Heliyon 9 (2023) e15535

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

CILP2: A prognostic biomarker associated with immune infiltration in colorectal cancer

Xueli Wang [a,1], Yu Zhang [b,1], Niping Song [c,1], Kaiqiang Li [d], Siyun Lei [a], Jianwei Wang [a], Zhen Wang [d,*], Wei Zhang [a,e,**]

[a] College of Pharmaceutical Science, Zhejiang University of Technology, Hangzhou 310032, China
[b] Department of Gastroenterology, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, China
[c] The Second Clinical Medical College, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China
[d] Center for Laboratory Medicine, Allergy Center, Department of Transfusion Medicine, Zhejiang Provincial People's Hospital, Affiliated People's Hospital, Hangzhou Medical College, Hangzhou, Zhejiang 310014, China
[e] Department of Gastrointestinal Surgery, The Second Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310005, China

ARTICLE INFO

Keywords:
Colorectal cancer
CILP2
Immune microenvironment
Prognosis
Immune infiltration

ABSTRACT

Purpose: The function played by cartilage intermediate layer protein 2 (CILP2) between colorectal cancer (CRC) progression and immune response remains unclear, especially with respect to immune cell infiltration and checkpoints. **Materials and Methods**: We examined *CILP2* expression in The Cancer Genome Atlas (TCGA) COAD-READ cohort and analyzed its relationship with clinicopathological features, mutations, survival, and immunity. Gene ontology, Kyoto Encyclopedia of Genes and Genomes pathway analysis, and gene set enrichment analyses (GSEA) were performed to determine *CILP2* related pathways. To further investigate the results of TCGA analysis, validation was performed using CRC cell lines, fresh pathological tissues, and a CRC tissue microarray (TMA). **Results:** In both TCGA and TMA cohorts, CILP2 expression was increased in CRC tissues and was associated with patient T stage (T3 and T4), N stage (N1), pathological stage (III and IV), and overall survival. Immune cell infiltration and checkpoint analysis revealed that *CILP2* expression is highly correlated with multiple immune marker genes, including PD-1. In addition, results of enrichment analysis indicated that *CILP2* related genes was mainly enriched in extracellular matrix related functions. **Conclusion:** Elevated *CILP2* expression is associated with adverse CRC clinical features and immune cells, it has potential as a biomarker detrimental to CRC survival.

1. Introduction

Colorectal cancer (CRC) is a common malignant tumor with an incidence of approximately 10%, ranking third globally, and a mortality rate of approximately 9.4%, ranking second [1,2]. China accounts for 28.8% of new CRC cases and 30.6% of CRC-related deaths worldwide [3]. Overall, the incidence and mortality rate of CRC are rapidly increasing, a cause of considerable concern for

* Corresponding author.
** Corresponding author. Department of Gastrointestinal surgery, The Second Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310005, China.
E-mail addresses: wangzhen@hmc.edu.cn (Z. Wang), zhangweils1968@163.com (W. Zhang).
[1] These are co-first authors.

https://doi.org/10.1016/j.heliyon.2023.e15535
Received 10 March 2023; Received in revised form 4 April 2023; Accepted 13 April 2023
Available online 20 April 2023

the healthcare system [4]. Immunotherapy has shown promising results in oncology studies. Immune checkpoint inhibitors, such as PD-1 and PD-L1, have shown promising results [5,6]. Therefore, discovering new treatments and therapeutic targets to expand CRC therapeutics is crucial for reducing CRC mortality rates.

Extracellular matrix (ECM) is involved in constituting the tumor microenvironment (TME). During cancer progression, immune cells are subjected to signals from the ECM to exert anti- or pro-tumor effects [7]. Cartilage intermediate layer protein (CILP2) is most abundantly expressed in the intermediate zone of articular cartilage. It plays a potential role in the structure and function of non-chondral tissue extracellular matrix [8]. A previous study revealed that ECM stiffness efficiently promotes chondrocyte differentiation by initiating the transforming growth factor beta (TGF-β) pathway [9]. Current studies regarding CILP2 have focused on its association with lipid metabolism diseases. Cartilage intermediate layer protein 1 (CILP1) can act as a mediator of ECM remodeling in the heart and affect TGF-β signaling [10]. The relationship between CILP2 and malignant tumors has not been studied thoroughly, with few reports describing this relationship. These studies report that CILP induces breast cancer brain metastases by affecting $CD4^+$ T immune cell function [11]. However, the exact pathway by which CILP2 plays a role in CRC, especially regarding the immune response, and its link to immune cell infiltration and immune checkpoints within the TME remain unclear. Therefore, this article focuses on the complex relationship between CILP2 and CRC and provide new insights to guide CRC diagnosis and treatment.

We comprehensively analyzed *CILP2* expression and its association with clinicopathological features and survival based on clinical data from multiple publicly available databases. Furthermore, we explored possible pathogenic mechanisms involving *CILP2* during CRC development by performing enrichment analysis. Since various components of TME are key factors in tumor progression and treatment, the relationship between CILP2 and immune is also discussed. Finally, we further verified the results in CRC cell lines, tissue samples, adjacent tissue microarray (TMA) and retrospective data. In conclusion, this study illustrates the importance of CILP2 in CRC and provides a new candidate gene for the treatment of CRC.

2. Material and methods

2.1. Clinical tissue samples and cell lines

HCT116, SW480, SW620, and RKO, and normal colonic epithelial cells (NCM460) were purchased from Pricella (Wuhan, China). Human CRC tissue samples were obtained from general surgical resections at the Zhejiang Provincial People's Hospital (including 180 CRC tissue specimens and paired normal adjacent tissues). Paraffin-embedded tissues were used to create a 360-point TMA [12]. This retrospective study was approved by the Institutional Review Board of Zhejiang Provincial People's Hospital (Protocol QT2022391) and followed the Declaration of Helsinki. The requirement for informed consent was waived owing to the anonymity of patient data.

2.2. Gene expression and survival analysis

Transcriptome RNA sequencing data and the corresponding clinical data of 698 CRC patients (51 with normal paracancerous tissue and 647 with tumor tissue) were downloaded from The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/). Normal tissue data were obtained from the Genotype-Tissue Expression (GTEx) project (http://commonfund.nih.gov/GTEx/). We investigated the correlation of CILP2 expression with the following clinical features using nonparametric tests: pathological stage (I and II vs. III and IV), T stage (T1 and T2 vs. T3 and T4), N stage (N0 vs. N1 and N2), and M stage (M0 vs. M1). Survival curves were analyzed using the Kaplan—Meier method. The statistical significance of survival curves was evaluated using the log-rank test. Significance was set at $p < 0.05$.

2.3. Genetic alteration analysis

The cBioPortal for Cancer Genomics (http://www.cbioportal.org) was used to evaluate the genetic alteration profile of *CILP2* in TCGA COAD-READ cohort patients, including the mutation type, alteration frequency, and copy number alteration. Mutation site information for *CILP2* was obtained from the Cancer Type Summary and Mutations modules. Copy number profiles were generated for these data along with p-values obtained from the Spearman and Pearson tests.

2.4. Immune correlation analysis

The abundance and distribution of Tumor-infiltrating immune cells (TIIC) in the TCGA COAD-READ cohort was determined by single sample genomic enrichment analysis using the R package GSVA. We calculated the abundance of *CILP2* expression and TIIC using the Tumor Immune Estimation Resource (TIMER) database (https://cistrome.shinyapps.io/timer/). Correlations between *CILP2* expression and 65 immune checkpoints (43 immunostimulants and 22 immunosuppressants) and 21 major histocompatability complex (MHC) molecules were determined using Spearman's correlation coefficient. The link between *CILP2* and various immune characteristics were obtained from the TISIDB database (http://cis.hku.hk/TISIDB/).

2.5. Functional enrichment analyses

Using the LinkedOmics databasse (http://www.linkedomics.org), genes positively or negatively co-expressed with *CILP2* were identified in the "LinkInterpreter" module, and GSEA was used to investigate the Panther pathway behind the biological functions

affecting *CILP2*. Adjusted *p*- and q-values of <0.05 were considered significant.

2.6. Western blotting and real-time quantitative polymerase chain reaction (RT-qPCR)

Total protein was extracted using radioimmunoprecipitation assay (RIPA) lysis solution (Beyotime, Shanghai, China). Protein samples were separated on 8% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. After milk closure, the strips were incubated with the corresponding antibody CILP2 (Santa Cruz Biotechnology, Dallas, TX, USA) and GAPDH (Huabio, Hangzhou, China) overnight at 4 °C before incubation with secondary antibodies (Huabio, Hangzhou, China). Bands were acquired using an enhanced chemiluminescence solution (Verde Bio, Shanghai, China) with a Bio-Rad (Hercules, CA, USA) image system.

RNA was extracted using TRIzol (Ambion, Austin, TX, USA). RT-qPCR was performed using the EVO M-MLV RT and SYBR Green Kit (Accurate Biology, Hunan, China). Relative *CILP2* mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. *CILP2* primer: forward; 5′- AGGGCGACTTTACCATTGAGG -3′ and reverse; 5′- GTCCATGAACTCACCGCTG -3′.

2.7. Immunohistochemical staining

Immunohistochemistry was performed to determine CILP2 expression in the TMA sections and paraffin-embedded tissue pairs, as

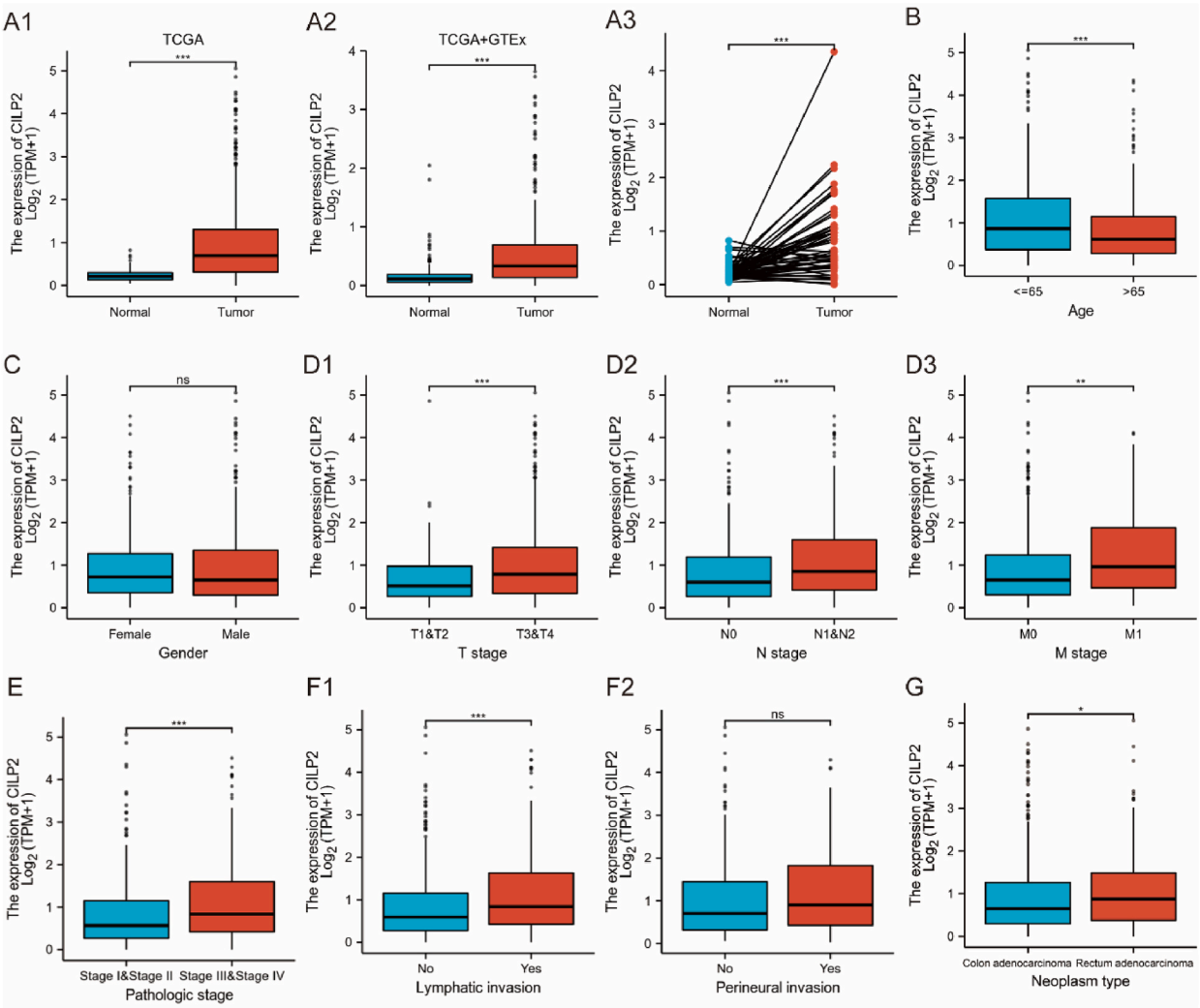

Fig. 1. Expression levels of cartilage intermediate layer protein 2 (*CILP2*) mRNA in the Cancer Genome Atlas (TCGA) CODA-READ cohort and its correlation with clinicopathological features. (A1-A3) *CILP2* mRNA expression was upregulated in colorectal cancer (CRC) tumor tissues. (A1) TCGA tumor and normal tissues. (A2) TCGA + (GTEx) tumor and normal tissues. (A3) TCGA paired tissues. (B–G) The mRNA expression level of *CILP2* was analyzed using TCGA COAD-READ data sets according to (B) age, (C) gender, (D1) T, (D2) N, (D3) M, (E) pathologic stage, (F1) lymphatic invasion, (F2) perineural invasion, and (G) neoplasm type. ns, no significant difference; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

described in previous studies [13]. Briefly, tissue sections were dewaxed, repaired, closed, and then incubated with anti-CILP2 (Invitrogen, UAS) overnight at 4 °C. Brown immunoreactive signals were obtained using an Ultra-View Universal DAB assay kit (Roche, Mannheim, Germany). Staining results were independently evaluated by two pathologists. The TMA staining score was based on a previously reported semi-quantitative scoring system of staining percentage and staining intensity [14]. The percentage of positive cells was indicated (0, 0%; 1, ≤25%; 2, 25%–50%; 3, 51%–75%; 4, ≥75%). Scoring from 0 to 3 according to staining intensity. The two scores were then multiplied by 0–6 (for low CILP2 expression) or 7–12 (representing high CILP2 expression).

2.8. Statistical analysis

CILP2 expression in CRC tissues was detected by one-way regression analysis. Significant differences between categorical variables were analyzed using the Chi-squared and Fisher's exact tests. The Kaplan—Meier curve and Cox regression analysis were used to assess the prognostic potential of CILP2 for overall survival. Spearman's correlation coefficient was used to analyze the correlation between CILP2 expression and TIICs in CRC. Statistical and bioinformatics analyses were performed using R software version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria) and SPSS version 26.0 (IBM, Armonk, NY, USA). GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. Results were considered statistically significant with a two-sided p value of <0.05.

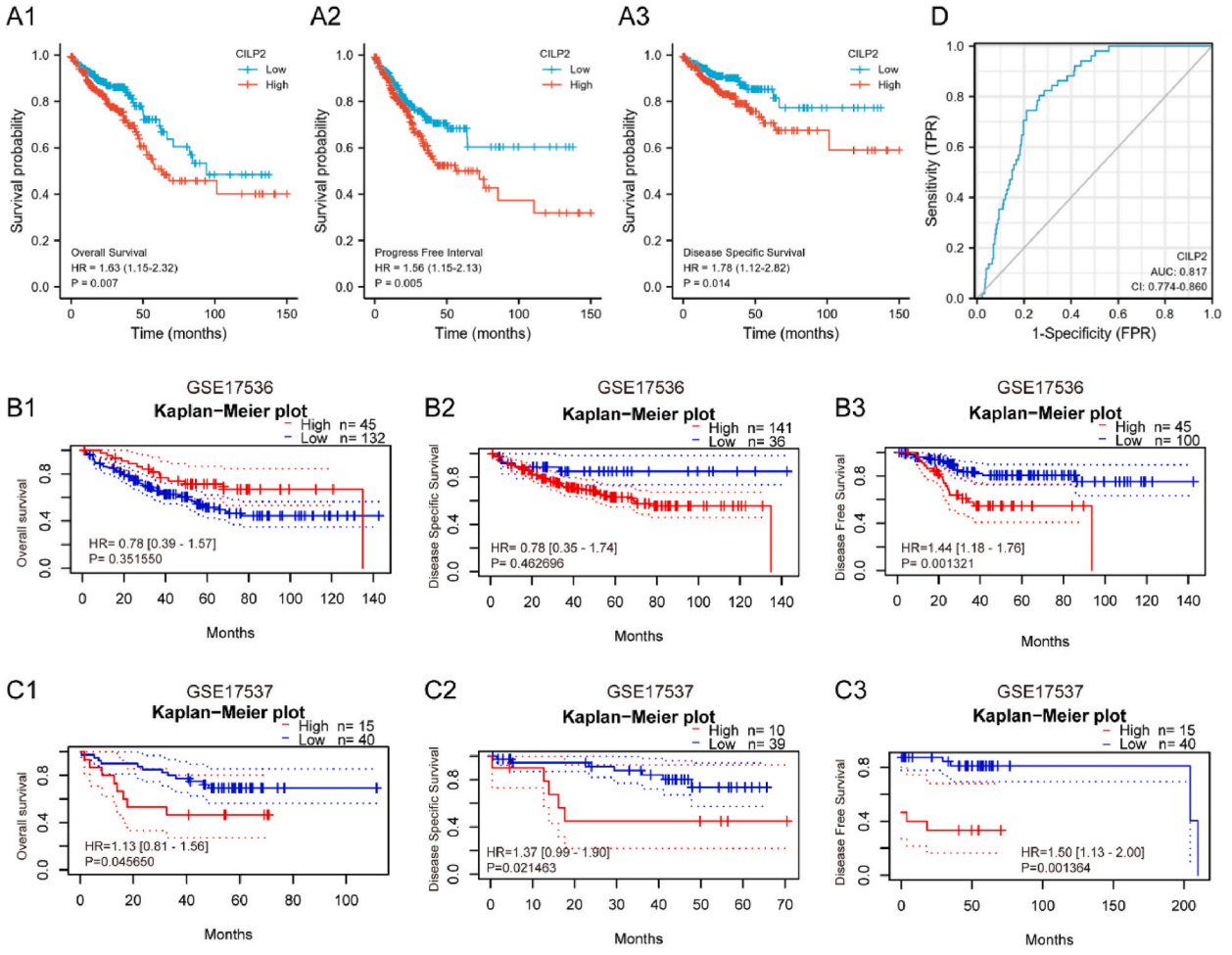

Fig. 2. Prognostic value of cartilage intermediate layer protein 2 (*CILP2*) expression in The Cancer Genome Atlas (TCGA) COAD-READ. Survival curves showing (A1) overall survival (OS), (A2) progression-free interval (PFI) and (A3) disease-specific survival (DSS) rates in colorectal cancer (CRC) patients with low and high *CILP2* expression in TCGA COAD-READ dataset. (B–C) Kaplan—Meier survival curve analysis of OS, disease-free survival (DFS) and DSS in the PrognoScan database (GSE17536 and GSE17537). (D) receiver operating characteristic (ROC) curve analysis indicated an area under the curve (AUC) of 0.817 (95% CI = 0.774–0.860) for the TCGA COAD-READ dataset. ns, no significant difference; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3. Results

3.1. Findings based on TCGA data

3.1.1. Abnormal overexpression of CILP2 is associated with tumor stage and metastasis

We observed from TCGA and GTEx databases that *CILP2* expression was higher in CRC tissues than in normal tissues (Fig. 1 A1 and A2, $p < 0.001$). In addition, *CILP2* expression was higher in CRC tissues than in paired non-tumor tissues (Fig. 1 A3, $p < 0.001$). Based on the median level of *CILP2* mRNA expression in the TCGA COAD-READ cohort, samples were divided into two groups. The results showed a significant correlation between high *CILP2* expression and age (>65 years) ($p < 0.001$), T stage (T3 and T4) ($p < 0.001$), M stage (M1) ($p < 0.01$), N stage (N1 and N2) ($p < 0.001$), and pathologic stage (Stage III and Stage IV) ($p < 0.001$), but not with sex ($p > 0.05$) (Fig. 1B–E). These results strongly suggest that the higher expression of *CILP2* has a more severe tumor stage. In addition, high

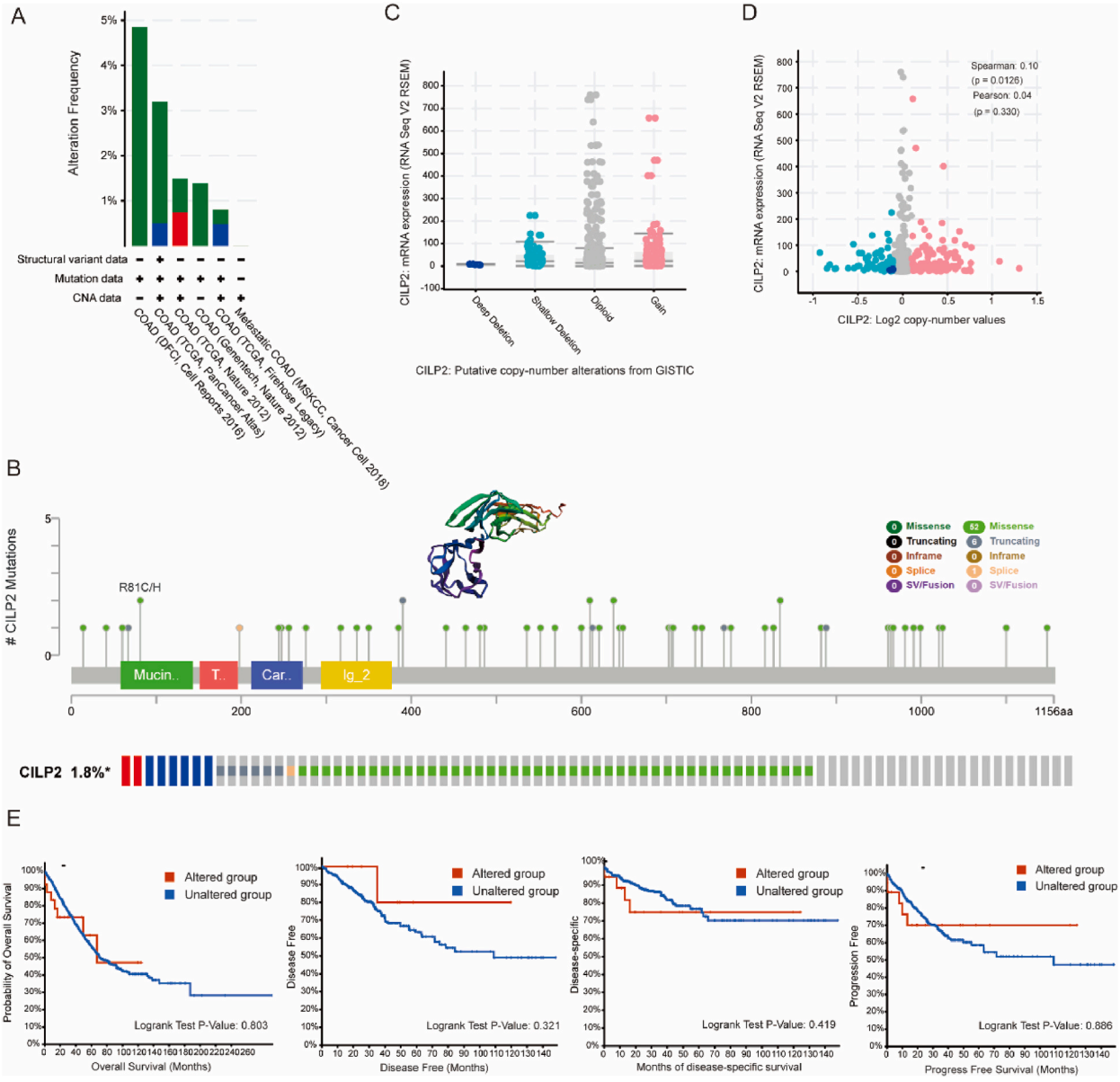

Fig. 3. Correlation of cartilage intermediate layer protein 2 (CILP2) gene alterations with prognosis in The Cancer Genome Atlas (TCGA) COAD-READ cohorts. (A) Bar graph of *CILP2* alteration frequencies in different databases. (B) Schematic representation of gene mutation sites of *CILP2* in the coding chain, where 1.8% of *CILP2* showed structural variants. The association between *CILP2* copy number and mRNA expression are shown in the dot (C) and correlation plot (D) by cBioPortal. (E) Kaplan—Meier curves of overall survival (OS), disease-free survival (DFS), disease-specific survival (DSS), and progression-free interval (PFS) in altered and unaltered CILP2 groups.

CILP2 expression is more likely to occur in rectal adenocarcinoma ($p < 0.05$), and tends to lymphatic invasion ($p < 0.001$) (Fig. 1F and G).

3.1.2. *Abnormal overexpression of CILP2 is associated with poor tumor prognosis*

Kaplan—Meier analysis showed that high *CILP2* expression endows patients with shorter overall survival (OS) (HR = 1.63, 95% CI = 1.15–2.32, $p = 0.007$, Fig. 2 A1), progression-free interval (PFI) (HR = 1.56, 95% CI = 1.15–2.13, $p = 0.005$, Fig. 2 A2), and disease-specific survival (DSS) (HR = 1.78, 95% CI = 1.12–2.82, $p = 0.014$, Fig. 2 A3). The PrognoScan database was used to further assess the prognostic value of *CILP2*, main parameters including OS, DSS, and disease-free survival (DFS). In the GSE17537 cohort, high *CILP2* expression predicted poorer OS (HR = 1.13, 95% CI = 0.81–1.56, $p = 0.046$, Fig. 2C1), DSS (HR = 1.37, 95% CI = 0.99–1.90, $p = 0.021$, Fig. 2C2), and DFS (HR = 1.50, 95% CI = 1.13–2.00, $p = 0.001$, Fig. 2C3) in CRC patients. However, in the GSE17536 cohort, high *CILP2* expression had a relatively poor DFS (HR = 1.44, 95% CI = 1.18–1.76, $p = 0.001$, Fig. 2 B3) in CRC patients without a statistically significant association with poorer DSS (HR = 0.78, 95% CI = 0.35–1.74, $p = 0.46$, Fig. 2 B2) and improved OS (HR = 0.78, 95% CI = 0.39–1.57, $p = 0.35$, Fig. 2 B1). Receiver operating characteristic curve analysis showed an area under the curve value of 0.817 (95% CI:0.774–0.860, Fig. 2D) for *CILP2* mRNA expression level. These results suggest that *CILP2* can affect the survival prognosis of CRC patients.

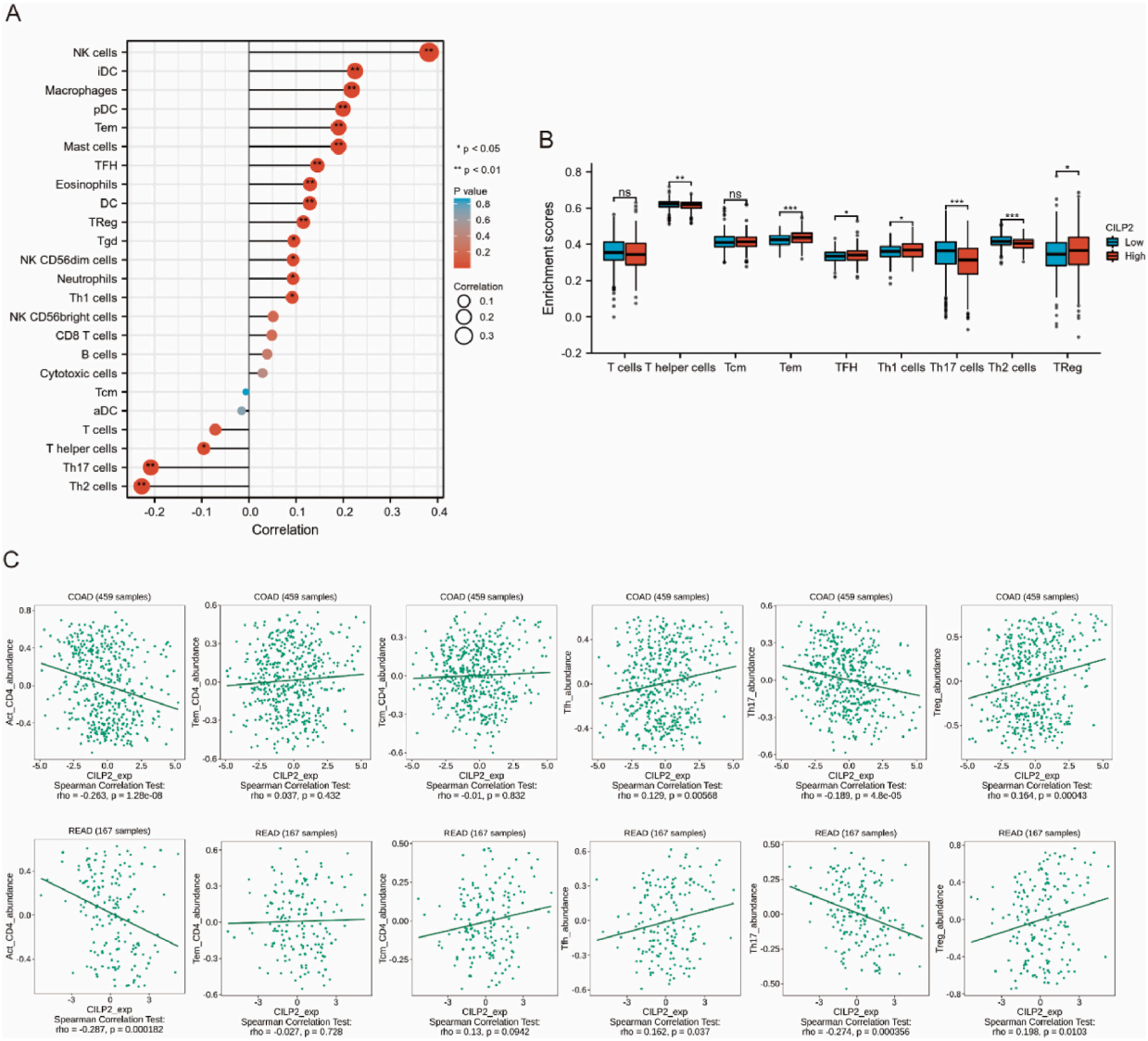

Fig. 4. Correlation analysis of cartilage intermediate layer protein 2 (CILP2) expression and immune infiltration. (A) Correlation of CILP2 expression with 17 immune infiltrating cells in The Cancer Genome Atlas (TCGA) COAD-READ cohorts. (B) Immunoscore of the CILP2 high and low expression groups. (C) Correlation of CILP2 expression levels with colorectal cancer (CRC) immune cell markers in the TISIDB database. ns, no significant difference; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.1.3. Association of CILP2 gene alterations with clinical outcomes of CRC patients

We queried the frequency and type of *CILP2* gene alterations in six databases and found that mutations were the most common gene alterations in CILP2, followed by *CILP2* deep deletion (Fig. 3A). In TCGA the total *CILP2* gene variation rate was 3.2% (2.69% mutations and 0.51% deep deletion) in 594 cases. A schematic representation of the type, location, and case number of genetic alterations in its coding chain showed that 1.8% of *CILP2* genes had structural variants (Fig. 3B). In the *CILP2* gene, we found copy number gain

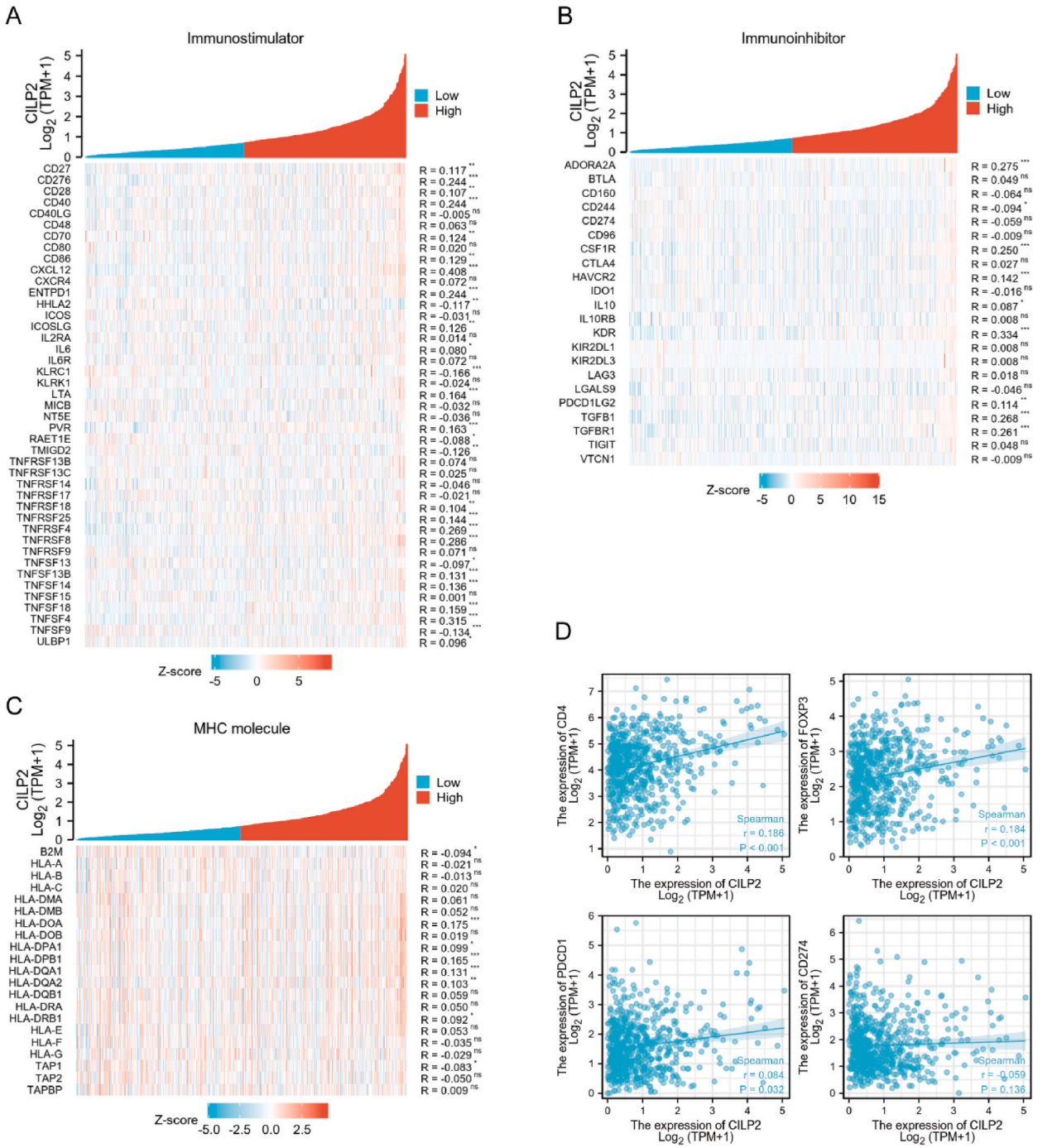

Fig. 5. Correlation of immune checkpoint and cartilage intermediate layer protein 2 (CILP2) expression; specifically, *CILP2* with 43 immunostimulators (A), 22 immunoinhibitors (B), and 21 major histocompatibility complex (MHC) molecules (C) in The Cancer Genome Atlas (TCGA) COAD-READ cohort. (D) PDCD1 (PD-1), CD274 (PD-L1), CD4, and FOXP3 expression correlation with CILP2 expression. ns, no significant difference; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

and deep deletion, with higher and lower mRNA expression levels, respectively (Fig. 3C). Interestingly, Spearman analysis showed a positive correlation between *CILP2* mRNA expression levels and copy number ($p = 0.0126$), while Pearson analysis showed no correlation ($p = 0.330$) (Fig. 3D). There was no significant correlation between OS ($p = 0.803$), DFS ($p = 0.321$), DSS ($p = 0.419$), or PFS ($p = 0.886$) (Fig. 3E) in patients with and without *CILP2* alteration, indicating that CILP2 gene alteration is not related to the prognosis of patients. Therefore, we focused on the association between *CILP2* expression and CRC occurrence.

3.1.4. Correlation of CILP2 expression with immune infiltration in the COAD-READ cohorts

It has been demonstrated that TIICs are involved in the formation of a complex TME, which influencs the treatment of patients with tumors [15]. Therefore, we explored the correlation between *CILP2* expression and TIICs using the TIMER database. The results showed that *CILP2* expression in CRC correlated with 17 TIICs, including NK cells, dendritic cells (DC), effector memory T (Tem) cells,

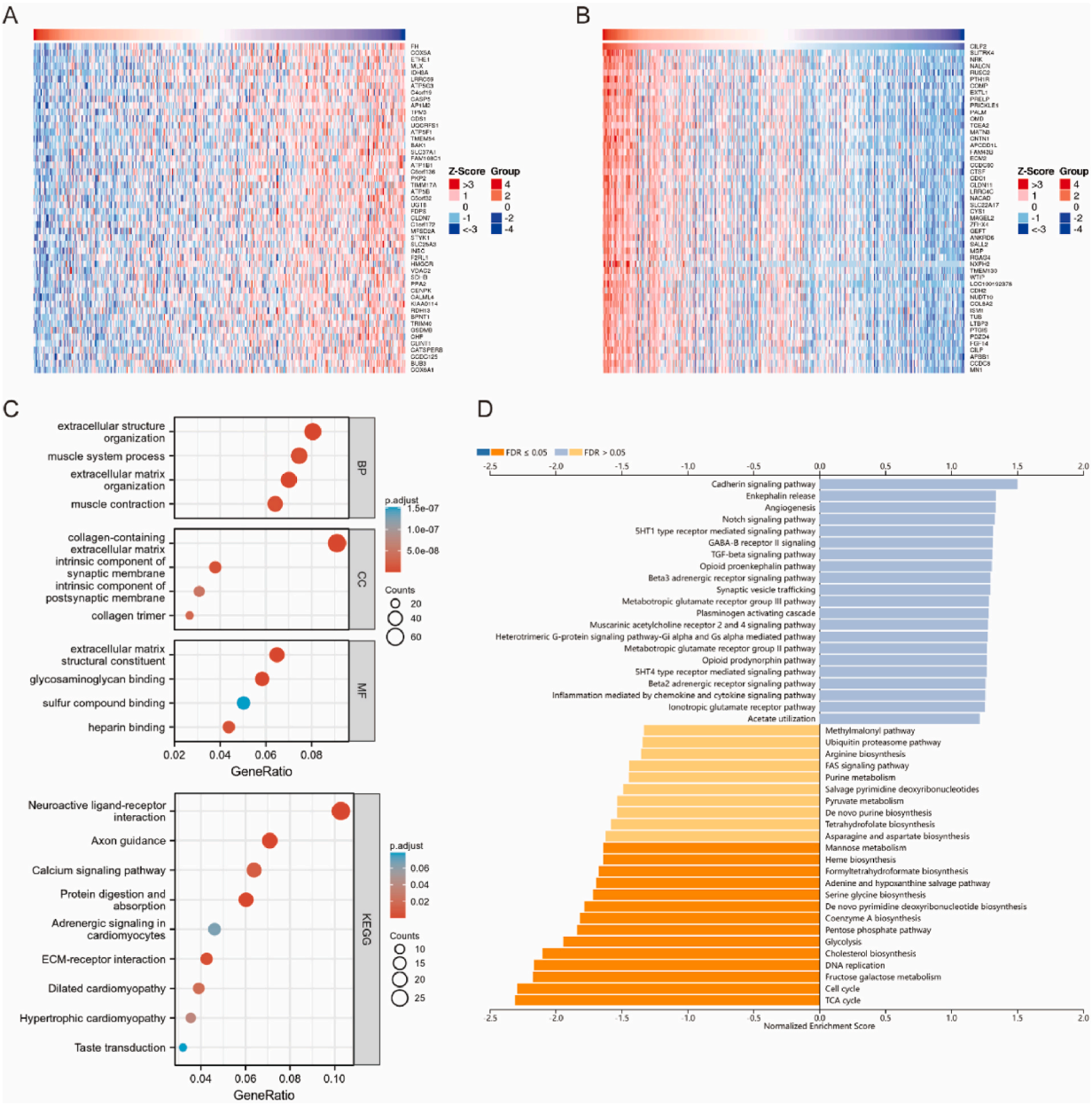

Fig. 6. Cartilage intermediate layer protein 2 (*CILP2*) gene enrichment analysis in The Cancer Genome Atlas (TCGA) COAD-READ dataset. (A–B) Heatmaps showing genes positively and negatively correlated with *CILP2* in COAD-READ (top50). (C) Gene onthology (GO) analysis of *CILP2*-associated genes and prediction of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were performed. (D) Gene Set Enrichment Analysis (GSEA) related to CILP2 expression. Terms with p values of <0.05 were considered significantly enriched.

and regulatory T (Treg) cells (Fig. 4A). Next, we further analyzed the different functional T cells. The expression of Treg, Tem, and Th1 cells was higher in the *CILP2* overexpression group (Fig. 4B). Additionally, Act_CD4, Ffh, and Th17 were significantly negatively correlated with *CILP2* expression, while Tregs were significantly positively correlated with *CILP2* expression (Fig. 4C).

3.1.5. Correlation of CILP2 expression with immune checkpoints in COAD-READ cohorts

Next, we focused on immune checkpoint genes. We selected 86 immunomodulators containing 43 immunostimulatory factors, 22 immunosuppressive factors, and 21 MHC molecules. In the COAD-READ cohort, *CILP2* expression was significantly correlated with 27 immunostimulators (Fig. 5A), nine immunoinhibitors (Fig. 5B), and eight MHC molecules (Fig. 5C). In particular, it was significantly and positively correlated with PDCD1 (PD-1), CD4, and FOXP3 expression (Fig. 5D). Therefore, we speculate that CILP2 may affect the prognosis of CRC patients by regulating immune cells.

3.1.6. Functions of CILP2 in TCGA COAD-READ cohorts

LinkedOmics data mining was used to identify genes positively or negatively co-expressed with CILP2, and a heatmap of the top 50 was drawn (Fig. 6A and B). Next, we analyzed the biological functions that might involve CILP2, and selected genes co-expressed with *CILP2* for enrichment analysis. The GO module identifies the biological processes (BP) term of the extracellular matrix organization, and the cellular components (CC) term of the collagen-containing extracellular matrix are mainly enriched. Molecular functional (MF) analysis showed that the structural constituents of the extracellular matrix were significantly enhanced. KEGG analysis showed that ECM-receptor interaction was also enriched (Fig. 6C). Using GSEA, we identified that *CILP2* expression was closely associated with the

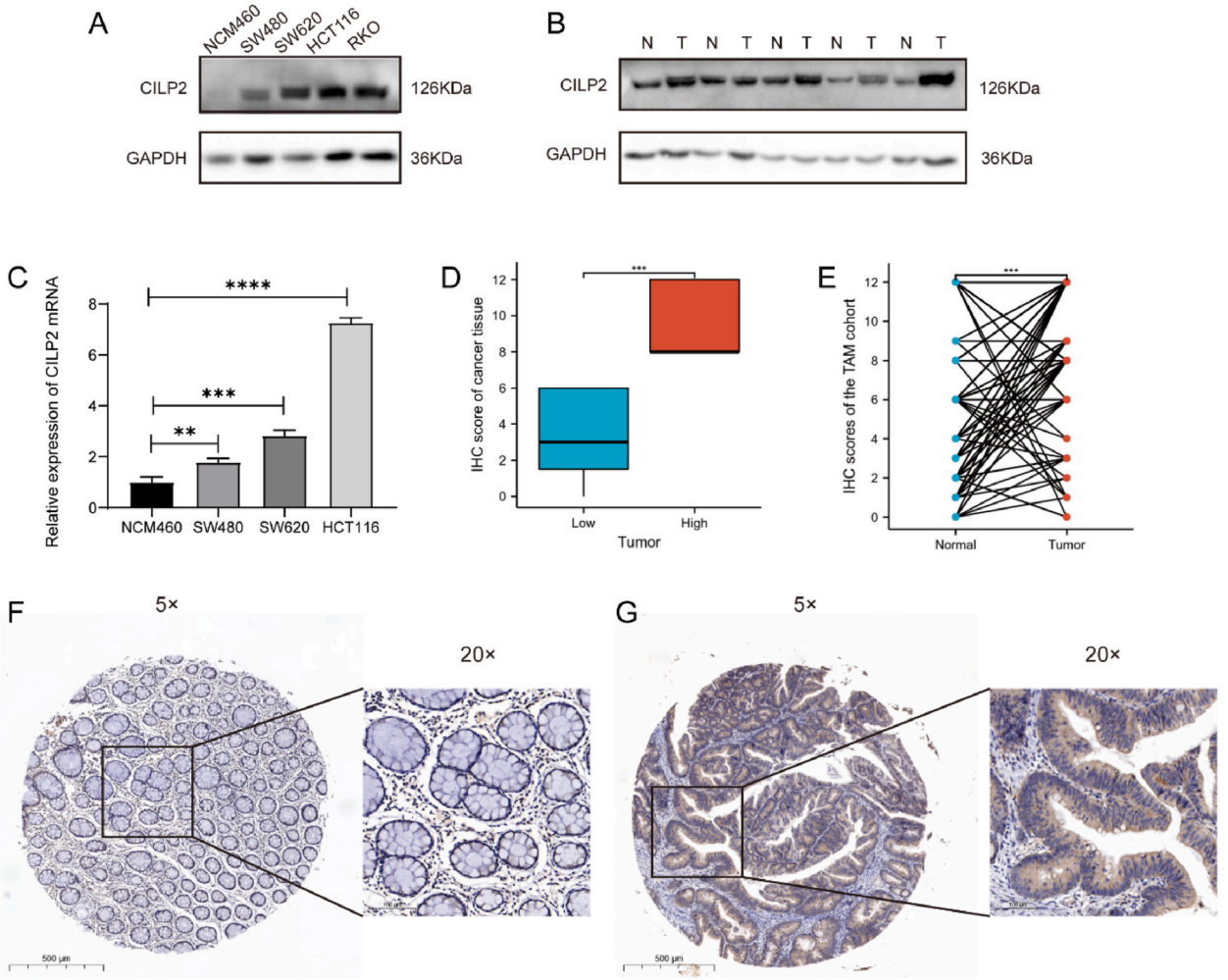

Fig. 7. Elevated cartilage intermediate layer protein 2 (CILP2) expression was in colorectal cancer (CRC) tissues. Western blot (A) and quantitative RT-PCR (C) analysis of CILP2 expression in intestinal epithelial (NCM460) and CRC cells. (B) Western blotting analysis of CRC and adjacent normal tissue samples from five pairs of randomly selected CRC patients. (D) CILP2 expression levels in CRC tissues in the tissue microarray (TMA) cohort. (E) Paired graphs of CRC and paired normal tissues IHC assays in the TMA cohort. (F–G) Representative immunohistochemistry images of CILP2 in normal and CRC tissues.

calmodulin, and the TGF-β signaling pathways (Fig. 6D). As far as the results of the analysis are concerned, CILP2 may alter CRC cell biological behavior by affecting the structure function of the ECM.

3.2. Validation results based on TMA cohort

3.2.1. Validation of high CILP2 expression in CRC

To get more reliable results, we determined CILP2 expression in NCM460 and four CRC cell lines at both the protein and mRNA levels. The results showed that CILP2 expression was higher in CRC cells than in NCM460, no differences were observed between mRNA and protein expression (Fig. 7A and C). Five patients' cancer tissues and their paracancerous tissues were randomly selected to detect their protein expression of CILP2. The expression of CILP2 was higher in CRC tissues (Fig. 7B). A similar result was obtained in the TMA cohort ($p < 0.001$) (Fig. 7E). CILP2 protein was highly expressed in 82.68% (148/179) (1 clinical information is missing) of CRC tissue samples ($p < 0.001$) (Fig. 7D). Representative images of CILP2 immunohistochemical staining (Fig. 7F and G).

3.2.2. Correlation of CILP2 expression with clinicopathological parameters in the TMA cohort

To further determine clinical relevance, we examined and analyzed CILP2 expression in a TMA cohort of 180 CRC patients (1 clinical information is missing) and their paired normal CRC tissues. According to immunohistochemical scoring guidelines, a score of 7–12 in tumor tissue was considered high CILP2 expression (n = 148), while a score of 0–6 indicated low CILP2 expression (n = 31). The Chi-squared test showed that high CILP2 expression was significantly associated with T stage T3+T: (T1+T2, 57.89%; T3+T4, 89.36%; $P < 0.001$), N stage N1+N2 (N0,74.45%; N1+N2, 91.76%; $p = 0.002$), and Pathologic stage III + IV (I + II, 74.19%; III + IV, 91.86%; $p = 0.002$). There were only five patients with M1 stage in the TMA cohort; therefore, no correlation between CILP2 and M stage was observed. There was no correlation between CILP2 expression and age ($p = 0.252$), sex ($p = 0.252$), tumor size ($p = 0.718$), vascular involvement ($p = 0.234$), neurological involvement ($p = 0.00.939$), or lymphatic involvement ($p = 0.242$) in the TMA cohort, probably due to the limited sample size (Table 1).

3.2.3. High CILP2 expression predicts poor prognosis in CRC patients in the TMA cohort

Kaplan—Meier analysis of our cohort showed that the OS of patients at different times, with the CILP2 high expression group at 5 years (HR = 0.25, 95% CI = 0.08–0.81, $p = 0.02$, Fig. 8A), 7 years (HR = 0.29, 95% CI = 0.10–0.79, $p = 0.016$, Figs. 8B), 10 years (HR = 0.46, 95% CI = 0.21–1.00, $p = 0.049$, Fig. 8C), and end date of follow-up (HR = 0.45, 95% CI = 0.21–0.98, $p = 0.045$, Fig. 8D) had lower OS than the low expression group. Further univariate and multivariate Cox regression analyses showed that CILP2 expression level ($p = 0.046$) and age ($p = 0.003$) were associated with poor prognosis at 7 years in CRC patients (Table 2).

4. Discussion

CILP2 is a secreted glycoprotein [16]. CILP2 was confirmed to be associated with atherosclerosis, according to Hu et al. (2020), who

Table 1
Association between cartilage intermediate layer protein 2 (CILP2) expression and clinical characteristics of colorectal cancer (CRC) patients in the TAM cohort.

Characteristic	CILP2 expression			
		High (n = 148)	Low (n = 31)	*p*
Age (years)	≤65	74 (79.56%)	19 (20.43%)	0.252
	>65	74 (86.05%)	12 (13.95%)	
Gender	Male	96 (85.71%)	16 (14.29%)	0.166
	Female	52 (77.61%)	15 (22.39%)	
Location	Colon	79 (84.95%)	14 (15.05%)	0.405
	Rectum	69 (80.23%)	17 (19.77%)	
T stage	T1+T2	22 (57.89%)	16 (42.11%)	**< 0.001**
	T3+T4	126 (89.36%)	15 (10.64%)	
N stage	N0	70 (74.45%)	24 (25.54%)	**0.002**
	N1+N2	78 (91,76%)	7 (8.24%)	
M stage	M0	144 (82.76%)	30 (17.24%)	0.872
	M1	4 (80.00%)	1 (20.00%)	
Pathologic stage	I + II	69 (74.19%)	24 (25.81%)	**0.002**
	III + IV	79 (91.86%)	7 (8.14%)	
Vascular recidivism	Yes	17 (73.91%)	6 (26.09%)	0.234
	No	131 (83.97%)	25 (16.03%)	
Neurological recidivism	Yes	15 (83.33%)	3 (16.67%)	0.939
	No	133 (82.61%)	28 (17.39%)	
Lymphatic recidivism	Yes	44 (88.00%)	6 (12.00%)	0.242
	No	104 (80.62%)	25 (19.38%)	
Tumor size	≤3 cm	38 (84.44%)	7 (15.56%)	0.718
	>3 cm	110 (82.09%)	24 (17.91%)	

Values shown in bold are statistically significant ($p < 0.05$).

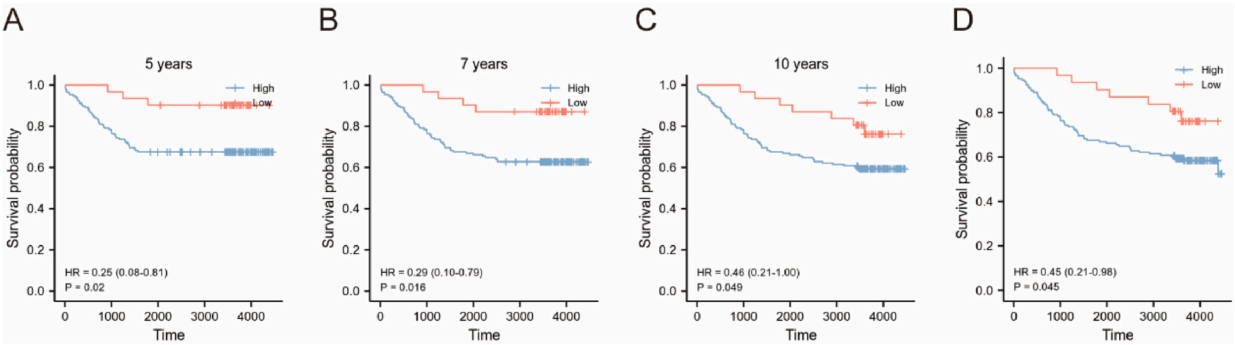

Fig. 8. Kaplan—Meier analysis of cartilage intermediate layer protein 2 (CILP2) expression and overall survival (OS) in the tissue microarray (TMA) cohort. OS of colorectal cancer (CRC) patients with low and high CILP2 expression in the TMA cohort after (A) 5, (B) 7, and (C)10 years, and (D) at the end of follow-up.

Table 2
Univariate and multivariate Cox regression analyses of clinicopathological factors associated with overall survival.

Characteristics	Univariate analysis			Multivariate analysis		
	p	HR	95% CI	*p*	HR	95% CI
CILP2 expression (Low vs. High)	**0.016**	0.287	0.104–0.793	**0.046**	0.349	0.124–0.982
Age (≤65 vs. > 65)	**0.003**	0.440	0.258–0.750	**0.003**	0.440	0.255–0.762
M stage (M1 vs. M0)	**0.022**	3.303	1.193–9.147	0.372	1.630	0.558–4.765
Pathologic stage (III + IV vs. I + II)	**<0.001**	2.799	1.620–4.836	0.08	1.755	0.934–3.295
Neurological recidivism (Yes vs. No)	**0.048**	1.989	1.006–3.930	0.190	1.607	0.791–3.266
Lymphatic recidivism (Yes vs. No)	**0.001**	2.365	1.409–3.970	0.061	1.747	0.975–3.128

Values shown in bold are statistically significant ($p < 0.05$). CILP2, cartilage intermediate layer protein 2.

revealed that CILP2 regulates the transcription of *CD36* through the peroxisome proliferator-activated receptor gamma (PPARγ) pathway, thereby increasing the uptake of lipids by cells and resulting in atherosclerosis [17]. Luo et al. (2017) reported that the rs16996148 genetic locus in *CILP2* strongly influenced the risk of dyslipidemia in the Chinese population [18]. The *CILP2* gene is associated with lipid and cardiovascular diseases in the Asian Malay population [19]. Furthermore, Li et al. (2022), reported for the first time that *CILP2* expression in the serum of obese individuals was higher than that in normal individuals [20]. In conclusion, many current studies suggest an association between CILP2 and lipid metabolism in cardiovascular disease. To our knowledge, the role of CILP2 in cancer has not been extensively studied. Since CILP2 is a specific component of the ECM synthesized by articular chondrocytes, it may regulate immune cells in the TME by remodeling the ECM, thus affecting tumor progression. Here, we further investigated the relationship between CILP2 and CRC immune cells to gain insight regarding the mechanisms underlying CRC development and progression.

We mined the TCGA database and found that CILP2 is highly expressed in CRC, which is consistent with our results in CRC cell lines, tissue specimens from CRC patients, and the TMA cohort. Further analysis of the samples from the TCGA database showed significant correlations between high *CILP2* expression and clinical parameters such as age (>65), T stage (T3 and T4), M stage (M1), N stage (N1 and N2), pathologic stage (III and IV), and survival. Regarding the TMA cohort results, CILP2 expression was associated with T stage (T3 and T4), N stage (N1 and N2), pathological stage (III and IV), and overall survival. These suggest that CILP2 is abnormally expressed in CRC and may adversely affect CRC progression.

Immune cells of the TME exhibit complex functions in CRC progression [21–23]. However, the role of CILP2 in immune cells in the TME has not yet been reported. Therefore, another important direction of this study is to discuss the interface between CILP2 and CRC immunity. There is evidence that in addition to genetic mutations, immune cells are involved in the development of CRC [24,25]. Goc et al. (2021) found that Type 3 innate lymphoid cells (ILC3) were downregulated in CRC, accompanied by T-cell imbalance, increased TH17 cells, and decreased TH1 cell mucosa, suggesting that ILC3s contribute to adaptive immune dysfunction and thus promote the development of CRC [26]. ECM can alter tumor growth and progression [27]. Collagen type XVI (col-16) plays a role in intestinal diseases by affecting ECM structure and inducing cell invasion [28]. Lysyl oxidase (LOX) upregulation enhances tumor ECM remodeling to promote CRC metastasis [29]. Overall, genetic alterations reshape immune cell function in the TME. This is in agreement with a study reported by Förster et al. (2008) [30]. Here, we demonstrate that CILP2 was correlated with a variety of immune cells, including NK, Tem, Th1, and Treg cells. Further studies revealed that *CILP2* expression levels were positively correlated with PD-1, CD4, and FOXP3. GO, KEGG, and GSEA analyses revealed that CILP2 was significantly enriched in EMC structures related functions and the TGF-β signaling pathway. Based on these results, CILP2 may manipulate immune cells in CRC to induce tumor response.

In conclusion, we have confirmed in databases and clinical retrospective data that aberrant expression of CILP2 plays a specific role in the clinically adverse pathological features of CRC and significantly affects the prognosis of patients. Furthermore, we focus on the

potential role of CILP2 and its relationship with immune cells, which provides a Promising opportunity for future CRC treatment. Nevertheless, our study has some limitations. Additional clinical data and experiments related to immunomolecular biology are required to verify the clinical value and function.

5. Conclusions

We elaborated on the possible role of CILP2 in CRC by analyzing the relationship between CILP2 expression and the clinical features, prognostic value, mutations, and immune cells of CRC. Further cellular and animal model experiments are required to explore the pathogenic role played by CILP2 in the altered biological behavior of CRC cells, and to provide improved support for establishing the clinical significance of CILP2.

Author contribution statement

Xueli Wang: Conceived and designed the experiments; Wrote the paper.
Yu Zhang: Analyzed and interpreted the data; Wrote the paper.
Niping Song: Performed the experiments; Wrote the paper.
Kaiqiang Li and Jianwei Wang: Conceived and designed the experiments.
Siyun Lei: Analyzed and interpreted the data.
Zhen Wang and Wei Zhang: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Funding

This work was supported by Natural Science Foundation of Zhejiang Province (no. LQ20H160061); Medical Health Science and Technology Project of Zhejiang Provincial Health Commission (no. 2021KY502); and Zhejiang Provincial Science and Technology Program of Traditional Medicine (no. 2022ZA016).

Data availability statement

All data generated in this study are available from the corresponding author upon reasonable request.

Declaration of interest's statement

The authors declare no conflict of interest.

Abbreviations

CRC	Colorectal cancer
CILP2	Cartilage intermediate layer protein 2
TMA	tissue microarray
TIIC	Tumor-infiltrating immune cell
ECM	extracellular matrix
TME	tumor microenvironment
OS	overall survival
DFS	disease-free survival
DSS	disease-specific survival
PFI	progression-free interval

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15535.

References

[1] Y. Xi, P. Xu, Global colorectal cancer burden in 2020 and projections to 2040, Transl Oncol 14 (2021), 101174, https://doi.org/10.1016/j.tranon.2021.101174.
[2] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA A Cancer J. Clin. 71 (2021) 209–249, https://doi.org/10.3322/caac.21660.
[3] N. Li, B. Lu, C. Luo, J. Cai, M. Lu, Y. Zhang, H. Chen, M. Dai, Incidence, mortality, survival, risk factor and screening of colorectal cancer: a comparison among China, Europe, and northern America, Cancer Lett. 522 (2021) 255–268, https://doi.org/10.1016/j.canlet.2021.09.034.
[4] C. Xia, X. Dong, H. Li, M. Cao, D. Sun, S. He, F. Yang, X. Yan, S. Zhang, N. Li, W. Chen, Cancer statistics in China and United States, 2022: profiles, trends, and determinants, Chin. Med. J. 135 (2022) 584–590, https://doi.org/10.1097/CM9.0000000000002108.

[5] K. Ganesh, Z.K. Stadler, A. Cercek, R.B. Mendelsohn, J. Shia, N.H. Segal, L.A. Diaz Jr., Immunotherapy in colorectal cancer: rationale, challenges and potential, Nat. Rev. Gastroenterol. Hepatol. 16 (2019) 361–375, https://doi.org/10.1038/s41575-019-0126-x.
[6] D.R. Almquist, D.H. Ahn, T.S. Bekaii-Saab, The role of immune checkpoint inhibitors in colorectal adenocarcinoma, BioDrugs 34 (2020) 349–362, https://doi.org/10.1007/s40259-020-00420-3.
[7] J. Huang, L. Zhang, D. Wan, L. Zhou, S. Zheng, S. Lin, Y. Qiao, Extracellular matrix and its therapeutic potential for cancer treatment, Signal Transduct. Targeted Ther. 6 (2021) 153, https://doi.org/10.1038/s41392-021-00544-0.
[8] B.C. Bernardo, D. Belluoccio, L. Rowley, C.B. Little, U. Hansen, J.F. Bateman, Cartilage intermediate layer protein 2 (CILP-2) is expressed in articular and meniscal cartilage and down-regulated in experimental osteoarthritis, J. Biol. Chem. 286 (2011) 37758–37767, https://doi.org/10.1074/jbc.M111.248039.
[9] J.L. Allen, M.E. Cooke, T. Alliston, ECM stiffness primes the TGFbeta pathway to promote chondrocyte differentiation, Mol. Biol. Cell 23 (2012) 3731–3742, https://doi.org/10.1091/mbc.E12-03-0172.
[10] F.A. Van Nieuwenhoven, C. Munts, R.C. Op't Veld, A. Gonzalez, J. Diez, S. Heymans, B. Schroen, M. Van Bilsen, Cartilage intermediate layer protein 1 (CILP1): a novel mediator of cardiac extracellular matrix remodelling, Sci. Rep. 7 (2017), 16042, https://doi.org/10.1038/s41598-017-16201-y.
[11] X. Sun, N. Yang, X. Zhou, H. Dai, Q. Li, A. Feng, G. Xu, Y. Liu, L. Xu, Z. Zhang, Z. Yang, X. Li, CILP, a putative gene associated with immune infiltration in breast cancer brain metastases, Front. Genet. 13 (2022), 862264, https://doi.org/10.3389/fgene.2022.862264.
[12] B. Glinsmann-Gibson, L. Wisner, M. Stanton, B. Larsen, L. Rimsza, A. Maguire, Recommendations for tissue microarray construction and quality assurance, Appl. Immunohistochem. Mol. Morphol. 28 (2020) 325–330, https://doi.org/10.1097/PAI.0000000000000739.
[13] Y. Zhang, X. Zhang, Z. Jin, H. Chen, C. Zhang, W. Wang, J. Jing, W. Pan, Clinical impact of X-ray repair cross-complementary 1 (XRCC1) and the immune environment in colorectal adenoma-carcinoma pathway progression, J. Inflamm. Res. 14 (2021) 5403–5417, https://doi.org/10.2147/JIR.S331010.
[14] W. Hu, S. Li, S. Zhang, B. Xie, M. Zheng, J. Sun, X. Yang, L. Zang, GJA1 is a prognostic biomarker and correlated with immune infiltrates in colorectal cancer, Cancer Manag. Res. 12 (2020) 11649–11661, https://doi.org/10.2147/CMAR.S235500.
[15] Y. Zhang, Z. Zhang, The history and advances in cancer immunotherapy: understanding the characteristics of tumor-infiltrating immune cells and their therapeutic implications, Cell. Mol. Immunol. 17 (2020) 807–821, https://doi.org/10.1038/s41423-020-0488-6.
[16] T. Wu, Q. Zhang, S. Wu, W. Hu, T. Zhou, K. Li, D. Liu, H.F. Gu, H. Zheng, Z. Zhu, L. Li, G. Yang, CILP-2 is a novel secreted protein and associated with insulin resistance, J. Mol. Cell Biol. 11 (2019) 1083–1094, https://doi.org/10.1093/jmcb/mjz016.
[17] W. Hu, K. Li, H. Han, S. Geng, B. Zhou, X. Fan, S. Xu, M. Yang, H. Liu, G. Yang, Y. Liu, Circulating levels of CILP2 are elevated in coronary heart disease and associated with atherosclerosis, Oxid. Med. Cell. Longev. 2020 (2020), 1871984, https://doi.org/10.1155/2020/1871984.
[18] H. Luo, X. Zhang, P. Shuai, Y. Miao, Z. Ye, Y. Lin, Genetic variants influencing lipid levels and risk of dyslipidemia in Chinese population, J. Genet. 96 (2017) 985–992, https://doi.org/10.1007/s12041-017-0864-x.
[19] E.S. Tai, X.L. Sim, T.H. Ong, T.Y. Wong, S.M. Saw, T. Aung, S. Kathiresan, M. Orho-Melander, J.M. Ordovas, J.T. Tan, M. Seielstad, Polymorphisms at newly identified lipid-associated loci are associated with blood lipids and cardiovascular disease in an Asian Malay population, J. Lipid Res. 50 (2009) 514–520, https://doi.org/10.1194/jlr.M800456-JLR200.
[20] Q. Li, D. Pu, X. Xia, H. Liu, L. Li, Serum concentrations of cartilage intermediate layer protein 2 were higher in overweight and obese subjects, BioMed Res. Int. (2022), 6290064, https://doi.org/10.1155/2022/6290064.
[21] P. Ge, W. Wang, L. Li, G. Zhang, Z. Gao, Z. Tang, X. Dang, Y. Wu, Profiles of immune cell infiltration and immune-related genes in the tumor microenvironment of colorectal cancer, Biomed. Pharmacother. 118 (2019), 109228, https://doi.org/10.1016/j.biopha.2019.109228.
[22] D. Wu, Y. Ding, T. Wang, P. Cui, L. Huang, Z. Min, M. Xu, Significance of tumor-infiltrating immune cells in the prognosis of colon cancer, OncoTargets Ther. 13 (2020) 4581–4589, https://doi.org/10.2147/OTT.S250416.
[23] L. Ye, T. Zhang, Z. Kang, G. Guo, Y. Sun, K. Lin, Q. Huang, X. Shi, Z. Ni, N. Ding, K.N. Zhao, W. Chang, J. Wang, F. Lin, X. Xue, Tumor-infiltrating immune cells act as a marker for prognosis in colorectal cancer, Front. Immunol. 10 (2019) 2368, https://doi.org/10.3389/fimmu.2019.02368.
[24] Y. Cao, N. Jiao, T. Sun, Y. Ma, X. Zhang, H. Chen, J. Hong, Y. Zhang, CXCL11 correlates with antitumor immunity and an improved prognosis in colon cancer, Front. Cell Dev. Biol. 9 (2021), 646252, https://doi.org/10.3389/fcell.2021.646252.
[25] Y. Yue, Q. Zhang, Z. Sun, CX3CR1 acts as a protective biomarker in the tumor microenvironment of colorectal cancer, Front. Immunol. 12 (2021), 758040, https://doi.org/10.3389/fimmu.2021.758040.
[26] J. Goc, M. Lv, N.J. Bessman, A.L. Flamar, S. Sahota, H. Suzuki, F. Teng, G.G. Putzel, J.R.I.L.C. Bank, G. Eberl, D.R. Withers, J.C. Arthur, M.A. Shah, G. F. Sonnenberg, Dysregulation of ILC3s unleashes progression and immunotherapy resistance in colon cancer, Cell 184 (2021) 5015–5030 e5016, https://doi.org/10.1016/j.cell.2021.07.029.
[27] M. Najafi, B. Farhood, K. Mortezaee, Extracellular matrix (ECM) stiffness and degradation as cancer drivers, J. Cell. Biochem. 120 (2019) 2782–2790, https://doi.org/10.1002/jcb.27681.
[28] C. Jensen, S.H. Nielsen, J.H. Mortensen, J. Kjeldsen, L.G. Klinge, A. Krag, H. Harling, L.N. Jorgensen, M.A. Karsdal, N. Willumsen, Serum type XVI collagen is associated with colorectal cancer and ulcerative colitis indicating a pathological role in gastrointestinal disorders, Cancer Med. 7 (2018) 4619–4626, https://doi.org/10.1002/cam4.1692.
[29] B. Wei, X. Zhou, C. Liang, X. Zheng, P. Lei, J. Fang, X. Han, L. Wang, C. Qi, H. Wei, Human colorectal cancer progression correlates with LOX-induced ECM stiffening, Int. J. Biol. Sci. 13 (2017) 1450–1457, https://doi.org/10.7150/ijbs.21230.
[30] R. Forster, A.C. Davalos-Misslitz, A. Rot, CCR7 and its ligands: balancing immunity and tolerance, Nat. Rev. Immunol. 8 (2008) 362–371, https://doi.org/10.1038/nri2297.