltration Analysis

The relative abundance of SPP1 high-low expression group and 24 kinds of immune cells in colorectal cancer samples was analyzed via GSVA of R language (Hänzelmann, Castelo, & Guinney, 2013), and the algorithm was ssGSEA. The relevance between SPP1 and infiltrating immune cells at colorectal cancer was studied through TIMER database to explore its possible

mechanism. Statistical deconvolution methods was used through TIMER database to conclude Gene expression profiling of tumor-infiltrating immune cells (B. Li et al., 2016). Moreover, The correlation between SPP1 and tumor purity was definite (Aran, Sirota, & Butte, 2015).

Besides, we discovered the relationship by the TIMER and GEPIA between SPP1 and tumor-infiltrating immune cell genetic markers at colorectal tumor samples，which have been cited in previous studies (Danaher et al., 2017; Siemers et al., 2017). The correlation between SPP1 and immune gene markers remained recognized by using the scatter plots. The correlation coefficient was confirmed via Spearman method. Gene expression levels are shown with Log2TPM.

2.5 Data Analysis

The R package (version 3.6.3) (https://www.r-project.org/) was picked for data analyzing and graphics. Statistical significance of SPP1 in two contrast tissues was respectively assessed by Wilcoxon rank test and paired-sample T-test. Correlations between SPP1 expression and clinical characteristics were also assessed by Wilcoxon rank test and logistic regression.

Survival curves were also generated by Progno Scan, and their results are shown together with HR. The correlation of genes express keep assessed through Spearman statistical significance, with the following absolute guidelines determining their correlation strength: 0.00-0.19 'very weak', 0.20-0.39 'weak', 0.40-0.59“Medium”, 0.60-0.79“Strong”, 0.80-1.0“Very strong”. P values were 2-sided and $p < 0.05$ was considered significant.

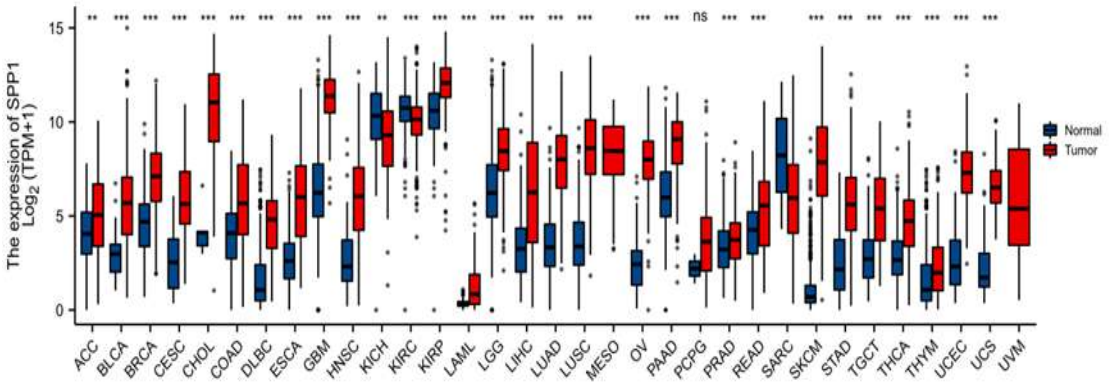
3. Results

3.1 Elevated Expression of SPP1 in Colorectal Cancer

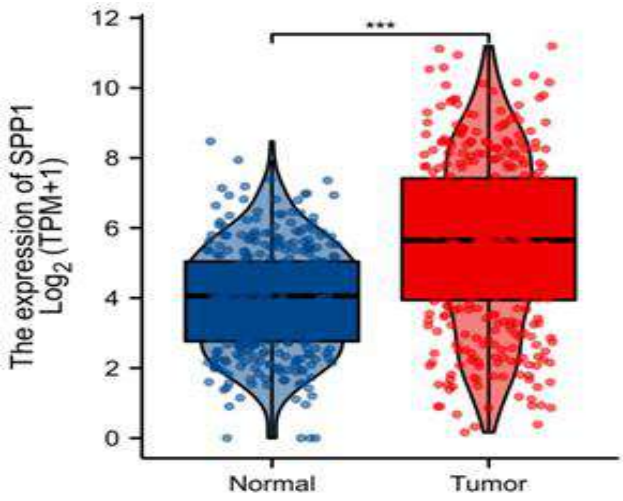
Our cohort included 619 colorectal cancer patients, of whom 50 matched normal tissues searched in the TCGA. Furthermore, for riching our samples, more gene statistics of normal colorectal tissues (n = 308) were collected from the GTEX. SPP1 differential expression in pan-cancer showed SPP1 remained high express in various types of cancers, such as adenocarcinoma, cervical squamous-cell carcinoma, urothelial carcinoma of bladder, cholangiocarcinoma (Figure 1A). SPP1 expression in CRC keep obviously higher than the other ($p<0.001$) (Figure 1B).

Moreover, SPP1 also show high express in 50 pairs of colorectal cancer and the normal matched ($p < 0.001$) (Figure 1C). The ROC curve value was 0.846(CI=0.808-0.885), which suggested that SPP1 expression had good predictive power and was used to distinguish cancer tissues from the normal (Figure 1D). Protein expression data provided by the HPA database were

compared for SPP1 protein expression differences in two different attributes of colorectal tissues by immuno histochemical staining using antibody HPA027541. The conclusion appeared ultimately that SPP1 made high expressing in colorectal cancer, but keep low in regular tissue (Figure 2).



1A



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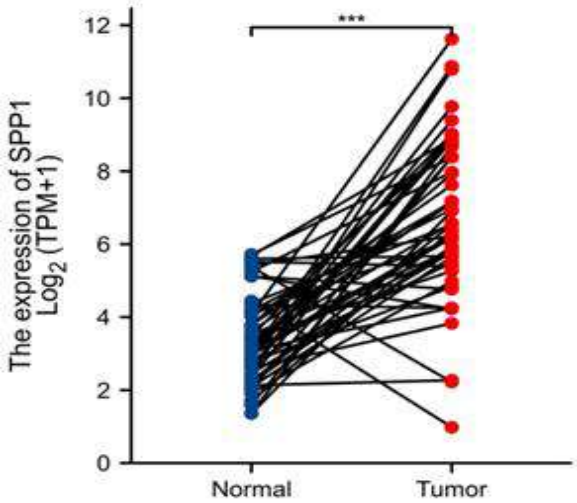

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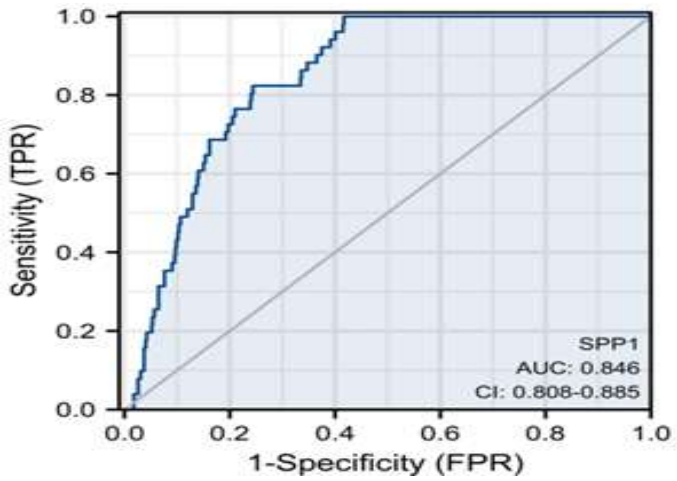


1D

Figure 1: Expression levels of SPP1 in different types of tumors and colorectal cancer. Expression of SPP1 (A) in different types of tumors compared with normal tissues in TCGA and GTEx databases, (B) in colorectal cancer and non-matched normal tissues in the TCGA and GTEx databases, and (C) in colorectal cancer and matched normal tissues in TCGA database. (D) ROC curves for classifying colorectal cancer versus normal colorectal tissues in the TCGA database. TCGA, The Cancer Genome Atlas; GTEx, Genotype Tissue Expression Project; ROC, receiver operating characteristic. *p < 0.05, **p < 0.01, and ***p < 0.001.

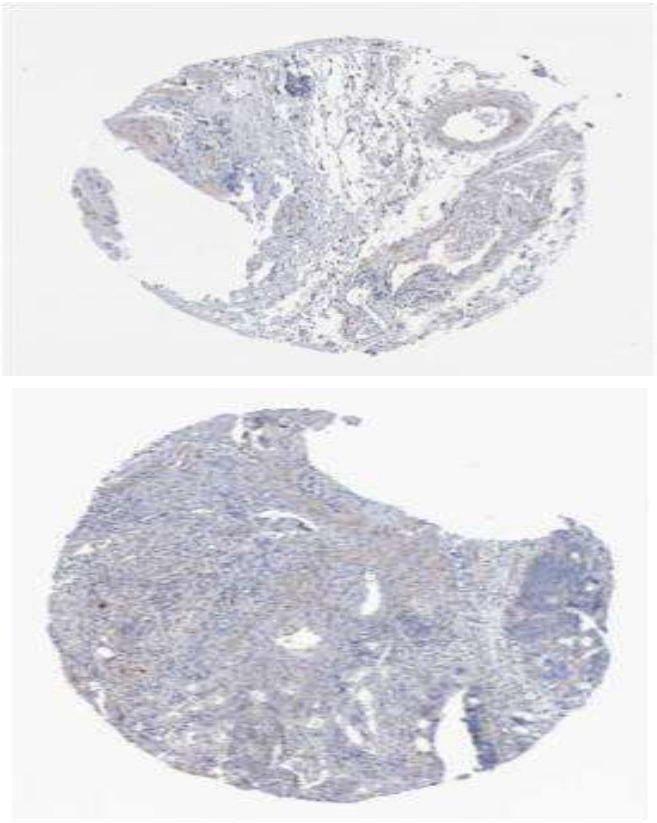
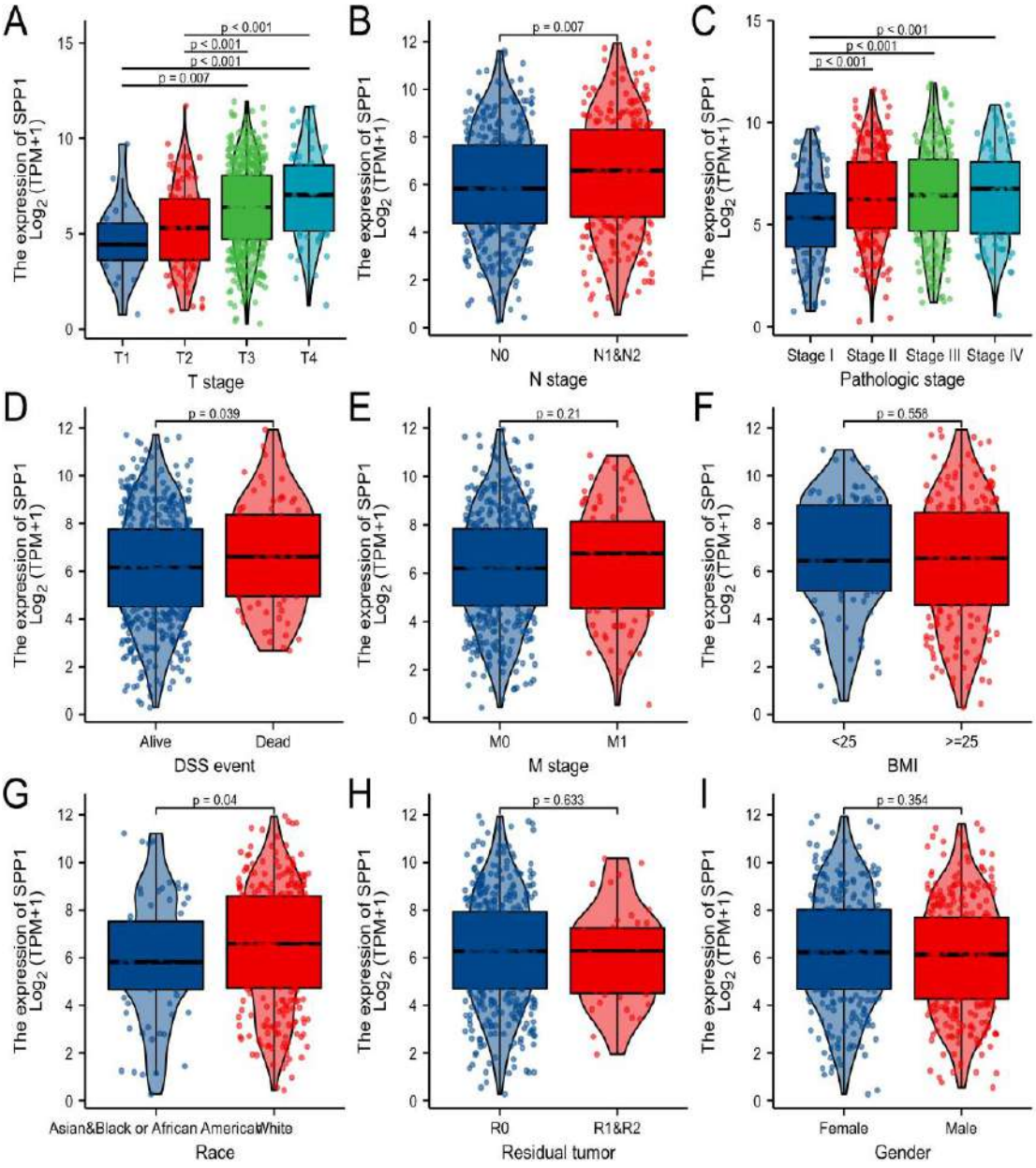


Figure 2: Immunohistochemistry images in normal (left) and colorectal cancer tissues (right) from the Human Protein Atlas (HPA) database. SPP1 protein expression was significantly higher in colorectal cancer tissues than normal tissues.

3.2 Relationship Between SPP1 and Clinico pathologic Variables

Highiy expressed SPP1 at colorectal cancer became significantly correlated with stage T (T 4 versus T 1, p < 0.001; T 4 versus T 2, p < 0.001; T 3 versus T 1, p = 0.007; T 3 versus T 2, p < 0.001), stage N (N 1 & N 2 versus N 0, p = 0.007), pathological stage (IV versus I, p < 0.001; III versus I, p < 0.001; II versus I, p < 0.001), race (p = 0.040), disease-specific survival (p = 0.039) (Figure 3). Furthermore, univariate Logistic regression analysis provided us clinicopathological differences between the two contrast groups, including T stage (odds ratio [OR] = 2.704,95% CI = 1.792-4.142, p < 0.001), N stage (OR = 1.734,95% CI = 1.258-2.397, p < 0.001), pathologic stage (OR = 1.654,95% CI = 1.196-2.292, p = 0.002), race (OR = 1.982,95% CI = 1.194-3.328, p = 0.009), Perineural invasion (OR = 2.000,95% CI = 1.093.3752, p = 0.027) and Lymphatic invasion (OR = 1.432,95% CI = 1.020-2.015, P = 0.038) (Table 1).



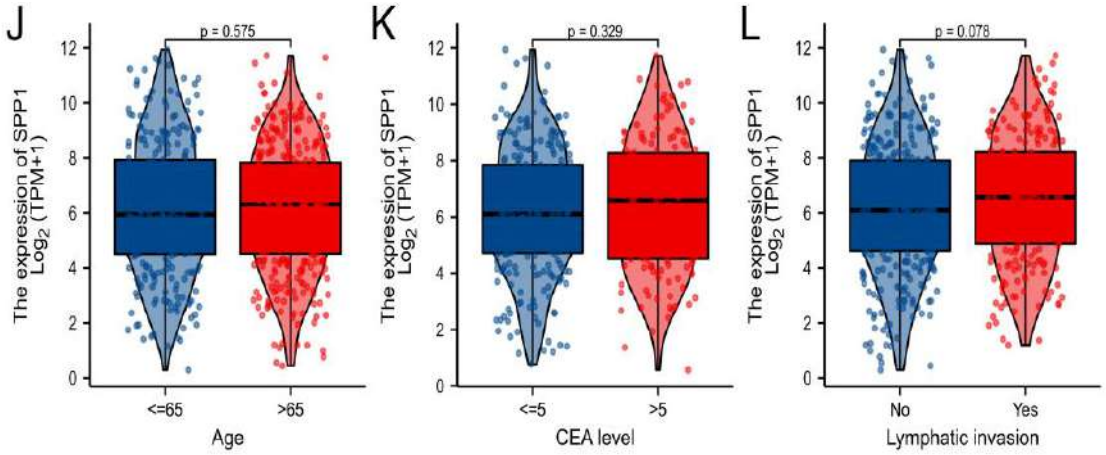


Figure 3: Associations between SPP1 expression and clinicopathological characteristics. Data are shown for (A) T stage; (B) N stage; (C) pathological stage; (D) DSS event; (E) M stage; (F) BMI; (G) Race; (H) Residual tumor; (I) Gender; (J) Age; (K) CEA level; and (L) Lymphatic invasion. DSS, disease-specific survival; BMI, body mass index.

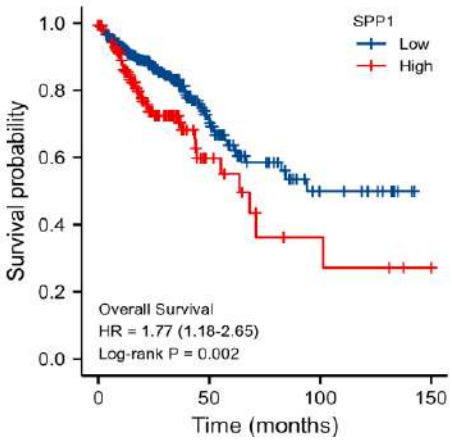
Table 1: Associations of SPP1 expression with clinicopathological characteristics of patients (n = 619).

CHARACTERISTICS	TOTAL(*N*)	ODDS RATIO(*OR*)	*P*
T STAGE (T3&T4 VS. T1&T2)	617	2.704 (1.792-4.142)	<0.001
N STAGE (N1&N2 VS. N0)	616	1.734 (1.258-2.397)	<0.001
M STAGE (M1 VS. M0)	546	1.423 (0.897-2.277)	0.137
PATHOLOGIC STAGE (STAGE III&STAGE IV VS. STAGE I&STAGE II)	599	1.654 (1.196-2.292)	0.002
AGE (>65 VS. <=65)	619	1.258 (0.915-1.731)	0.158
GENDER (MALE VS. FEMALE)	619	0.943 (0.687-1.293)	0.715
RACE (WHITE VS. ASIAN&BLACK OR AFRICAN AMERICAN)	369	1.982 (1.194-3.328)	0.009
RESIDUAL TUMOR (R1&R2 VS. R0)	492	0.907 (0.480-1.714)	0.762
LYMPHATIC INVASION (YES VS. NO)	558	1.432 (1.020-2.015)	0.038
PERINEURAL INVASION (YES VS. NO)	232	2.000 (1.093-3.752)	0.027
NEOPLASM TYPE (RECTUM ADENOCARCINOMA VS. COLON ADENOCARCINOMA)	619	0.887 (0.620-1.267)	0.509
CEA LEVEL (>5 VS. <=5)	397	1.349 (0.895-2.037)	0.153
BMI (>=25 VS. <25)	304	0.874 (0.536-1.419)	0.587
CEA LEVEL, CARCINOEMBRYONIC ANTIGEN LEVEL; BMI, BODY MASS INDEX.			

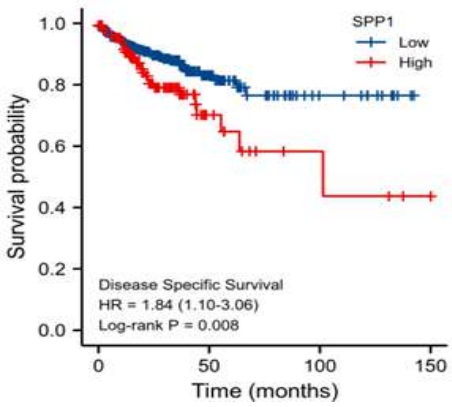
3.3 SPP1 Prognostic in Colorectal Cancer

We studied their relevance between SPP1 and its prognosis of such cancer patients. The sufferers were put into SPP1 highly expressed group and the other by the optimal cut point. Then, the Kaplan-meier means keep take to calculate the correlation between SPP1 and the colorectal cancer patient's

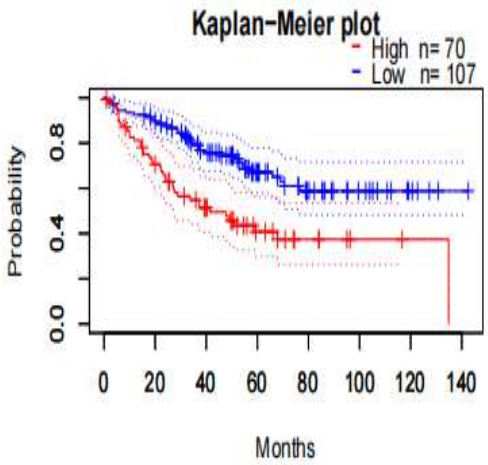
prognosis. The conclusions reveal the overall and disease-specific survival at highly expressed group were seriously worse than that in its contrast group (OS: hazard ratio [HR]=1.77,95%CI=1.18-2.65, p=0.002; DS: HR=1.84,95%CI=1.10-3.06, p=0.008) (Figure 4A、B). To furtheriy explore SPP1 prognostic condition at colorectal cancer, SPP1 prognosis expression was assist by the Progno Scan. Notably, the results reminded SPP1 significantly influenced the sufferer's prognosis. There were 177 and 226 samples in different stages for two cohorts (GSE17536, GSE14333) (Jorissen et al., 2009; Smith et al., 2010) in the Progno Scan database , then the results indicated that highly expressed one came associated with poorer prognosis(OS HR = 1.23, 95% CI = 1.03-1.46, Cox P = 0.020；DSS HR = 1.33，95% CI = 1.08-1.64，Cox P = 0.008；disease free survival [DFS] HR = 1.56，95% CI = 1.19 -2.05，Cox P = 0.001；DFS HR = 1.40，95% CI = 1.14 -1.71，Cox P = 0.001)(Figure 4C-F). Taken together, we can imagine that high SPP1 expression came a risk factor independently for poor prognosis among the sufferers. Meanwhile, the clinical relevance of SPP1 to patients with colorectal cancer was discovered through a multivariable Cox proportional hazards model, suggesting that SPP1 can be made as an independent prognostic marker with colorectal cancer (HR = 1.459; 95% CI, 1.030-2.067; P = 0.033;) (Table 2).



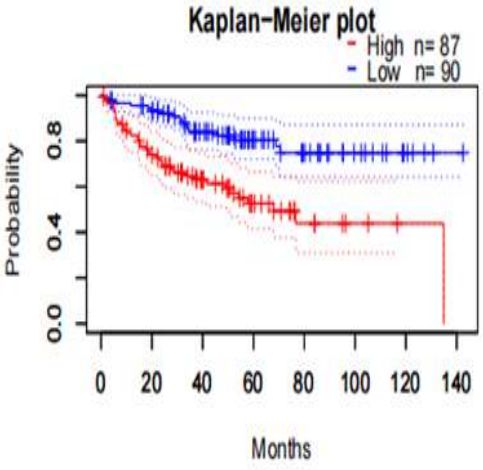
A: OS



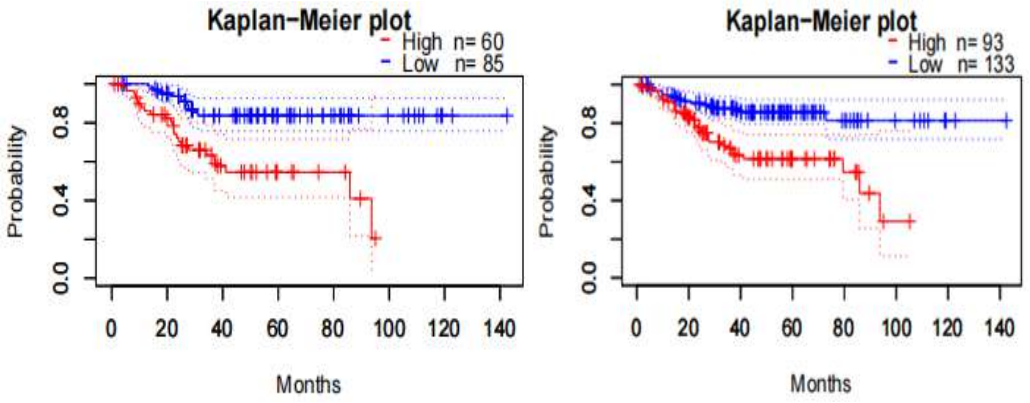
B: DSS



C: GSE17536, OS, HR=1.23, COX p=0.020



D: GSE17536, DSS, HR=1.33, COX p=0.008



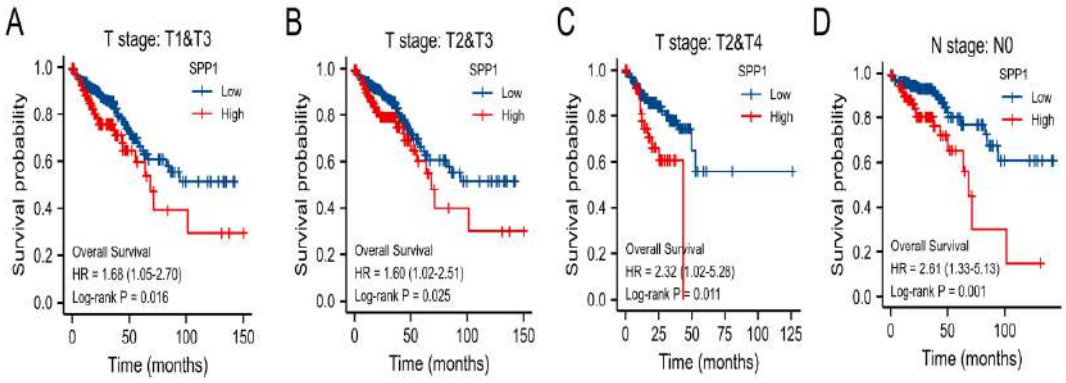
E: GSE17536, DFS, HR=1.56, COX p=0.001 **F:** GSE14333, DFS, HR=1.40, COX p=0.001

Figure 4: Kaplan-Meier survival curves comparing the high and low expression of SPP1 in colorectal cancer in the Kaplan-Meier method (A, B) and the Progno Scan database (C–F). (A, B) Survival curves of OS and DSS in the Kaplan-Meier method(n=619). (C–F) Survival curves of OS, DSS, and DFS in two colorectal cancer cohorts [GSE17536 (n = 177) and GSE14333 (n = 226)]. OS, overall survival; DSS, disease-specific survival; DFS, disease-free survival.

Table 2: Multivariate Cox proportional hazard regression analyses of the relationship between clinic pathological characteristics and overall survival in CRC from the Tumor Immune Estimation Resource (TIMER).

VARIABLES	HR	95% CI	P
AGE	1.150	1.040-1.272	**0.007**
GENDER (MALE)	0.742	0.152-3.629	0.712
STAGE2	0.097	0.007-1.290	0.077
STAGE3	0.404	0.053-3.093	0.383
STAGE4	0.430	0.057-3.218	0.411
PURITY	4.268	0.109-166.986	0.438
SPP1	1.459	1.030-2.067	**0.033**

Secondly, whether it's OS or DSS, patients with SPP1-overexpressing colorectal cancer had a significantly worse prognosis in the T1 and T3, T2 and T3, T2 and T4, N0, M0, over lapping stages, Residual tumor (-) and Lymphatic invasion (+) (all $p < 0.05$) (Figure 5).



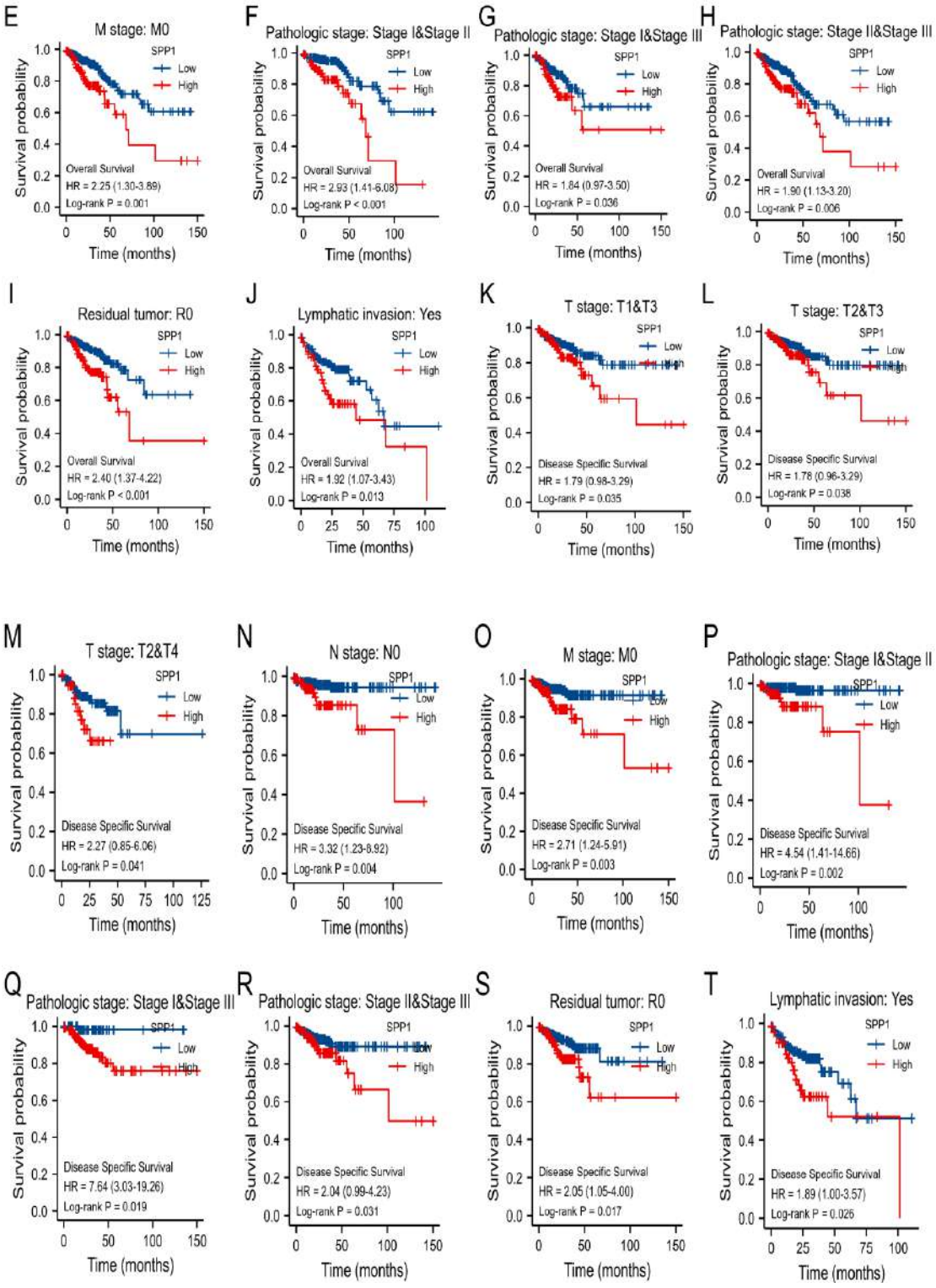
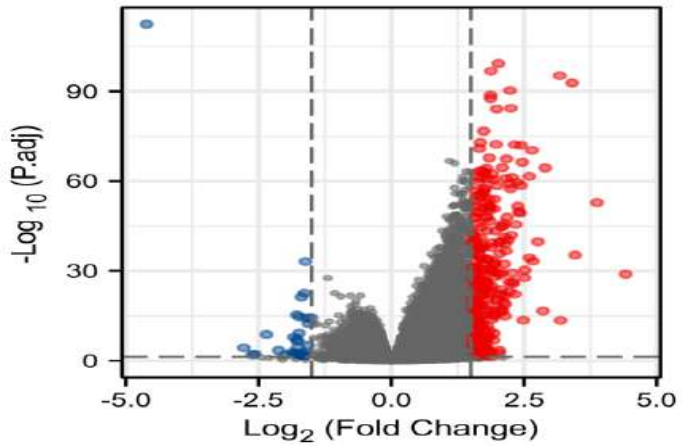


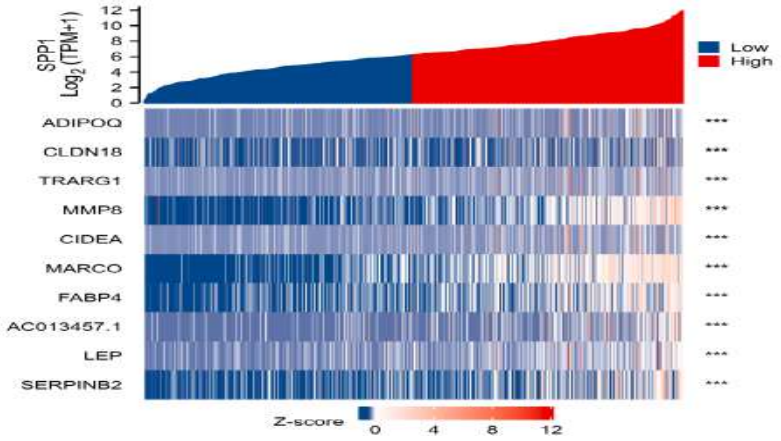
Figure 5: Prognostic values of SPP1 expression in patients with colorectal cancer evaluated by the Kaplan-Meier method in different subgroups. (A–J) OS survival curves of T1 and T3, T2 and T3, T2 and T4, N0, M0, stage I and II, stage I and III, stage II and III, Residual tumor (-) and Lymphatic invasion (+) between high- and low-SPP1 patients with colorectal cancer. (K–T) DSS survival curves of T1 and T3, T2 and T3, T2 and T4, N0, M0, stage I and II, stage I and III, stage II and III, Residual tumor (-) and Lymphatic invasion (+) between high- and low-SPP1 patients with colorectal cancer. OS, overall survival; DSS, disease-specific survival.

3.4 Identification of DEGs in Colorectal Cancer and Results of Functional Enrichment Analysis

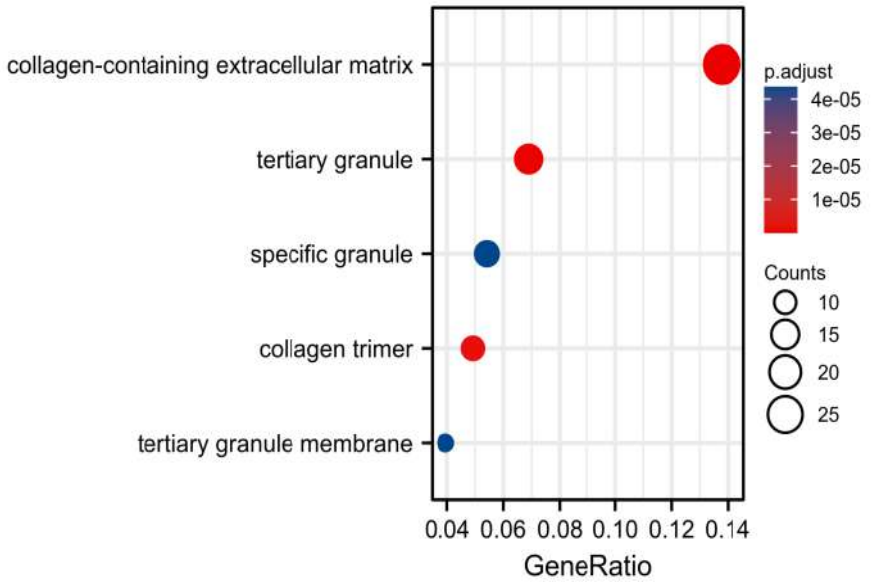
There were 330 DEGs in two contrast expressing groups, with 299(90.6%) upregulated genes and 31(9.4%) downregulated genes (adjusted p< 0.05, |log2-fc|>1.5) (Figure 6A). Their relationships between top 10 DEGs (including ADIPOQ, CLDN18, TRARG1, MMP8, CIDEA, Marco, FABP4, AC013457.1, LEP, and SERPINB2) and SPP1 are shown in Figure 6B. Setting p. adj < 0.1 and q < 0.2, the differentially expressing genes contained 253 bioprocess,57 cellular ingredients, 62 molecular effections and 7 KEGG, the first five information among them are showed by bubble graph. GO enrichment analysis indicated DEGs became enriched involved in collagen-containing extracellular matrix, extracellular organization, receptor ligand activity, or glycosaminoglycan binding (Figures 6C-E). Furthermore, KEGG pathway study showed that its pathways significantly enriched in DEGs mainly included phagosomes, Cytokine-Cytokine receptor interactions (Figure 6F). Next, we carried out GSEA between the two contrast parts and discovered the high was specialized enriched in immune-related biological development, suggesting that high SPP1 expression leads to an increased immuno phenotype in colorectal cancer (Figures 7A-D).



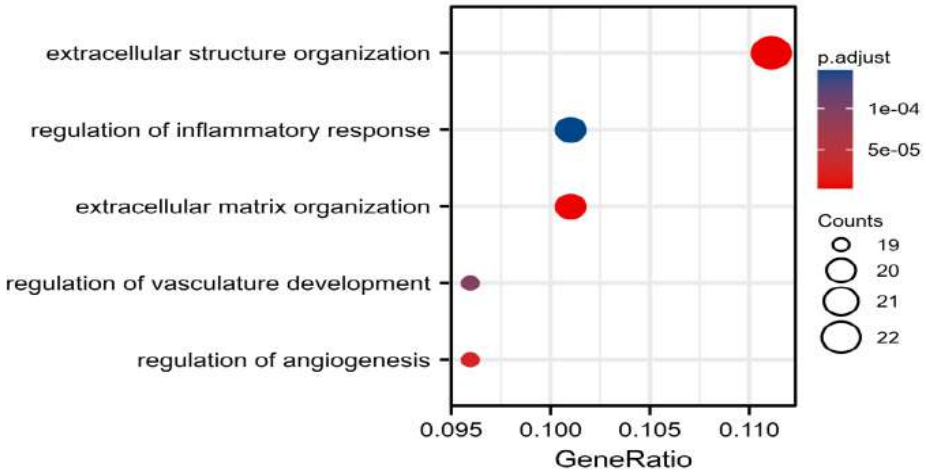
A



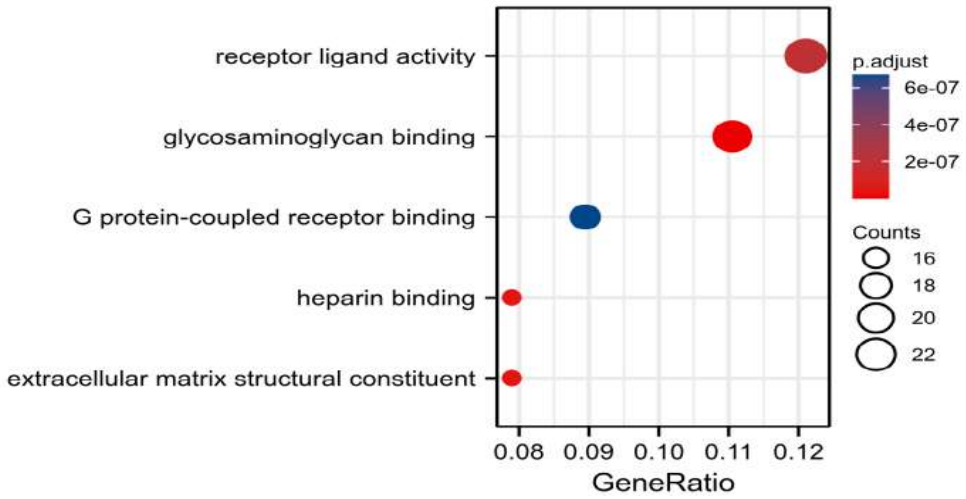
B


collagen-containing extracellular matrix
tertiary granule
specific granule
collagen trimer
tertiary granule membrane
0.04 0.06 0.08 0.10 0.12 0.14
GeneRatio
p.adjust
4e-05
3e-05
2e-05
1e-05
Counts
10
15
20
25

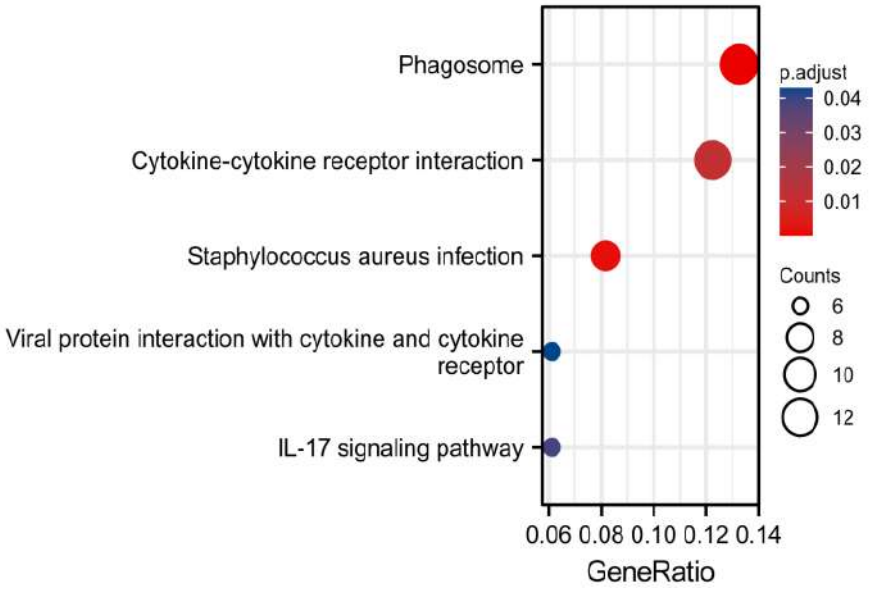
C: (cc)


extracellular structure organization
regulation of inflammatory response
extracellular matrix organization
regulation of vasculature development
regulation of angiogenesis
0.095 0.100 0.105 0.110
GeneRatio
p.adjust
1e-04
5e-05
Counts
19
20
21
22

D: (bp)

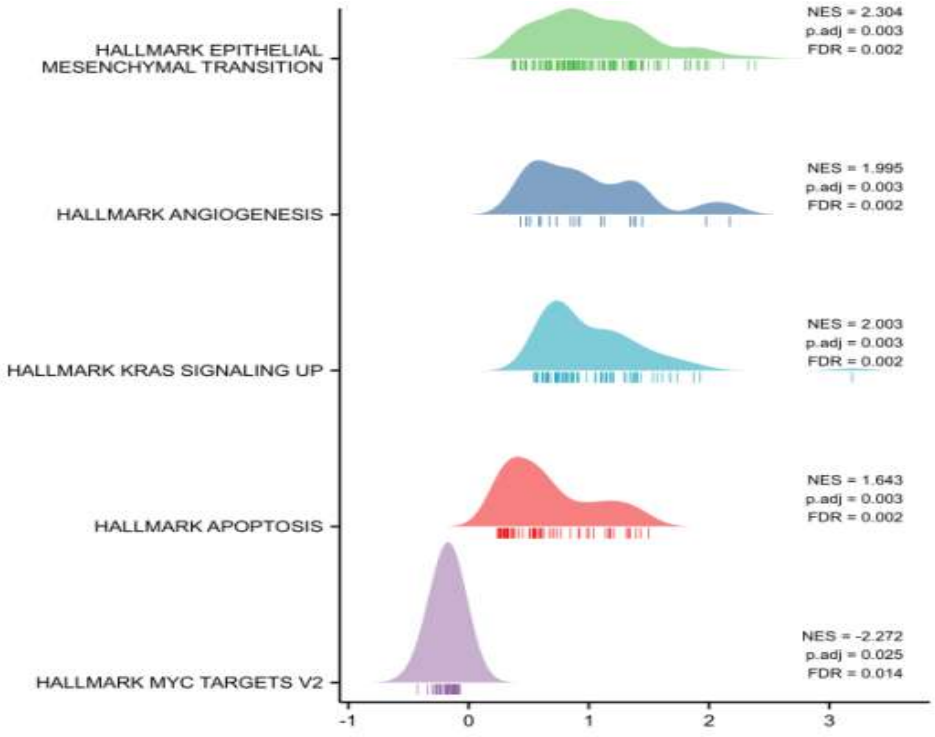

receptor ligand activity
glycosaminoglycan binding
G protein-coupled receptor binding
heparin binding
extracellular matrix structural constituent
0.08 0.09 0.10 0.11 0.12
GeneRatio
p.adjust
6e-07
4e-07
2e-07
Counts
16
18
20
22

E: (mf)

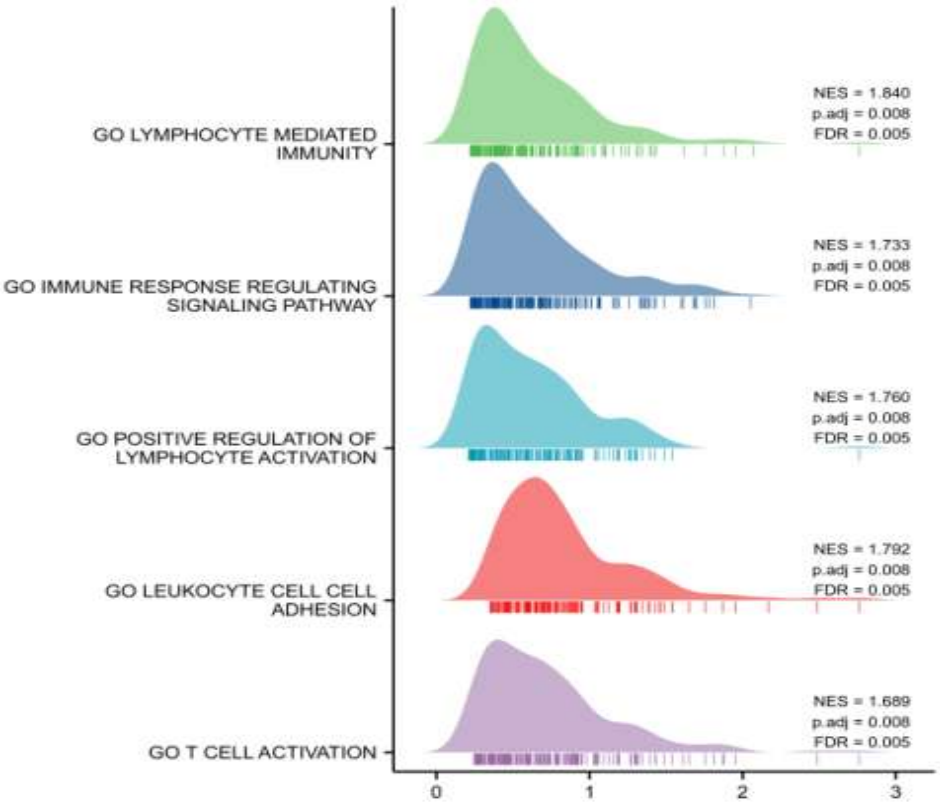


F: (kegg)

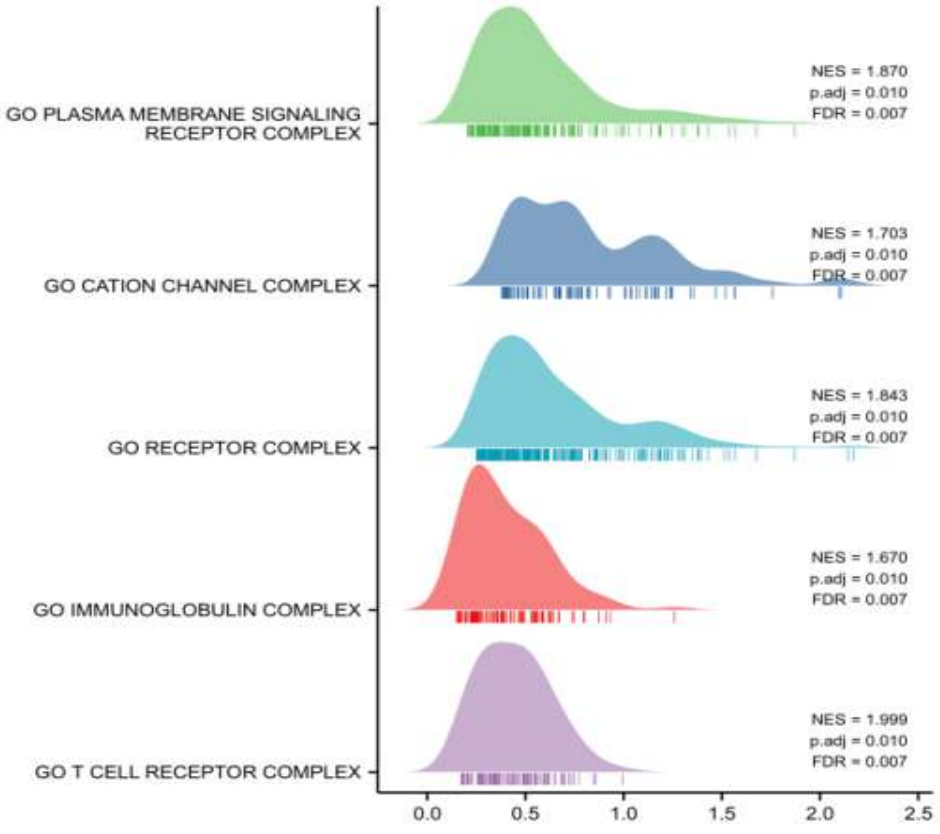
Figure 6: SPP1-related differentially expressed genes (DEGs) and functional enrichment analysis of SPP1 in colorectal cancer using GO and KEGG. (A) Volcano plot of DEGs. Blue and red dots indicate the significantly down-regulated and up-regulated DEGs, respectively. (B) Heatmap of correlation between SPP1 expression and the top 10 DEGs. (C–E) Enrichment analysis of GO terms for SPP1-related DEGs. (F) Enrichment analysis of KEGG terms for terms for SPP1-related DEGs. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.



A


GO LYMPHOCYTE MEDIATED IMMUNITY
NES = 1.840
p.adj = 0.008
FDR = 0.005
GO IMMUNE RESPONSE REGULATING SIGNALING PATHWAY
NES = 1.733
p.adj = 0.008
FDR = 0.005
GO POSITIVE REGULATION OF LYMPHOCYTE ACTIVATION
NES = 1.760
p.adj = 0.008
FDR = 0.005
GO LEUKOCYTE CELL CELL ADHESION
NES = 1.792
p.adj = 0.008
FDR = 0.005
GO T CELL ACTIVATION
NES = 1.689
p.adj = 0.008
FDR = 0.005
0
1
2
3

B


GO PLASMA MEMBRANE SIGNALING RECEPTOR COMPLEX
NES = 1.870
p.adj = 0.010
FDR = 0.007
GO CATION CHANNEL COMPLEX
NES = 1.703
p.adj = 0.010
FDR = 0.007
GO RECEPTOR COMPLEX
NES = 1.843
p.adj = 0.010
FDR = 0.007
GO IMMUNOGLOBULIN COMPLEX
NES = 1.670
p.adj = 0.010
FDR = 0.007
GO T CELL RECEPTOR COMPLEX
NES = 1.999
p.adj = 0.010
FDR = 0.007
0.0
0.5
1.0
1.5
2.0
2.5

C

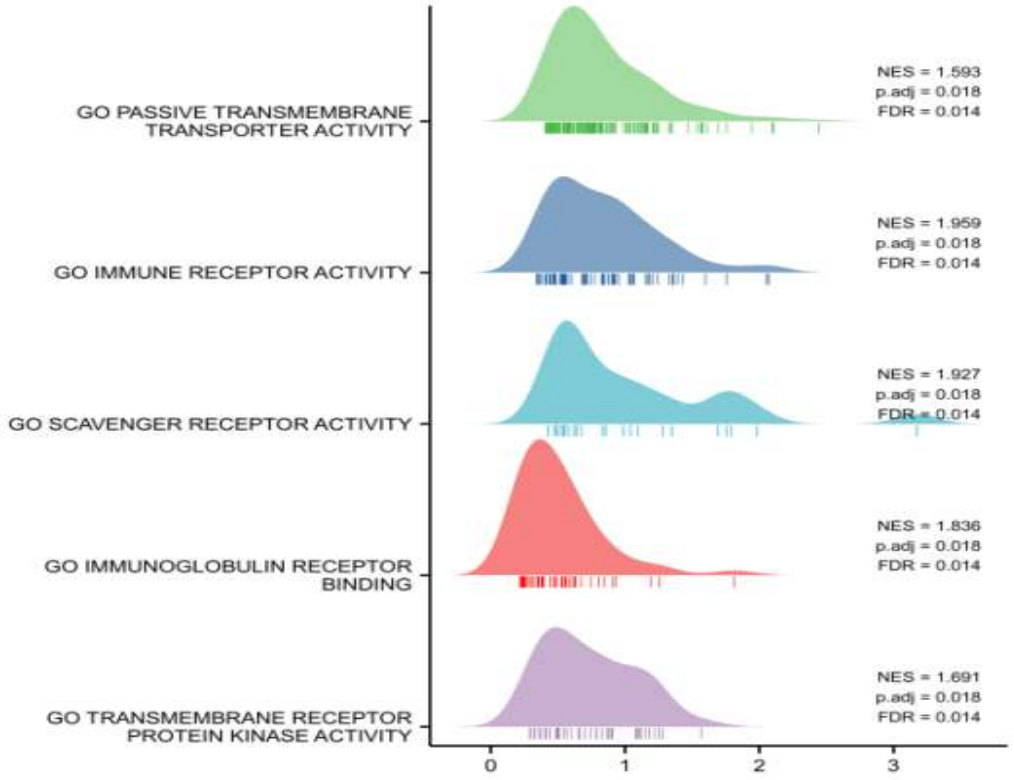


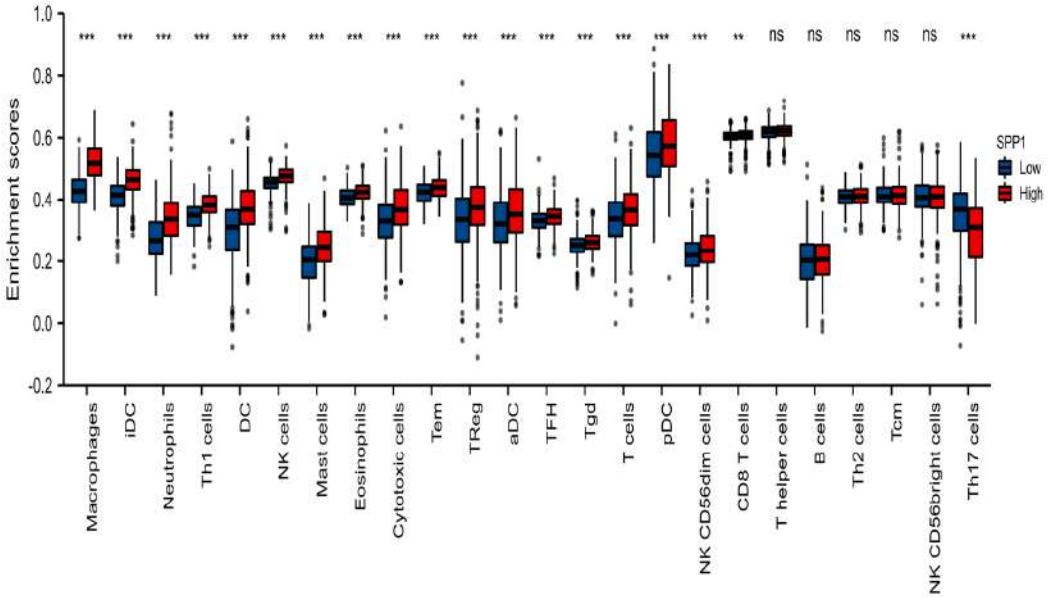
Figure 7: Gene set enrichment analysis (GSEA) of DEGs. (A) GSEA analysis of the Hallmark gene sets deposited in MSigDB. (B) GSEA analysis of the bp of Gene Ontology gene sets downloaded from MSigDB. (C) GSEA analysis of the cc of Gene Ontology gene sets downloaded from MSigDB. (D) GSEA analysis of the mf of Gene Ontology gene sets downloaded from MSigDB. GSEA, gene set enrichment analysis; DEGs, differentially expressed genes; MSigDB, Molecular Signatures database; NES, normalized enrichment score; FDR, false discovery rate.

3.5 Associations Between SPP1 and Tumor Immune Infiltrating Cells

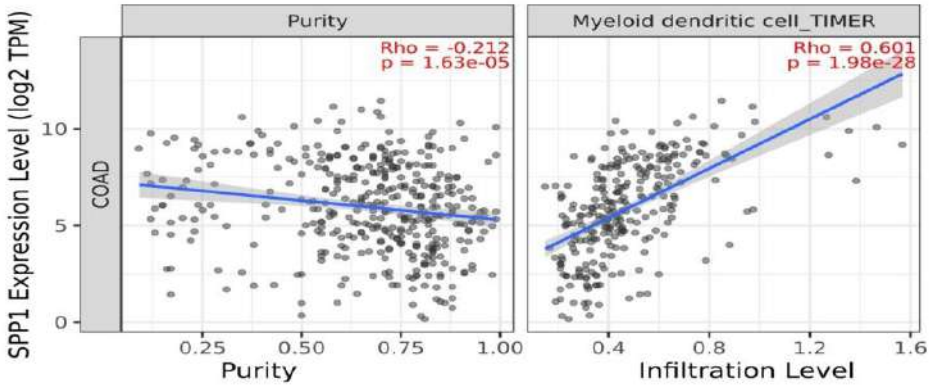
Tumor infiltrating lymphocytes can measure independently sentinel lymphnode state and cancer survival (Azimi et al., 2012; Ye et al., 2019). The immunein filtration between two expression groups was studied using SSGSEA algorithm. The conclusion showed that contrast in the low-expression one, DC ($p<0.001$)、macrophages ($p<0.001$)、neutrophils ($p<0.001$)、NK cells ($p<0.001$)、mast cells ($p<0.001$)、eosinophils ($p<0.001$)、cytotoxic cells ($p<0.001$)、Th1 cells ($p<0.001$)、Tem ($p<0.001$)、TReg ($p<0.001$)、TFH ($p<0.001$)、Tgd ($p<0.001$)、T cells ($p<0.001$)、aDC ($p<0.001$)、iDC($p<0.001$) 、pDC ($p<0.001$)、NK CD56dim cells ($p<0.001$)、CD8 T cells ($p=0.003$) became observably increased in the highly expressed one, however, Th17($p<0.001$) became obviously reduced (Figure 8A).

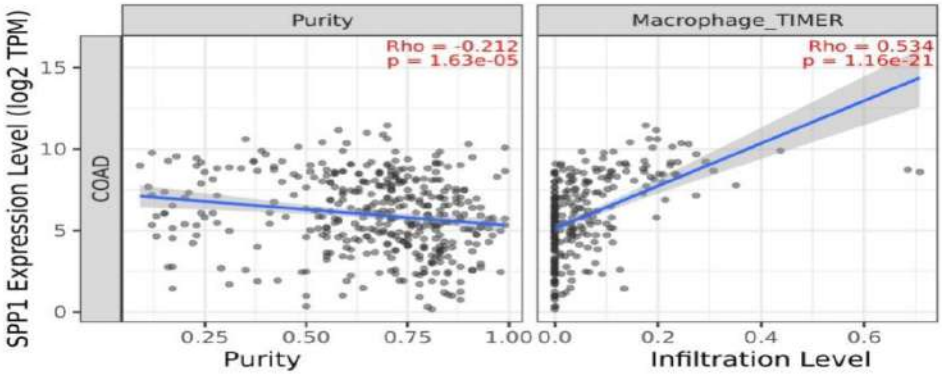
We also used the TIMER for analyzing their association including SPP1 and tumor infiltrating lymphocytes at colorectal cancer samples. After the

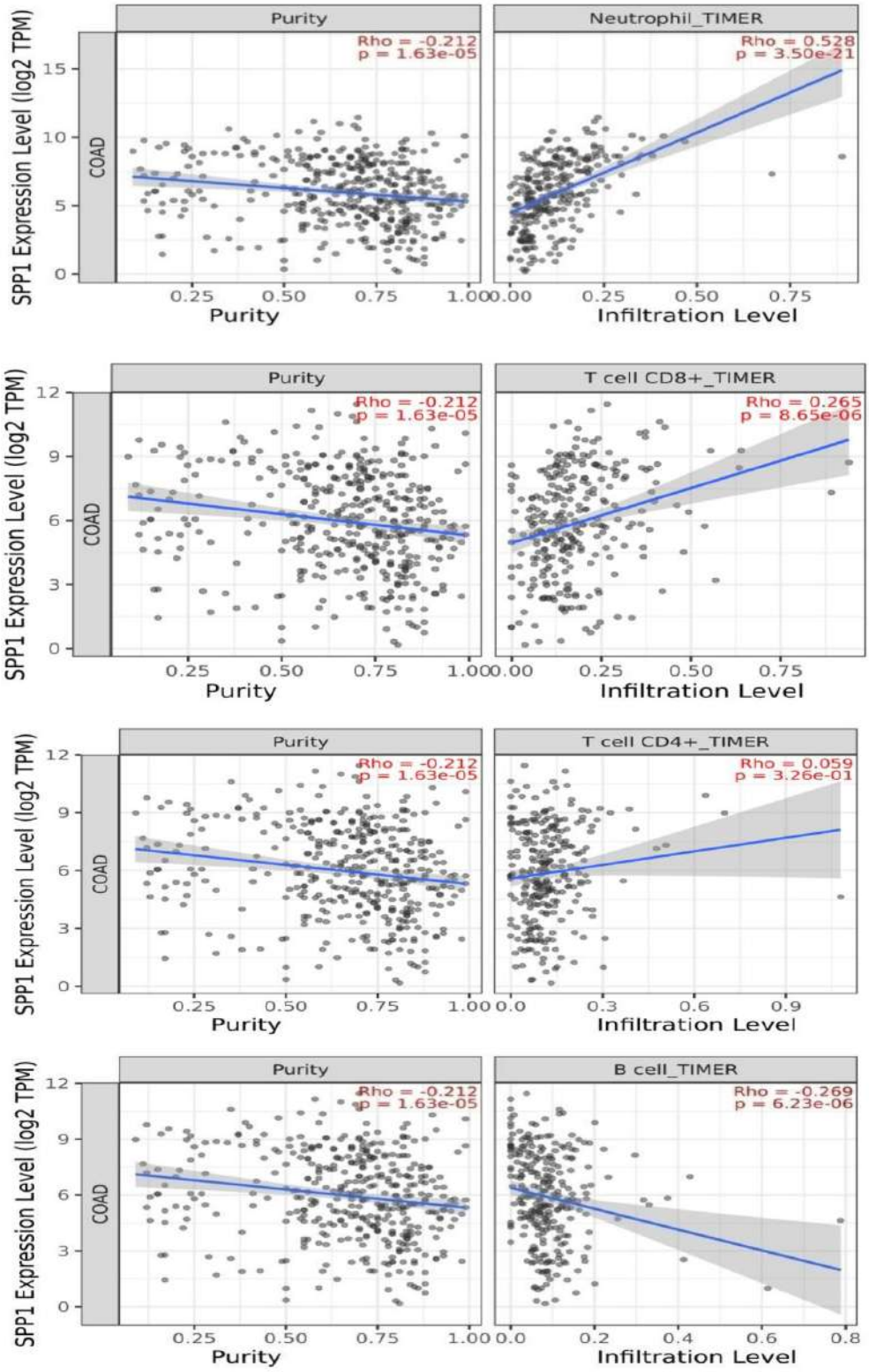
discovery， the SPP1 became positively correlated with their infiltration conditions for DC (r=0.601, p=1.98E-28), macrophages (r=0.534, p=1.16E-21, neutrophils（r=0.528, p= 3.50E-21）and CD8+ T cell（r=0.265, p= 8.65E-06）, this was then inversely correlated with B cell (r = –0.269, p = 6.23E-06) infiltration levels (Figure 8B).



A







B

Figure 8: Associations between SPP1 and Tumor Immune Infiltrating Cells. (A) Changes of 24 immune cell subtypes between high and low SPP1 expression groups in colorectal cancer. (B) Association between the expression level of SPP1 and immuno filtration cells (including dendritic cells, macrophages, neutrophils, CD8+ T cells, CD4+ T cells and B cells) in colorectal cancer. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, no significant.

3.6 Association assessment between SPP1 and gene markers of immune cells

By way of furtherly exploring its association between SPP1 and tumor immune infiltrating cells, we examined furthermore TIMER and GEPIA databases. Coincidentally, its result indicates that their expression levels in most of gene markers made significant related with SPP1 at colorectal cancer (Table3). Particularly, we found that CD86, CD115 of Monocyte, chemokine (C-C motif) ligand (CCL) -2, CD68, IL10 of Tams, PTGS2, IRF5 of M 1phenotype, CD163, VSIG4 and MS4A4A of M 2phenotype were associated to a large extent with SPP1 in colorectal cancer (all $p < 0.0001$; Figure 9A-D). In combination with the above findings, SPP1 was considered to participate in the control of macrophage polarization in colorectal cancer. It was still recognized that DC gene markers such as HLA-DPB1 remained significantly correlated with SPP1. Moreover, there were significant associations between SPP1 and gene markers of Treg and T cell exhaustion in colorectal cancer (Table 3). Previous relevant studies have shown that DC was capable of advancing tumor metastasis (Sawant et al., 2012). Whether SPP1 made a main role in the mechanism of DC and tumor metastasis needs further study. The genetic marker Foxp3 in Treg cells remained a key link in suppressing cytotoxic T cells that attack tumor cells (Facciabene, Motz, & Coukos, 2012).

Table 3(a): Correlation analysis between SPP1 and immune cell marker gene in TIMER and GEPIA database.

CELL TYPE	GENE MARKERS	TIMER				GEPIA	
		PURITY		TUMOR		TUMOR	
		Rho	*p*	Rho	*p*	Rho	*p*
CD8+ T CELL	CD8A	0.215	***	0.277	***	0.25	***
	CD8B	0.132	**	0.171	**	0.13	0.032
T CELL (GENERAL)	CD3D	0.104	0.036	0.194	***	0.21	**
	CD3E	0.174	**	0.254	***	0.29	***
	CD2	0.180	**	0.254	***	0.29	***
B CELL	CD19	-0.035	0.488	0.060	0.203	0.071	0.24
	CD79A	-0.004	0.933	0.109	0.020	0.17	*
MONOCYTE	CD86	0.625	***	0.649	***	0.67	***
	CD115(CSF1R)	0.544	***	0.592	***	0.63	***
TAM	CCL2	0.541	***	0.585	***	0.6	***
	CD68	0.511	***	0.538	***	0.6	***
	IL10	0.481	***	0.481	***	0.5	***

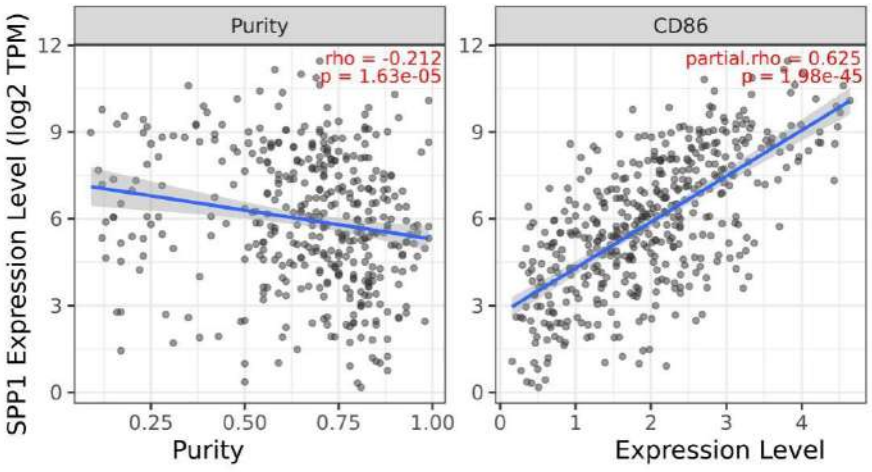
Table 3(b): Correlation analysis between SPP1 and immune cell marker gene in TIMER and GEPIA database.

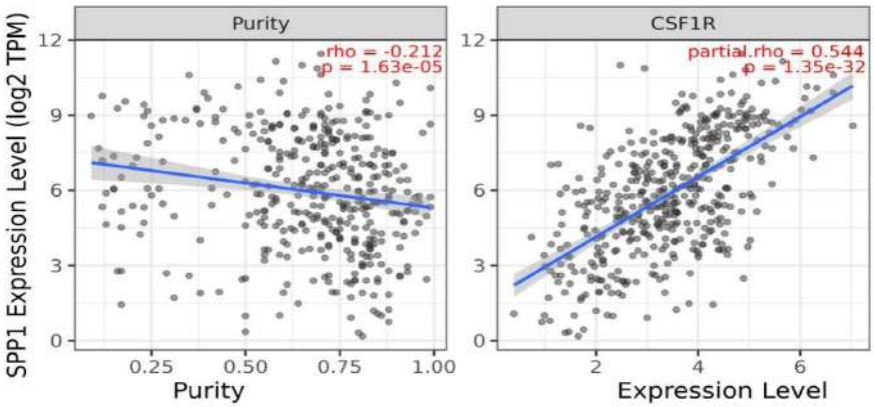
CELL TYPE	GENE MARKERS	TIMER				GEPIA	
		PURITY		TUMOR		TUMOR	
		Rho	*p*	Rho	*p*	Rho	*p*
M1 MACROPHAGE	INOS(NOS2)	-0.174	**	-0.143	*	-0.079	0.19
	IRF5	0.244	***	0.230	***	0.27	***
	COX2(PTGS2)	0.255	***	0.302	***	0.33	***
M2 MACROPHAGE	CD163	0.651	***	0.675	***	0.72	***
	VSIG4	0.665	***	0.688	***	0.7	***
	MS4A4A	0.635	***	0.659	***	0.68	***
NEUTROPHILS	CD66b(CEACAM8)	-0.076	0.124	-0.108	0.021	-0.16	*
	CD11b(ITGAM)	0.687	***	0.701	***	0.75	***
	CCR7	0.086	0.084	0.182	***	0.27	***
NATURAL KILLER CELL	KIR2DL1	0.178	**	0.210	***	0.21	**
	KIR2DL3	0.189	**	0.209	***	0.23	***
	KIR2DL4	0.150	*	0.195	***	0.13	0.029
	KIR3DL1	0.244	***	0.283	***	0.02	**
	KIR3DL2	0.175	**	0.211	***	0.25	***
	KIR3DL3	0.070	0.161	0.074	0.112	0.09	0.14
	KIR2DS4	0.150	*	0.183	***	0.18	*
DENDRITIC CELL	HLA-DPB1	0.436	***	0.479	***	0.51	***
	HLA-DQB1	0.271	***	0.339	***	0.32	***
	HLA-DRA	0.397	***	0.450	***	0.45	***
	HLA-DPA1	0.404	***	0.459	***	0.46	***
	BDCA-1(CD1C)	0.141	*	0.222	***	0.29	***
	BDCA-4(NRP1)	0.617	***	0.647	***	0.7	***
	CD11C(ITGAX)	0.644	***	0.664	***	0.69	***
TH1	T-bet(TBX21)	0.205	***	0.260	***	0.32	***
	STAT4	0.209	***	0.265	***	0.28	***
	STAT1	0.284	***	0.324	***	0.34	***
	IFN-γ(IFNG)	0.143	*	0.176	**	0.19	*
	TNF-α(TNF)	0.209	***	0.242	***	0.33	***

Table 3(c): Correlation analysis between SPP1 and immune cell marker gene in TIMER and GEPIA database.

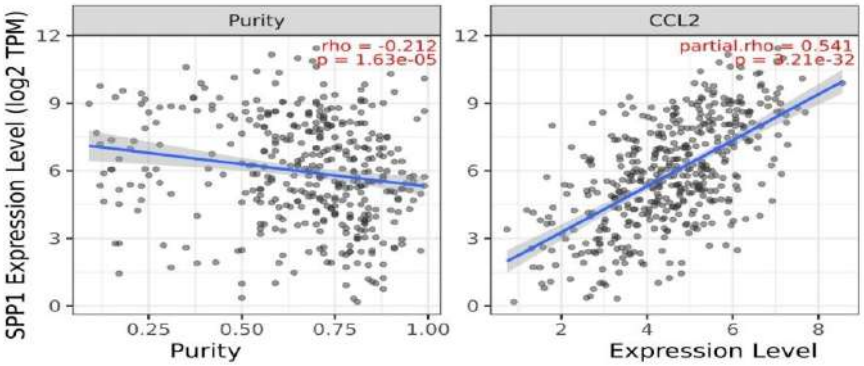
CELL TYPE	GENE MARKERS	TIMER				GEPIA	
		PURITY		TUMOR		TUMOR	
		Rho	*p*	Rho	*p*	Rho	*p*
TH2	GATA3	0.227	***	0.276	***	0.34	***
	STAT6	-0.047	0.341	-0.015	0.742	0.046	0.45
	STAT5A	0.186	**	0.196	***	0.31	***
	IL13	0.202	***	0.260	***	0.25	***
TFH	BCL6	0.416	***	0.459	***	0.54	***
	IL21	0.143	*	0.192	***	0.2	**
TH17	STAT3	0.175	**	0.225	***	0.33	***
	IL17A	-0.335	***	-0.303	***	-0.35	***
TREG	FOXP3	0.306	***	0.369	***	0.42	***
	CCR8	0.358	***	0.417	***	0.47	***
	STAT5B	0.099	0.047	0.112	0.016	0.23	**
	TGFβ(TGFB1)	0.532	***	0.569	***	0.62	***
T CELL EXHAUSTION	PD-1(PDCD1)	0.215	***	0.281	***	0.33	***
	CTLA4	0.269	***	0.320	***	0.4	***
	LAG3	0.218	***	0.286	***	0.25	***
	TIM-3(HAVCR2)	0.673	***	0.684	***	0.71	***
	GZMB	0.022	0.666	0.058	0.219	0.023	0.71

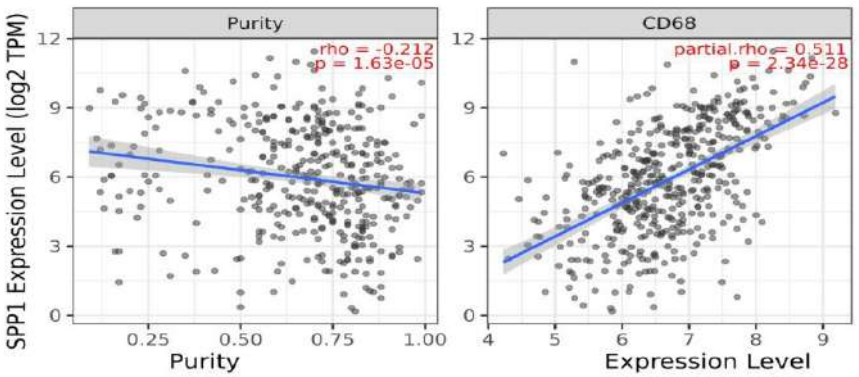
*PURITY, CORRELATION ADJUSTED BY PURITY; TAM, TUMOR-ASSOCIATED MACROPHAGE; TH, T HELPER CELL; TFH, FOLLICULAR HELPER T CELL; TREG, REGULATORY T CELL. (*P < 0.01, **P < 0.001, ***P < 0.0001).*

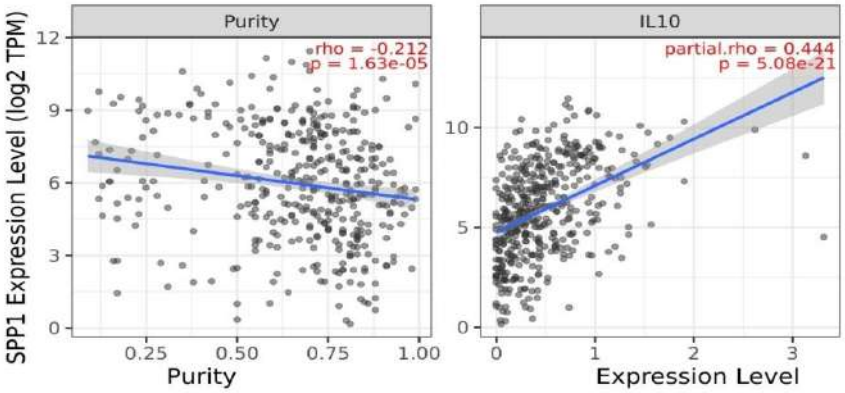




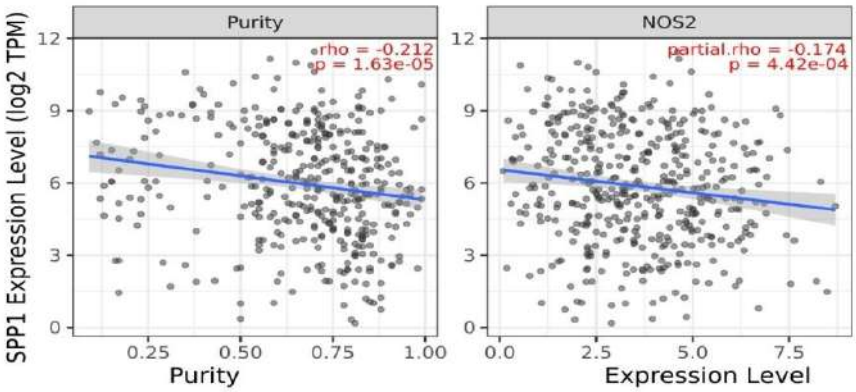
Monocytes

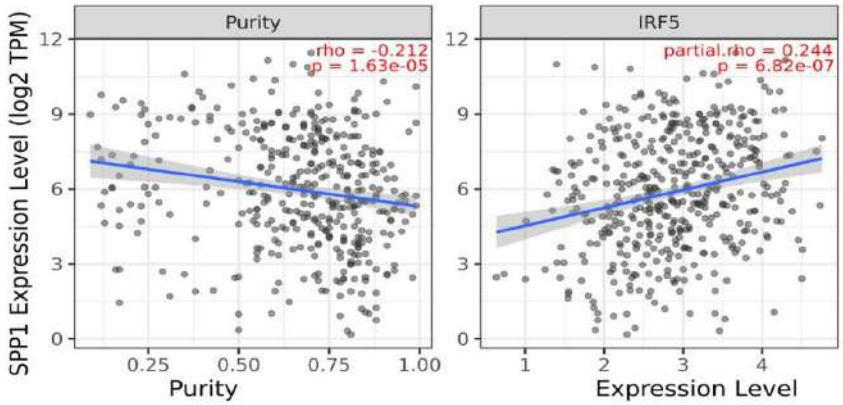


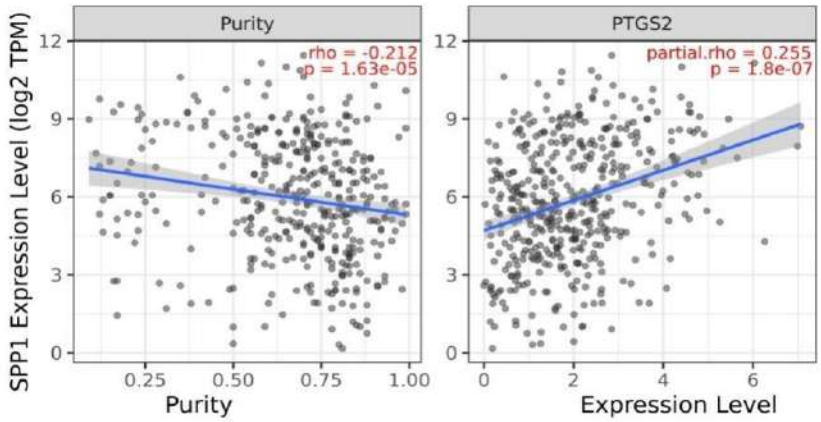




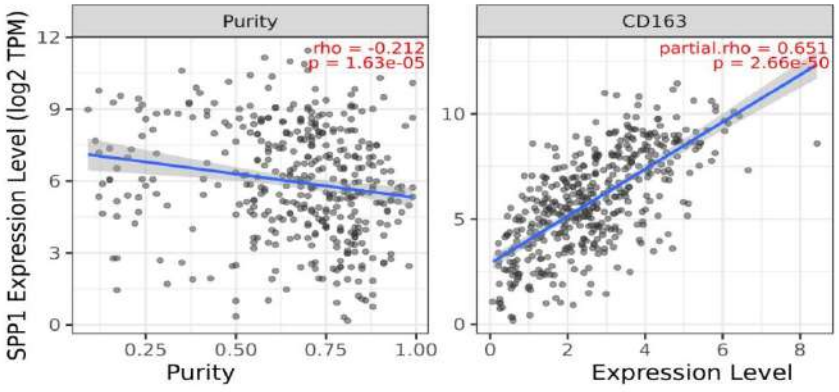
TAMs

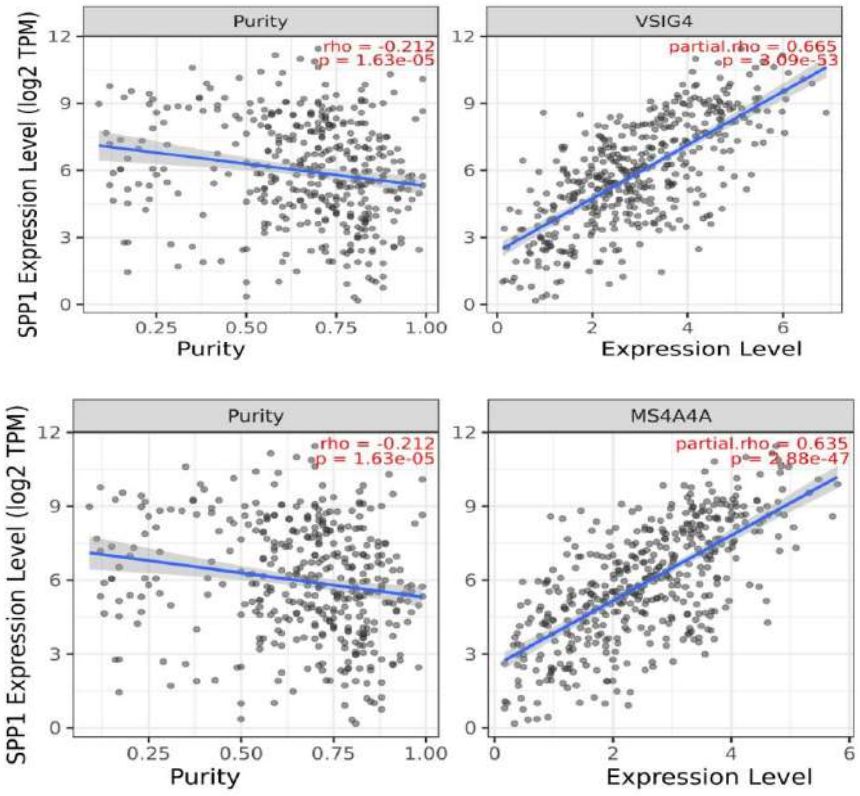






M1 macrophages





M2 macrophages

Figure 9: SPP1 expression correlated with macrophage polarization in colorectal cancer. Markers include CD86 and CSF1R of monocytes; CCL2, CD68, and IL10 of TAMs (tumor-associated macrophages); NOS2, IRF5, and PTGS2 of M1 macrophages; and CD163, VSIG4, and MS4A4A of M2 macrophages. (A–D) Scatterplots of correlations between SPP1 expression and gene markers of monocytes (A), TAMs (B), and M1 (C) and M2 macrophages (D) in colorectal cancer.

4. Discussion

SPP1, as well as called Osteopontin, belongs to a glycosylated phosphoprotein containing abundant acidic amino acid that is involved in the regulation of various carcinogenic and angiogenic factors, it is related to promoting tumor development. Multiple researches indicated that SPP1 attributed related to the evolving of malignant tumors to a large extent, and its expression is rising in esophageal cancer, gastric and lung cancer. SPP1 expression was significantly increased in high malignant tumors with the development of tumor and was identified as a prognostic factor (L.-z. Chen et al., 2018; Guo et al., 2020; Lin et al., 2015; Zhao, Ma, & Zhang, 2022). SPP1 remains high express at plasma and tumor tissues of head and neck cancer, which is closely related to high malignancy and poor prognosis (Qin et al., 2018). Thus, SPP1, which remains high expression in multitudinous tumors and closely relevance with tumor development, can be identified as a prognostic biomarker and regarded as a potential factor to cancer-targeted therapeutic method. Nevertheless, few research has been done on SPP1 in colorectal

cancer. In this project, we sought to explore the potential mechanisms by which SPP1 promotes colorectal cancer progression and to determine whether it can serve as a prognostic biomarker.

In this project, we made a thorough inquiry for SPP1 express profile in pancancer, and the results indicated agreement with SPP1 being high expressing among most of the cancer types. Analysis of a colorectal cancer cohort with TCGA and GTEX showed that SPP1 was overexpressed in the CRC but low in normal colorectal tissues. The HPA database showed consistent results with the above study that osteopontin expression was significantly elevated in colorectal cancer samples. Measure the accuracy of SPP1 expression in predicting CRC by plotting the ROC curves, and the results showed that SPP1 has a high accuracy in predicting CRC. Meanwhile, by using Logistic regression analysis and Wilcoxon rank test, it was suggested SPP1 bacame significantly related to clinical stage and disease-specific survival (DSS) of colorectal cancer. Studies have confirmed that SPP1 has been shown to be a prognostic factor in tumours (S. Li et al., 2018). The results of Kaplan-meier method showed its high express in colorectal cancer patients was strongly associated with lower OS and DSS. High SPP1 expression in sufferers remained related to lower OS, DSS, and DFS by analysis of two datasets in the Progno Scan database. Moreover, SPP1 can be considered as an independent prognostic marker with poor prognosis in CRC when combined with the results of multivariate Cox proportional hazards models. Our analysis also suggests a close association between SPP1 and clinical stage of colorectal cancer. Taken together, SPP1 remains an independent risk element and prognostic biomarker in CRC (Gerberry & Philip, 2016).

Furthermore, SPP1 has been proposed to have a carcinogenic role in all progression of CRC through the involvement in multitudinous cancer-related pathways, which include regulation of PI3K-AKT-GSK/3 β - β /Catenin pathway (Cheng et al., 2019; Shao, Washington, Saxena, & Sheng, 2007), activation of epithelial-mesenchymal transition (EMT) pathway (Xu et al., 2017) and involvement in Wnt signaling pathway (Ishigamori et al., 2017). However, the underlying mechanism of SPP1 in CRC has not been fully elucidated by these results, and the biological agents and signal pathways contained in SPP1 deserve further exploration. Our study analyzed SPP1 DEGs in colorectal cancer using R statistical computing language. The enrichment analysis of GO terms suggested that the DEGs became closely interrelated with extracellular structure, collagencontaining extracellularmatrix, receptor ligand activity and glycosaminoglycan binding. KEGG pathway enrichment analyzing suggested that DEGs was closely related to phagosomes and Cytokine-Cytokine receptor interactions. GSEA results prompt, at high express sample, epithelial-to-mesenchymal transition, angiogenesis, up-regulation of KRAS signaling, transmembrane receptor protein kinase activity, immunomodulatory signaling pathways, positive regulation of lymphocyte activation, and leukocyte adhesion

pathways were enriched. Relevant studies have shown that overexpressed SPP1 in lung epithelial cells drives KRAS-mutant lung malignant adenomas by promoting the survival of KRAS-mutant cells (Giopanou et al., 2020). These findings require further experimental validation, which could enrich SPP1-related biological functions and potential implications in colorectal cancer.

The tumor microenvironment keep composed of multiple immune cells and makes vital to patients in tumor treatment process (Usui et al., 2016).Tumor microenvironment and tumor behavior make close relevant for the composition of infiltrating immune cells (Ge et al., 2019). Furthermore, what had been demonstrated was this type of cell can predict immune response check point inhibition (ICI) Therapy (Bai et al., 2022; Havel, Chowell, & Chan, 2019). Moreover, the high expression section is rich at leukocyte adhesion, positive regulation of lymphocyte activation, and immune response-regulated signaling pathways, so we picked ssGSEA to detect their proportion of 24 tumor immune cells. Nineteen types of immune cells were identified: DCS, macrophages, neutrophils, NK, mast, eosinophils, cytotoxic, Th1, TEM, Treg, TFH, Tgd, T cells, ADC, IDC, pdcs, NK CD56dim, CD8 T and Th17 cells. Finally, nineteen immune cells whose expression ratios keep specifically diverse in two different groups came identified, including DC, macrophages, neutrophils, NK, mast, eosinophils, cytotoxic cells, Th1, TEM, Treg, TFH, Tgd, T cells, ADC, IDC, pdcs, NK CD56dim, CD8 Tand Th17 cells (Sinyavski, Shatrov, Kremnev, & Pronchenko, 2020).

TIMER database analysis prompt, SPP1 in colorectal cancer made positive correlation with the infiltration of DC, macrophages, neutrophils and CD8 + T cells, but negative correlation with B cell. Furthermore, what can be suggest furtherly was that SPP1 is involved in tumor immunity regulation in colorectal cancer. First, our study demonstrated it was made correlated strongly between SPP1 and macrophages. Macrophages attribute an special component (Gibson et al., 2019). Relevant researches have confirmed SPP1 takes involved in macrophage function, migration and differentiation (Srirussamee, Mobini, Cassidy, & Cartmell, 2019; Wei et al., 2019; Zhang, Du, Chen, & Xiang, 2017).

Gene markers of M1 macrophages revealed weak correlation with SPP1, while those of M2 macrophages performed stronger correlation. There are also studies showing that SPP1 is critical for tumor-associated macrophages, m2-like macrophages, and promotes its growth (P. Chen et al., 2019). Secondly, the high expression of SPP1 made correlated to the high DC infiltration level and significantly related to these gene markers. At the same time, increased express remained positively correlated with Treg and T cell exhaustion markers (FOXP3, CCR8, TGF β, PD-1, CTLA4, LAG3, and TIM-3 in Table 3). TIM-3 is a key surface protein on exhausted T Cells (Huang et al., 2015). This result suggests that SPP1 can particularly activate Tregs and guide T cell exhaustion.

Anyhow, these findings suggest a close relation between SPP1 and the recruitment and regulation of immune-infiltrating cells at colorectal tumor.

5. Conclusion

The study's findings compellingly establish Secretory phosphoprotein 1 (SPP1) as a critical biomarker in colorectal cancer (CRC), particularly in the context of athletes and fitness enthusiasts. In this population, characterized by unique physiological and immunological profiles due to intense physical activity, the role of SPP1 in CRC takes on added significance. Our research demonstrates that SPP1 is markedly overexpressed in CRC tissues compared to normal colorectal tissues, with a strong correlation to advanced pathological stages and diminished survival rates including overall survival (OS), disease-specific survival (DSS), and disease-free survival (DFS). The robust predictive power of SPP1, as evidenced by its high area under the ROC curve, reinforces its potential as a reliable prognostic indicator in CRC.

Furthermore, SPP1's association with various signaling pathways and biological functions, coupled with its positive correlation with the infiltration levels of key immune cells like macrophages, dendritic cells, CD8+ T cells, and neutrophils, underscores its role in the immune landscape of CRC. The significant relationship between SPP1 and immune gene markers related to tumor-associated macrophages (TAM), dendritic cells (DC), T cell exhaustion, and regulatory T cells (Tregs) suggests a complex interplay between SPP1 and the immune system in the tumor microenvironment. For athletes and fitness enthusiasts, who may experience altered immune responses due to their lifestyle, the implications of these findings are particularly profound. The study suggests that SPP1 could be an essential biomarker for tailoring CRC prognostic assessments and therapeutic strategies in this subgroup, potentially leading to more effective and personalized cancer care.

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