Heliyon 10 (2024) e28794

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Cell cycle associated protein 1 associates with immune infiltration and ferroptosis in gastrointestinal cancer

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ARTICLE INFO

Keywords:
CAPRIN1
Ferroptosis
Immune infiltrates
Prognosis
Gastrointestinal cancer

ABSTRACT

Background: Cell Cycle-Associated Protein 1 (CAPRIN1) play an important role in cell proliferation, oxidative stress, and inflammatory response. Nonetheless, its role in tumor immunity and ferroptosis is largely unknown in gastrointestinal cancer patients.
Methods: Through comprehensive bioinformatics, we investigate CAPRIN1 expression patterns and its role in diagnosis, functional signaling pathways, tumor immune infiltration and ferroptosis of different gastrointestinal cancer subtypes. Besides, immunohistochemistry (IHC) and immune blot were used to validate our esophagus cancer clinical data. The ferroptotic features of CAPRIN1 *in vitro* were assessed through knockdown assays in esophagus cancer cells.
Results: CAPRIN1 expression was significantly upregulated, correlated with poor prognosis, and served as an independent risk factor for most gastrointestinal cancer. Moreover, CAPRIN1 overexpression positively correlated with gene markers of most infiltrating immune cells, and immune checkpoints. CAPRIN1 knockdown significantly decreased the protein level of major histocompatibility complex class I molecules. We also identified a link between CAPRIN1 and ferroptosis-related genes in gastrointestinal cancer. Knockdown of CAPRIN1 significantly increased the production of lipid reactive oxygen species and malondialdehyde. Inhibition of CAPRIN1 expression promoted ferroptotic cell death induced by RAS-selective lethal 3 and erastin in human esophagus cancer cells.

Abbreviations: CAPRIN1, Cell Cycle-Associated Protein 1; ESCA, Esophageal carcinoma; ESCC, esophageal squamous cell carcinoma; EA, Esophageal adenocarcinoma; STAD, Stomach adenocarcinoma; LIHC, Liver Cancer; CHOL, Cholangiocarcinoma; PAAD, Pancreatic adenocarcinoma; COAD, Colon adenocarcinoma; READ, Rectum cancers; CRC, Colorectal cancer; TME, tumor microenvironment; FRGs, ferroptosis-related genes; TCGA, The Cancer Genome Atlas; CPTCA, Clinical Proteomic Tumor Analysis Consortium; TISIDB, Tumor-immune interaction database; GEO, Gene Expression Omnibus; OS, Overall survival; RFS, Relapse-free survival; DSS, Disease-specific-survival; PFS, progression-free survival; GSEA, Gene-set enrichment analysis; ATM, Ataxia-telangiectasia mutated; MDA, Malondialdehyde; HMGB1, high mobility group protein B1; PD-1, Programmed death-1; PD-L2, Programmed Cell Death Protein 1 Ligand 2.

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https://doi.org/10.1016/j.heliyon.2024.e28794
Received 19 July 2023; Received in revised form 23 March 2024; Accepted 25 March 2024
Available online 27 March 2024

Conclusion: Collectively, our results demonstrate that CAPRIN1 is aberrantly expressed in gastrointestinal cancer, is associated with poor prognosis, and could potentially influence immune infiltration and ferroptosis.

1. Introduction

Gastrointestinal cancer is a group of most common tumors, including esophageal (ESCA), gastric (STAD), liver (LIHC), gallbladder (CHOL), pancreatic (PAAD), colon (COAD), and rectum cancers (READ) [1,2]. Despite the advancement made in the improvement of therapeutic strategies, colorectal (CRC), LIHC, and STAD are accounting over 2.5 million deaths annually, and globally still ranked 2nd, 3rd, and 4th leading causes of cancer-related deaths, respectively [3–5]. Therefore, the identification of biomarkers for patient diagnosis and prognosis with high sensitivity and specificity is an unmet clinical need. The development of novel clinical therapies is also necessary for effective management. Several pieces of evidence indicate that genetic alterations, including amplification, deletions, mutations, or chromosomal translocation regulate tumorigenesis, tumor progression, and therapy response [6]. With the ongoing advances in high-throughput sequencing, microarray and genome technologies, comprehensive bioinformatic analysis tools with large-scale cancer omics data and experimental approaches are widely available. Consequently, this has provided comprehensive clinical and molecular data for patients with different cancer types [7,8]. These findings have promoted the efficient identification of valuable prognostic biomarkers and therapeutic targets that improve gastrointestinal cancer outcomes.

Increasing evidence suggests that immune cells as well as the cross-talk between cancer cells and the proximal immune cells in tumor microenvironment (TME) have a profound impact on the progression of malignant tumors [9–11]. Tumor-infiltrating immune cells comprise innate immune cells, i.e., neutrophils, natural killer cells, monocytes, macrophages, and dendritic cells, adaptive immunity, including T cells, B cells, and T lymphocytes (Treg) [12]. High monocyte-derived macrophages infiltration of most tumor types including gastric cancer, lung cancer, hematoma, and other malignancies correlated with a negative prognosis [13,14]. $CD8^+$ T cells could mediate the recognition of cancer cell through the major histocompatibility complex I (MHC-I)/T-cell receptor (TCR) complex and exert an anti-cancer effect by releasing IFNγ and TNFα [15,16]. In addition, immunotherapy using immune checkpoint inhibitors (ICIs) (e.g., PD-1 and PD-L1 CTLA4 mAb) significantly revolutionize the treatment of patients with multiple solid tumors [17,18]. The expression levels of immune checkpoint-related genes dictate the treatment responses of immune checkpoint inhibitors [19]. However, the current immunotherapies yet remain unsatisfied in metastatic esophagogastric adenocarcinoma and colorectal cancers, and the failure is attributed to the heterogeneity of tumors [20,21]. Therefore, it is urgently required to identify novel immune-related therapeutic strategies and targets in gastrointestinal cancer to improve tumor management.

Cell cycle-associated protein 1 (CAPRIN1) belongs to the RNA-binding proteins family [22]. It is a stress granules protein that participates in the cell cycle, apoptosis, and ferroptosis [23]. Several studies have demonstrated that CAPRIN1 is overexpressed in several gastrointestinal cancers, including liver, gastric, and colon cancers [24–27], where it may contribute to tumor initiation, growth, metastasis, and differentiation. Our recent study identified CAPRIN1 as a key regulator in ESCA cell proliferation and glycolysis by regulating METTL3 and WTAP *in vitro* and *in vivo* [28]. CAPRIN1 has significant impacts on immune cell function and it is significantly upregulated in dividing bone marrow cells and resting splenic T or B lymphocytes [29,30]. This subsequently initiates tumors and supports progression and metastasis during mutations. Meanwhile, experimental analyses revealed that CAPRIN1 was involved in different stress response pathways [23]. However, its regulatory roles and mechanisms in tumor immunity, and ferroptosis is largely unknown in gastrointestinal tumor progression. This may provide novel strategies to predict the prognosis of gastrointestinal cancer patients.

In this research, we employed bioinformatics tools to comprehensively investigate patterns of CAPRIN1 expression and its prognostic value using publicly available gastrointestinal cancer datasets. Next, functional and pathway enrichment analyses were performed to assess potential functional profiles of CAPRIN1 and its underlying mechanisms in gastrointestinal cancer. Moreover, we performed correlation analyses to explore the relationship of CAPRIN1 expression with tumor immunity and ferroptosis-related genes (FRGs), in gastrointestinal cancer. Thereafter, these results were validated *in vitro* using human ESCA cells. Collectively, the present findings suggest that CAPRIN1 is a novel diagnostic and prognostic biomarker, as well as a therapeutic target for gastrointestinal cancer.

2. Methods

2.1. Analysis of CAPRIN1 expression and prognostic value in public datasets

Analysis of CAPRIN1 mRNA expression was performed using datasets obtained from TCGA and gene expression profiling interactive analysis 2 (GEPIA2, http://gepia2.cancer-pku.cn/#general); large-scale proteome data from the Clinical Proteomic Tumor Analysis Consortium (CPTCA), the Cancer Cell Line Encyclopedia (CCLE) databases. Immunohistochemistry (IHC) images of CAPRIN1 sections were downloaded from the Human Protein Atlas database (HPA). Profiles of CAPRIN1 mRNA expression were correlated with clinical data from gastrointestinal cancer patients downloaded from the TCGA website.

Next, we generated Kaplan-Meier survival curves to assess the relationship between expression levels of different genes with survival rates in the Kaplan-Meier Plotter website, GEPIA2 database, and the PrognoScan database [31] across several publicly available cancer datasets. The overall survival (OS), relapse-free survival (RFS), progression-free survival (PFS) and

disease-specific-survival (DSS) were selected to analyze the relevance between gene expression and prognosis. To further determine the predictive accuracy of CAPRIN1 in distinguishing gastrointestinal cancer patients from healthy controls, the receiver operating characteristic (ROC) curves were generated and the area under the curve (AUC) using the pROC package [1.18.0] in R, with the censored data of TCGA cancers.

2.2. IHC and immunofluorescence stain assay

Retrospectively, we reviewed 43 ESCA patients, surgically treated at Taihe Hospital between 2015 and 2017. IHC was performed on the esophagus tissue in line with the previously described protocols [32,33]. Briefly, tissues were incubated with antibodies against CAPRIN1 (1:500, Proteintech) overnight, and subsequently with HRP secondary antibody. They were finally stained with 3,3′-Diaminobenzidine (DAB). Subsequently, the nuclei staining was performed with hematoxylin. All IHC results were independently evaluated by two pathologists. The staining intensity was defined as 0 (negative expression), 1 (weak expression), 2 (moderate expression), and 3 (strong expression) points.

For immunofluorescence staining, the cultured cells on slides were washed with PBS and fixed with 4% paraformaldehyde. The cells were overnight stained with CAPRIN1 (1:400, Proteintech) and MHC-1 primary antibody (1:100, Proteintech) at 4 °C, then with Donkey anti-rabbit Alexa Fluro 488 (1:300, Abcam) and Alexa Fluor 568 conjugated donkey anti-mouse IgG (1:300 dilution, Abcam) at 37 °C in constant darkness. The slides were subsequently mounted with DAPI (Thermo Fisher Scientific) to stain cell nuclei and then observed under a confocal fluorescence microscope (LEICA TCS SP8).

2.3. Functional annotation and gene set enrichment analysis (GSEA) of CAPRIN1 across different cancers

Interactive networks of CAPRIN1 were analyzed using the STRING online tool (string-db.org). Next, Xiantao bioinformatics toolbox (https://www.xiantao.love/products) was explored to conduct Gene ontology (GO, www.geneontology.org) knowledgebase and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) signaling pathway enrichment analyses of CAPRIN1 co-expression genes, with the level of significance set at $P \leq 0.01$. Differentially expressed genes between high and low CAPRIN1 expression group were performed using the DESeq2 package [1.36.0] in R. Genes with $P < 0.01$ were considered significantly enriched. Whereas GSEA analysis was conducted using the Xiantao bioinformatics toolbox with clusterProfiler package [4.4.4] c2.cp.kegg.v7.0.symbols.gmt. The default of 1000 permutations was used to correct for multiple testing. Significance gene sets were affirmed with $|NES| > 1$, NOM p-val <0.05, and FDR (qvalue) < 0.25.

2.4. Immune estimations

The Tumor Immunity Estimation Resource (TIMER) database was used to estimate the abundance of immune infiltration in different cancers [34]. In addition, the resulting correlation modules were used to evaluate the correlations between CAPRIN1 expression and immune marker sets of immune cells (innate immunity and adaptive immunity cells), as well as tumor purity in gastrointestinal cancer. Additionally, immune cell enrichment analysis was conducted by CIBERSORT function in the immunedeconv package [35]. Further, the gene- and tumor-immune interaction database (TISIDB, http://cis.hku.hk/TISIDB/index.php) was used to investigate the expression profile of CAPRIN1 across different immune subtypes. Eventually, we performed co-expression analysis between CAPRIN1 expression and 8 critical immune checkpoint blockade-related genes (i.e., SIGLEC15, IDO1, CD274, PDCD1, etc.) through Spearman analysis in gastrointestinal cancer [36,37]. Tumor Immune Dysfunction and Exclusion (TIDE) is a machine-learning approach that could predict the immune-checkpoint blockade (ICB) response [38]. Based on TCGA's transcriptional data, the TIDE algorithm was applied to predict the potential response to immune therapy of patients with CAPRIN1 high and low expression groups.

2.5. Correlation between CAPRIN1 expression with ferroptosis-related genes

The Xiantao bioinformatics toolbox was used to evaluate the correlation between CAPRIN1 with FRGs in gastrointestinal cancer in TCGA database. 249 ferroptosis-related genes (FRGs) from FerrDB (http://www.zhounan.org/ferrdb/) [39,40]. Also, the differentially expressed FRGs in tumor and paracancer groups were explored using the R package DESeq2 [1.36.0]. Genes with $P < 0.05$ were considered significantly differentially expressed. Then, the mRNA expression levels of FRGs, and their association with CAPRIN1 mRNA and with the overall survival (OS) were investigated from TCGA cancer patients. The overlapping genes that significantly correlated with CAPRIN1 expression and survival time, and DEGs in tumor and paracancer groups were the key hub genes in ESCA, COAD, and LIHC.

2.6. Cell cultures and treatment assays

Human cell lines, HEK239T, liver cancer cells (HepG2 and MHCC-97H), Hela, colon cancer cells (HCT116, SW480, and HT29), normal squamous epithelial cell line HET1A, ESCA cells (KYSE150, KYSE30) were purchased from iCell Bioscience Inc (China). The lentiviral particles of CAPRIN1 (shCAPRIN1) or control shRNA (shCtrl) were produced by Gene Chemical Technology Co. Ltd. The shRNA sequence targeting CAPRIN1 was as follows: 5' -CCAGGAAGTCACAAATAAT- 3'. For the gene knockdown experiment, KYSE30 cells were infected with lentivirus, followed by puromycin (3 μg/ml) selection for 10 days. Then CAPRIN1 stable knockdown cells (pooled clones) were subsequently validated by Western blot analysis, and expanded for experiments. Ferroptosis was triggered by

treating cells with RAS-selective lethal 3 (RSL3, HY-100218A, MCE) and erastin (HY-15763, MCE). The CAPRIN1 stable knockdown cells were seeded in 96-well plates, and cell viability assay was conducted after treatment with RSL3 and erastin at the indicated concentration for 48 h.

2.7. Reverse transcription quantitative real-time PCR (RT-qPCR) and Western blot analysis

Total RNA was isolated from different cells using the Trizol reagent according to the manufacturer' instructions (Thermo Fisher Scientific) and quantified using Nanodrop 2000 spectrophotometer. Then 2.0 μg RNA samples were reverse transcribed to complementary DNA (cDNA) using a reverse transcription kit (TaKaRa). The qPCR was conducted using the SYBR Green Master Mix Kit (TaKaRa) on ABI Vii7 real-time PCR instrument (Life technologies). The reaction mixture contains 2 × SYBR Taq Master Mix, each of the primer, cDNA, rox mix dye. The cycling parameters were 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 34 s for 40 cycles. β-actin was used as the inner control for qPCR. Primer sequences of target genes are outlined in Supplementary Table 1. The 14 ESCA tumor and matched non-cancerous fresh-frozen tissues were snap-frozen in liquid nitrogen. The lysates of tissues or whole cells were generated with RIPA lysis buffer (CST), quantified by BCA reagent (Beyotime), separated on SDS-PAGE, and transferred to PVDF membranes. The PVDF membranes were probed with antibodies of GAPDH (1:6000, Cell Signaling Technology), CAPRIN1 (1:5000, Proteintech), Schnurri-2 (Shn2, 1:2000, Bioss), MHC-I (1:4000, Proteintech Group), CD45 (1:1000, Proteintech, China), CD11b (1:1000, Abcam), PD-L1 (1:4000, Proteintech) overnight at 4 °C. They were incubated with HRP-conjugated secondary antibodies goat-and rabbit or anti-mouse (1:6000, Cell Signaling Technology) at room temperature for 1 h.

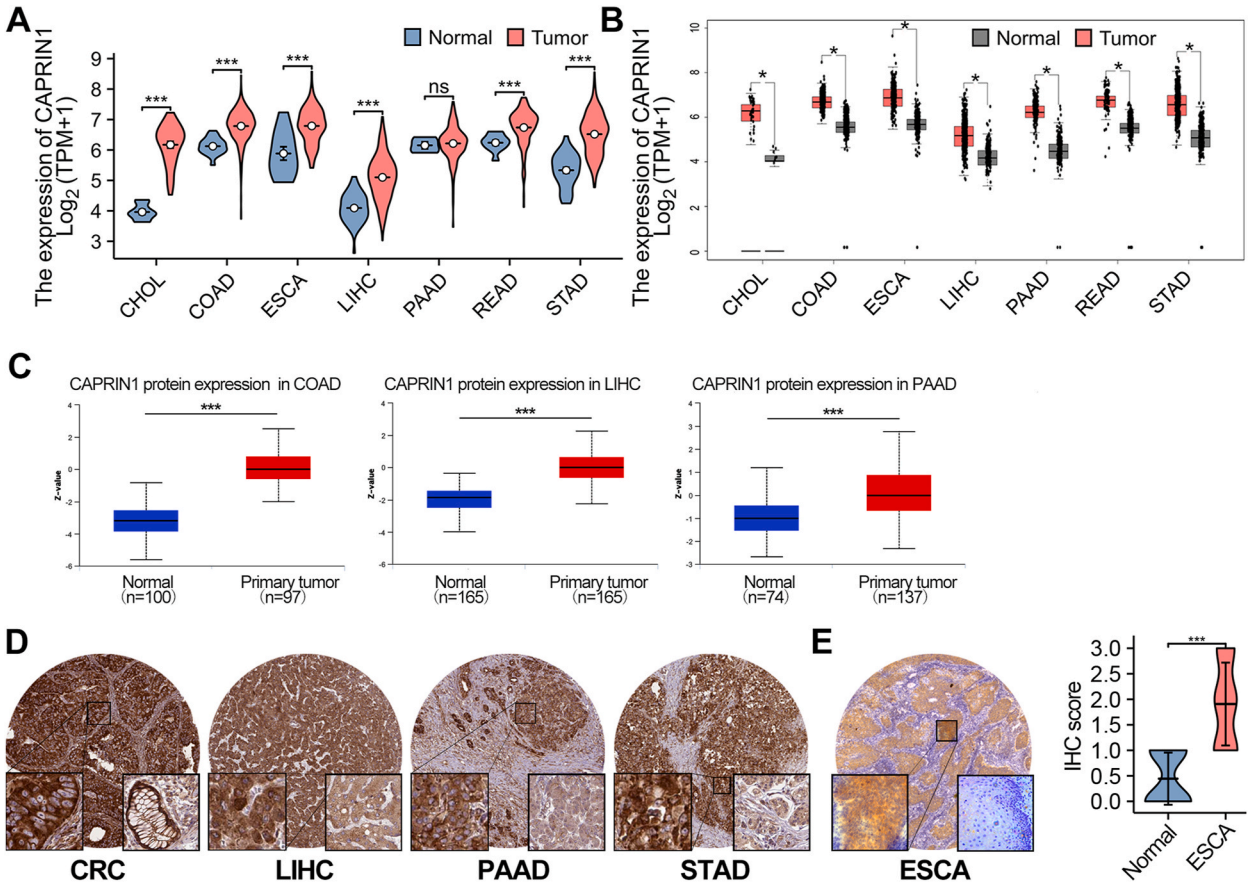

Fig. 1. The CAPRIN1 expression profiles across different gastrointestinal cancer types. (A) The expression profile of human CAPRIN1 in various types of tumors and normal tissues from the TCGA dataset. (B) Transcripts levels of CAPRIN1 across cancers (with tumor and normal samples) in the GEPIA2 database. (C) Protein expression of CAPRIN1 in COAD, LIHC, and PAAD (with tumor and normal samples) in the CPTAC samples. (D) Immunohistochemistry results showing CAPRIN1 expression in gastrointestinal cancers (colorectal, liver, pancreas, stomach) and corresponding normal tissues from the HPA database. (E) Representative immunohistochemical images and quantification of CAPRIN1 expression in ESCA tumor tissues and matched adjacent normal tissues. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

2.8. Measurement of reactive oxygen species (ROS) and lipid peroxidation

The cells were incubated with 20 μM H2DCFDA (MCE) at 37 °C for 30 min. For each sample, 10000 events were acquired and the ROS production was detected using the BD FACSCalibur flow cytometer [41]. Lipid peroxidation was evaluated using the malondialdehyde (MDA) assay kit (Beyotime, China) following the manufacturer's instructions.

2.9. Cell proliferation assay

To determine cellular toxicity, the CellTiter 96 Aqueous One Solution Kit (MTS Kit, Promega) was used according to the manufacturer's protocol. The treated cells were seeded on 96-well culture plates (5000 per well). The absorbance in each well was determined at 490 nm using a spectrophotometer. Results were expressed as relative viability of treated cells compared to untreated controls.

2.10. Statistical analysis

All the data analyses were performed using packages implemented in R software [version 4.0.3] in Xiantao bioinformatics toolbox (https://www.xiantao.love/products) [42]. The genes that correlated with CAPRIN1 expression were screened using the DESeq2 R package [1.36.0] [43]. Gene expression analyses and generation of graphs were produced in R software using the ggplot2 package [3.3.6]. Survival and ROC analyses were carried out using survival [3.3.1], survminer [0.4.6], and pROC [1.18.0] packages in R, whereas bivariate analyses were conducted using $\chi 2$, or Fisher's exact tests. Immune infiltration estimation across different groups was conducted using the CIBERSORT package [4.0.3] in R. In addition, differences in the composition of immune cell types were determined using the Mann-Whitney test to reveal their significance. Correlation between the two variables was determined using Spearman's or Pearson's test; the results were visualized with the ggplot2 [3.3.6] and pheatmap [1.0.10] packages in R.

3. Results

3.1. The clinical landscape of CAPRIN1 expression in human gastrointestinal cancer

We first investigated the CAPRIN1 expression in human gastrointestinal cancer tissues. Compared with normal tissues, CAPRIN1 mRNA was upregulated in CHOL, COAD, ESCA, LIHC, READ, and STAD tumor tissues in TCGA database (Fig. 1A). Matched TCGA normal and GTEx database analyses in GEPIA2 revealed CAPRIN1 mRNA upregulation in the COAD, ESCA, LIHC, PAAD, READ, and STAD tumor tissues (Fig. 1B). RNAseq results from the CCLE database revealed a 5-6-fold upregulation in CAPRIN1 mRNA expression

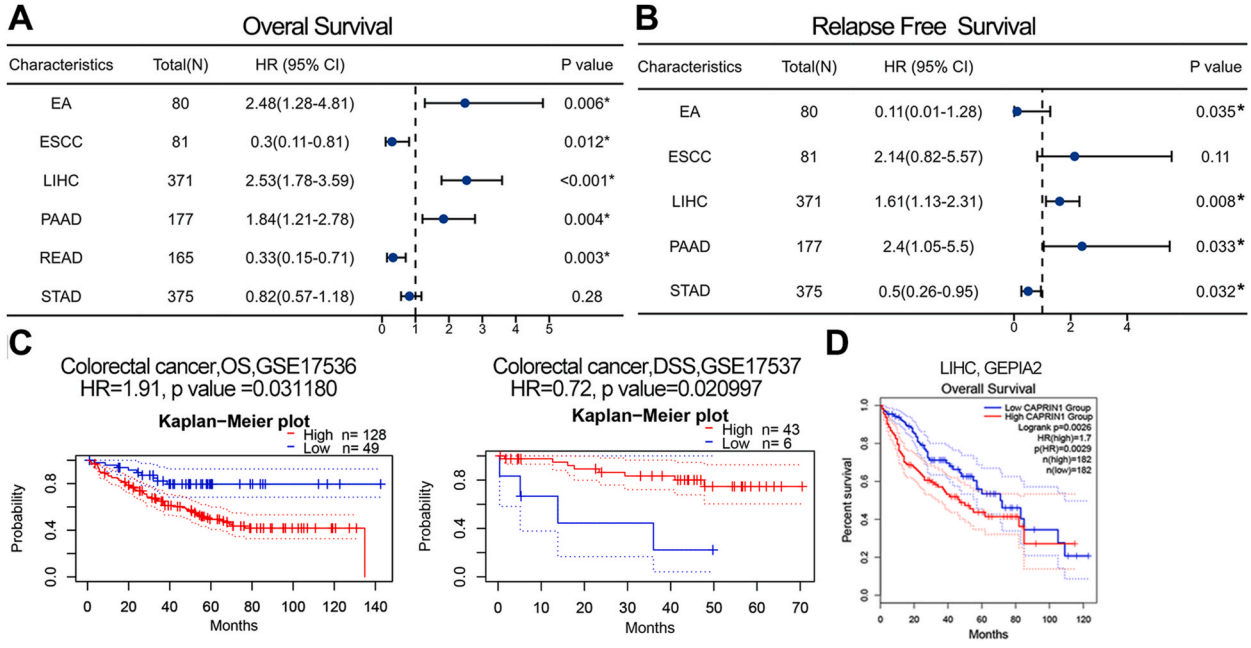

Fig. 2. Prognostic value of CAPRIN1 in human cancers. (A) Forest plot showing the relationship between CAPRIN1 expression and overall survival and relapse -free survival (B) in the Kaplan-Meier curves database. (C) Kaplan-Meier survival curves stratified in the PrognoScan database according to CAPRIN1 mRNA expression in colorectal cancer. DSS, disease-specific survival. (D) Prediction of OS of LIHC from the GEPIA2 database based on the CAPRIN1 expression. $^*P < 0.05$.

in a majority of cancer cells originating from the large intestine relative to the esophagus and liver cell lines (Supplementary Fig. 1A). CPTAC database analysis revealed that CAPRIN1 protein levels were significantly increased in colon cancer, pancreatic cancer, and liver cancer tissues (Fig. 1C). IHC staining results indicated that CAPRIN1 was significantly and highly expressed in gastrointestinal tumor (CRC, LIHC, PAAD, and STAD) tissues, relative to normal ones (Fig. 1D). Moreover, IHC results revealed upregulation of

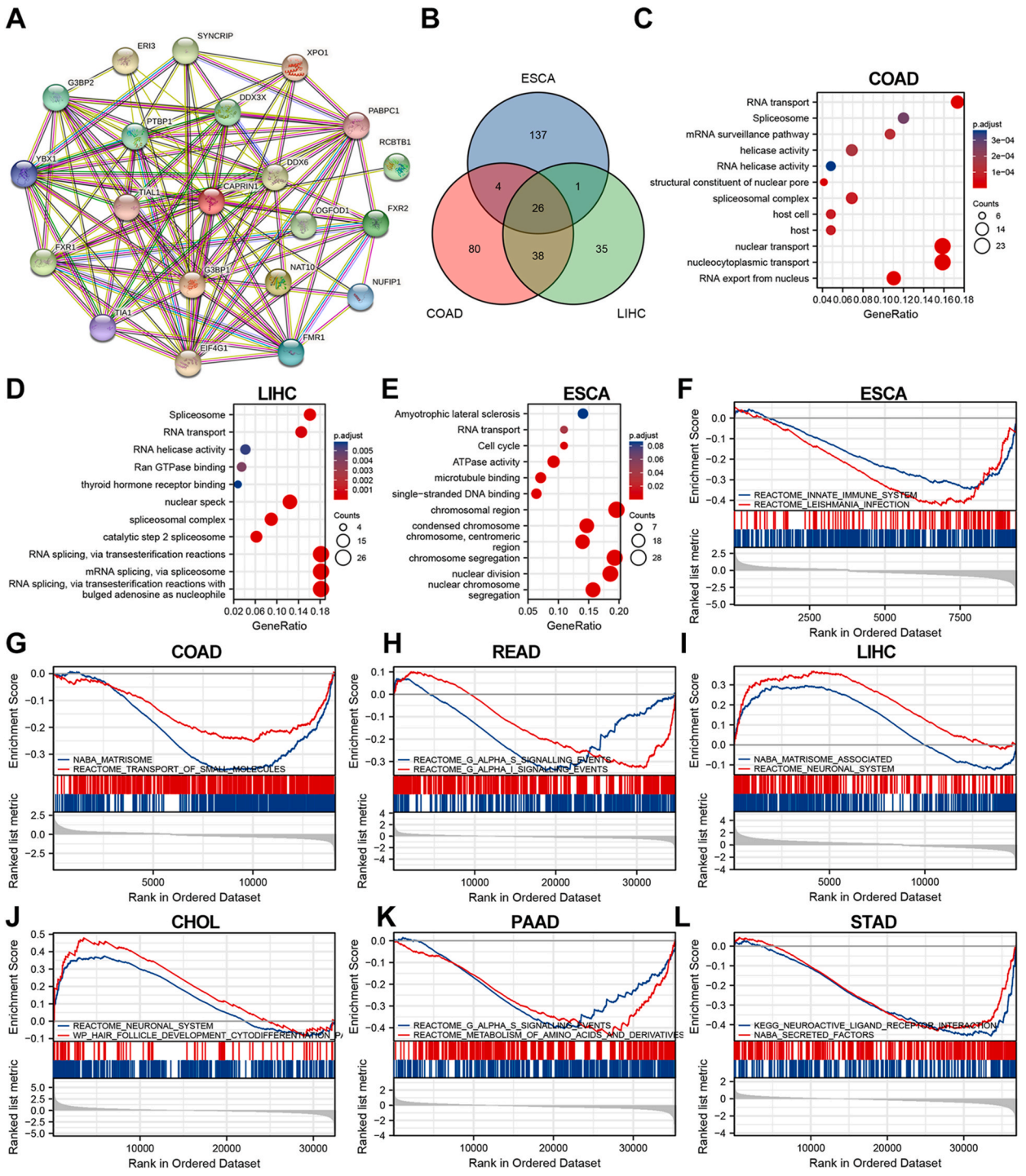

Fig. 3. Functional annotations of predicted genes and enriched pathways of CAPRIN1. (A) PPI network of CAPRIN1 obtained from the string database. (B) Venn diagram showing significantly enriched GO terms and KEGG pathways in COAD, LIHC, and ESCA. (C) Bubble plot illustrating the top 12 enriched GO terms in COAD, LIHC (D), and ESCA (E). (F–L) The 2 major pathways significantly enriched in high and low CAPRIN1 expression groups as identified by GSEA in the gastrointestinal cancer.

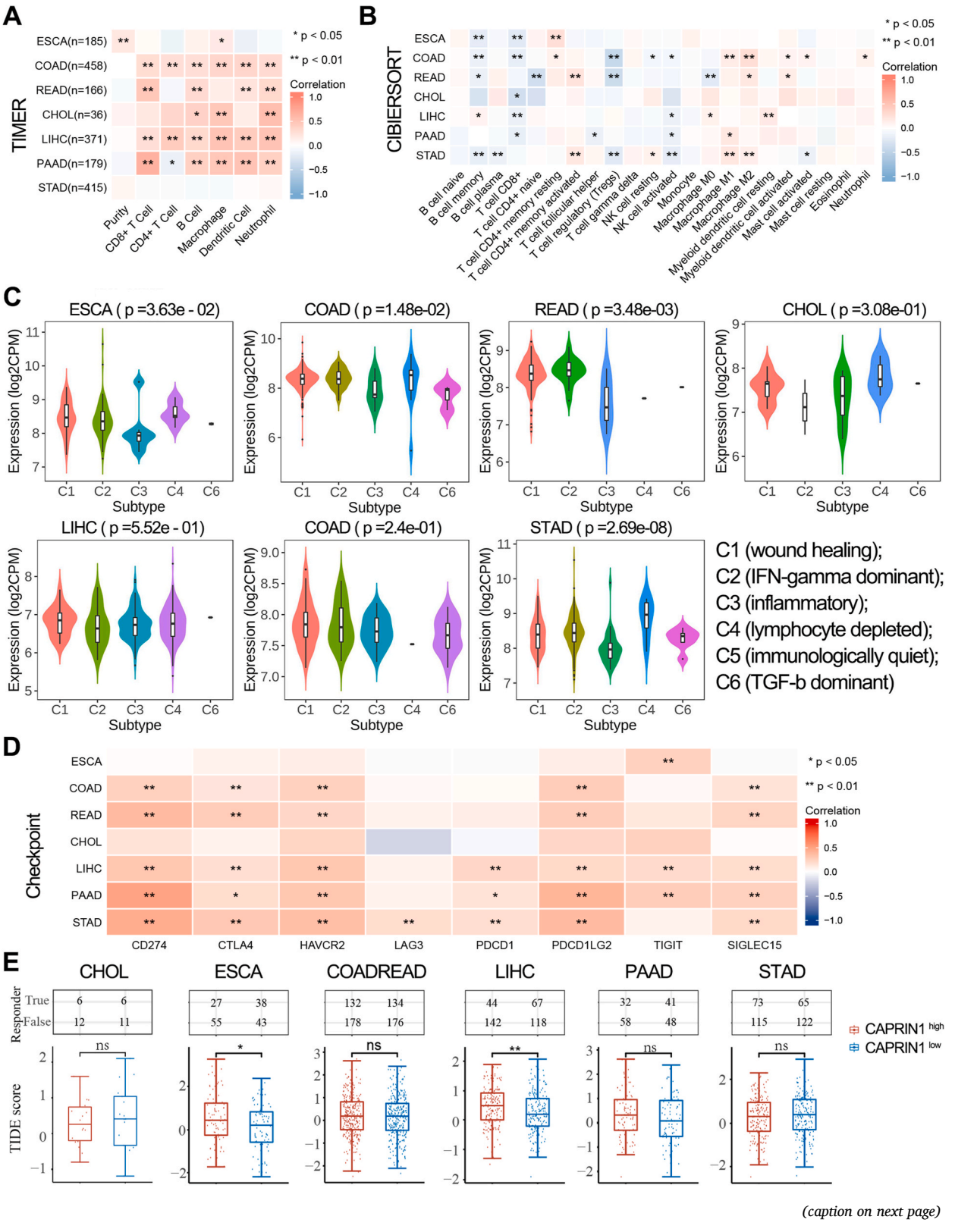

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Fig. 4. Correlation between CAPRIN1 expression and immune infiltration levels in seven types of gastrointestinal cancer. (A) Heatmap showing the relationship between CAPRIN1 expression and tumor purity, as well as immune cell infiltration in gastrointestinal cancers samples from the TIMER database. (B) Immune cell component analysis of 22 immune cells types using CIBERSORT R package in TCGA datasets. (C) Vioplot illustrating the association of CAPRIN1 expression levels and five immune subtypes across human gastrointestinal cancers samples via TISIDB. (D) Correlation matrix showing the relationship between immune checkpoints and CAPRIN1 expression in gastrointestinal cancer. (E) The distribution of potential ICB response scores in CAPRIN1 high and low groups using TIDE algorithm in TCGA cohorts. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

CAPRIN1 protein in ESCA relative to matched adjacent non-tumor tissues (Fig. 1E).

Further, we examined the relationship between CAPRIN1 expression with pathological features in gastrointestinal cancer patients, including age, tumor-node-metastasis (TNM) stage, histologic grade, etc. Consequently, CAPRIN1 expression was significantly different in the subgroups of clinical stage, including tumor stage in STAD, node stage in CRC, metastasis stage in ESCA, and histological grade in LIHC and PAAD ($P < 0.05$, Supplementary Table 2, Supplementary Table 3). Besides, CAPRIN1 expression was significantly associated with age in cancers, including ESCA, STAD, and LIHC ($P < 0.05$). CAPRIN1 protein expression was significantly and highly expressed in patients with COAD at stage 1 than at stage 3, and in obese than extreme weight and extremely obese patients (Supplementary Fig. 1B). For instance, CAPRIN1 protein expression in younger patients (21–40 years) was significantly higher than that in older patients (41–80 years) with LIHC ($P < 0.05$). The results were consistent with those based on TCGA databases. Nevertheless, the protein expression of CAPRIN1 was weakly expressed in younger patients with PAAD ($P < 0.05$).

In conclusion, our results revealed that CAPRIN1 mRNA and protein levels were unregulated, and correlated with the stages, grades, age, and clinical features of several tumors including most gastrointestinal cancers.

3.2. High CAPRIN1 expression is an independent prognostic factor in gastrointestinal cancer

Integrated analysis showed the significant relationship of CAPRIN1 expression with poor clinical prognosis of gastrointestinal cancer in Kaplan-Meier curves database ($P < 0.05$). Among them, CAPRIN1 significantly associated with the OS time of EA, ESCC, LIHC, PAAD and READ (Fig. 2A, logrank $P < 0.05$). High CAPRIN1 expression significantly associated with poor relapse-free survival (RFS) in patients with EA, LIHC, PAAD, and STAD (Fig. 2B). Furthermore, downregulated CAPRIN1 expression predicted poor OS and DSS in CRC as determined by PrognoScan (Fig. 2C). According to analysis in GEPIA2 database, OS time was markedly reduced in patients with increased CAPRIN1 mRNA levels in LIHC (Fig. 2D, logrank $P < 0.05$). Among the seven types of gastrointestinal cancer evaluated in more than 1900 patients, the AUC of the ROC curve was greater than 0.8 across all samples (Supplementary Fig. 2). Notably, CAPRIN1 displayed superior diagnostic accuracy (AUC >0.9) in distinguishing patients with STAD, PAAD, and CHOL from the normal controls. Moreover, CAPRIN1 showed high diagnostic value in the other 4 cancer types, including ESCA (0.836), COAD (0.880), READ (0.819), colorectal cancer (0.866) and LIHC (0.870). These results suggested that CAPRIN1 may be a valuable predictor for survival for gastrointestinal cancer.

3.3. Functional annotations and signaling pathways regulated by CAPRIN1

The potential co-expression genes with CAPRIN1 were screened by PPI (Fig. 3A). Summarily, GAP SH3 domain-binding protein 1 (G3BP1) was the gene with the most outstanding connectivity, followed by N-Acetyl transferase 10 (NAT10), G3BP2. GO terms revealed that 26 commonly gene sets were enriched in CAPRIN1-correlated genes in ESCA, COAD, and LIHC, including RNA transport, RNA localization, chromosomal region, and DNA-dependent ATPase activity, among others (Fig. 3B and Supplementary Table 4). A bubble g raph of these findings demonstrated that co-expressed genes of CAPRIN1 were mainly involved in RNA transport, nuclear transport, nucleocytoplasmic transport in COAD, RNA splicing via transesterification reactions, mRNA splicing, and RNA transport in LIHC, chromosome segregation, nuclear division, Cell cycle, and ATPase activity in ESCA (Fig. 3C–E). GSEA analysis implied that the primary signaling pathways which CAPRIN1 is involved in 7 gastrointestinal cancer subtypes (Fig. 3F-L), especially the immune system, including REACTOME_innate immune_system (NES = −1.577, $P < 0.001$) and REACTOME_leishmania_infection (NES = −1.818, $P < 0.001$) in ESCA, NABA_ matrisome in COAD (NES = - 2.206, P = 0.001) and LIHC (NES = 1.498, $P = 0.001$), REACTOME_ neuronal in LIHC (NES = 1.786, $P = 0.001$), CHOL (NES = 1.686, $P = 0.002$), and REACTOME_G _alpha_s_signalling_events in READ (NES = −1.499, $P = 0.001$), PAAD (NES = −1.654, $P = 0.001$). The impact of CAPRIN1 on tumor immune infiltrations varies across different tumor types with varying immunogenicity, providing a novel perspective on therapeutic strategy for gastrointestinal cancers.

3.4. CAPRIN1 expression positively correlates with immune cell infiltration in gastrointestinal cancer

TIMER analysis results showed that CAPRIN1 upregulation is significantly and positively correlated with tumor purity (r = 0.201, $P = 6.73e-03$) and macrophages (r = 0.169, $P = 2.31e-02$) in ESCA patients (Fig. 4A), as well as infiltration degrees of $CD8^+$ T cells, $CD4^+$ T cells, B cells, macrophages, neutrophils and dendritic cells in COAD, LIHC and PAAD. Nevertheless, a heatmap generated after CIBERSORT analysis revealed that CAPRIN1 has a significant negative correlation with over half of the marker sets involved in different immune cells in ESCA, COAD, READ, LIHC, and STAD (Fig. 4B). Besides, expression level of CAPRIN1 was significantly different in five immunophenotypes, comprising wound healing, IFN-gamma dominance, inflammation, lymphocyte depletion, and TGF-β dominance, in ESCA, COAD, READ, and STAD, but not in CHOL, LIHC, and PAAD (Fig. 4C). Results from correlating CAPRIN1 with checkpoint genes showed a strong positive association with five immune checkpoint inhibitors, such as PD-L1 (CD274), CTLA4,

TIM3 (HAVCR2), PD-L2 (PDCD1LG2), and SIGLEC15 in COAD, READ, LIHC, PAAD, and STAD (Fig. 4D). To determine whether CAPRIN1 expression could influence response to immunotherapy, patient data analysis were analyzed using the TIDE tool. The results showed that TIDE score in patients with high CAPRIN1 expression was significantly higher in ESCA and LIHC, but not in other cancer types (Fig. 4E).

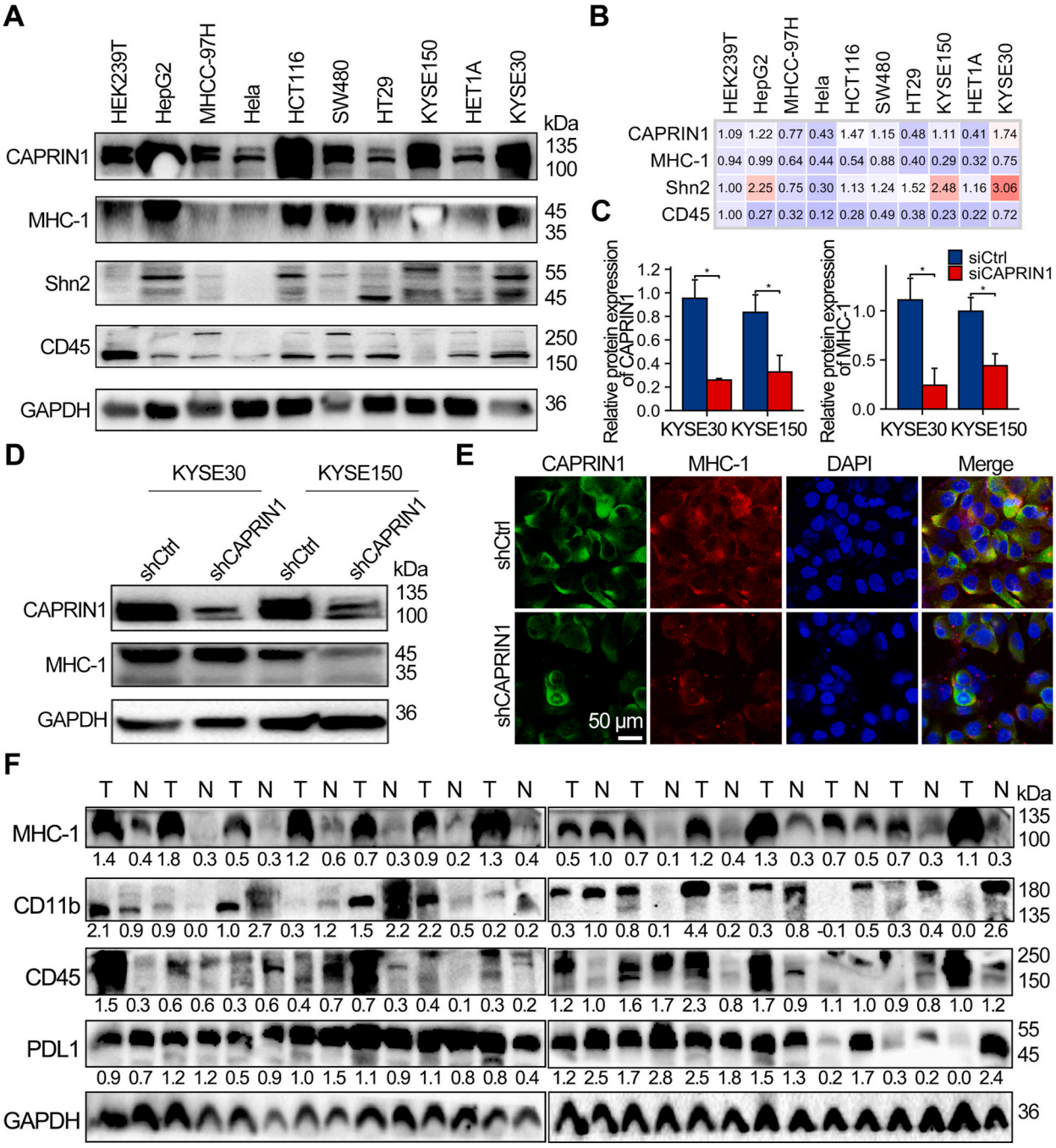

Fig. 5. The expression levels of CAPRIN1 and immune cells markers in gastrointestinal cancer cells and ESCA tumor tissues. (A–B) CAPRIN1, MHC-1, Shn2, CD45 proteins were expressed in different cells, including HEK239T, liver cancer cells (HepG2 and MHCC-97H), Hela, colon cancer cells (HCT116, SW480 and HT29), normal squamous epithelial cell line HET1A, ESCA cells (KYSE150, KYSE30). (C–D) Western blot analysis showing the expression of CAPRIN1 and MHC-1 in KYSE30, KYSE150, and MHCC-97H cells following CAPRIN1 knockdown. (E) Representative immunofluorescence images showing CAPRIN1 and MHC-1 expression levels following stable CAPRIN1 knockdown in KYSE150 cells. (F) The expression level of different immune markers (MHC-1, CD11b, CD45, and PD-L1) as determined by Western blotting in 14 paired ESCA tissues and adjacent tissues. T, ESCA tissues; N, paired adjacent normal tissues. *$P < 0.05$.

Further, we analyzed the relationship between CAPRIN1 expression levels with different gene markers involved in innate (Supplementary Table 5) and adaptive (Supplementary Table 6) immune cells in ESCA, COAD, and LIHC across the TIMER database. As a result, CAPRIN1 strongly correlated with the expression of most immune cell markers in COAD and LIHC. Notably, the association changed dramatically along with purity adjustment in ESCA, however, it was not apparent in COAD and LIHC. Innate immunity gene markers involving cells affected by CAPRIN1 expression after purity adjustment in ESCA included CD16 of monocytes, CCL2 of TAMs, CD206, CD163, and IL10 of M2 macrophages, CD11b and CD15 of neutrophils, as well as BDCA-4 and CD11c of dendritic cells (Supplementary Table 5). On the other hand, adaptive immunity gene markers in ESCA, affected by CAPRIN1 expression, included Th1 (STAT1 and STAT4), Th2 (IL13, STAT5A, and STAT6), Th17 (STAT3), and Tfh (CD278). Additionally, Treg-associated markers

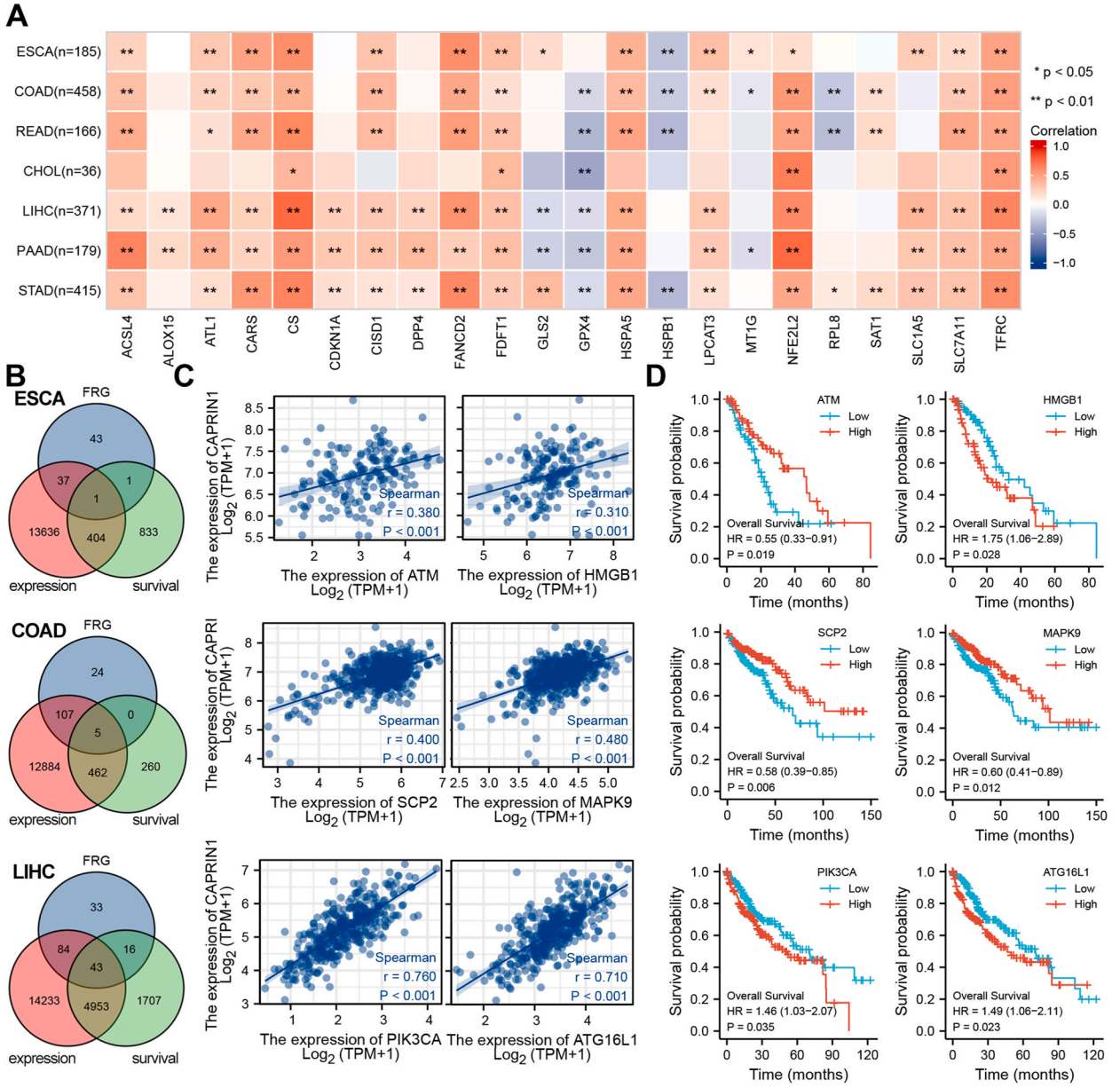

Fig. 6. The distribution of FRGs expression in patients with high- and low- CAPRIN1 expression from TCGA-gastrointestinal cancer cohort. (A) Positive (red) and negative (blue) correlation between 22 FRGs and CAPRIN1 expression. The thicker the line, the stronger the correlation between the two genes. (B) A Venn diagram showing the number of overlapping FRGs that were significantly correlated with CAPRIN1 expression, (Person's correlation coefficient), differentially expressed between tumor and paracancer groups, and survival analysis in TCGA-ESCA, TCGA-COAD, TCGA-LIHC. (C) Scatter plot indicating the co-expression between CAPRIN1 with ATM and HMGB1 in ESCA, MYB, and MAPK9 in COAD, PIK3CA, and ATG16L1 in LIHC. (D) Kaplan-Meier survival curves showing that the overall survival curves of the overlapped FRGs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(FOXP3, CCR8, STAT5B, and CD25) and T cell exhaustion markers (CTLA4 and TIM-3) positively correlated with CAPRIN1 expression levels in ESCA (Supplementary Table 6).

The expression profiles of different gastrointestinal cancer cell lines were evaluated to further assess the function of CAPRIN1 in immune infiltration. Consequently, CAPRIN1 protein in tumor cells was highly expressed in liver cancer cells (HepG2), colon cancer cells (HCT116, SW480), ESCA cells (KYSE150, KYSE30), particularly in HCT116 and KYSE30 (Fig. 5A and B). High expression of MHC-1 was found in HepG2, SW480, and KYSE30 cells. In addition, Shn2 was highly expressed in HepG2, HCT116, SW480, KYSE150, and KYSE30 cancer cell lines. However, CD45 was ubiquitously expressed in most cell lines, except in Hela and KYSE150. The knockdown efficiency of CAPRIN1 protein was evident in KYSE30 and KYSE150 cells (Fig. 5C and D). CAPRIN1 knockdown significantly decreased the protein level of MHC-1 by the immunoblot (Fig. 5C and D) and immunofluerence staining analysis (Fig. 5E). As shown in Fig. 5F, MHC-1, CD11b, and CD45 were significantly upregulated in tumor tissues of ESCA patients, compared to normal tissues, whereas the PD-L1 expression remained unchanged. Collectively, these findings indicate that CAPRIN1 can be a novel immunological biomarker in gastrointestinal cancer.

3.5. *High CAPRIN1 expression correlates with ferroptosis in gastrointestinal cancer*

Consensus clustering analysis of FRGs on the TCGA-gastrointestinal cancer in CAPRIN1-high expression tumors (Fig. 6A) revealed a significantly positive correlation between CAPRIN1 expression with ACSL4, ATL1, CARS, CS, CISD1, FANCD2, FDFT1, HSPA5, NFE2L2, SLC7A11, and TFRC genes in all cancer types, except CHOL ($P < 0.05$). Meanwhile, CAPRIN1 expression had a significant negative correlation with GPX4 and HSPB1 genes in most cancer types ($P < 0.05$). Overlapping CAPRIN1-correlated FRGs with differentially expressed genes and survival analysis data revealed 1 (high mobility group protein B1, HMGB1), 5 (MAPK9, MYB, SCP2, DRD4, and CDKN2A), and 43 (PIK3CA, NRAS, ATG16L1, ZFP69B, ATG13, TFRC, ATG7, and EIF2S1, among others) hub genes in ESCA,

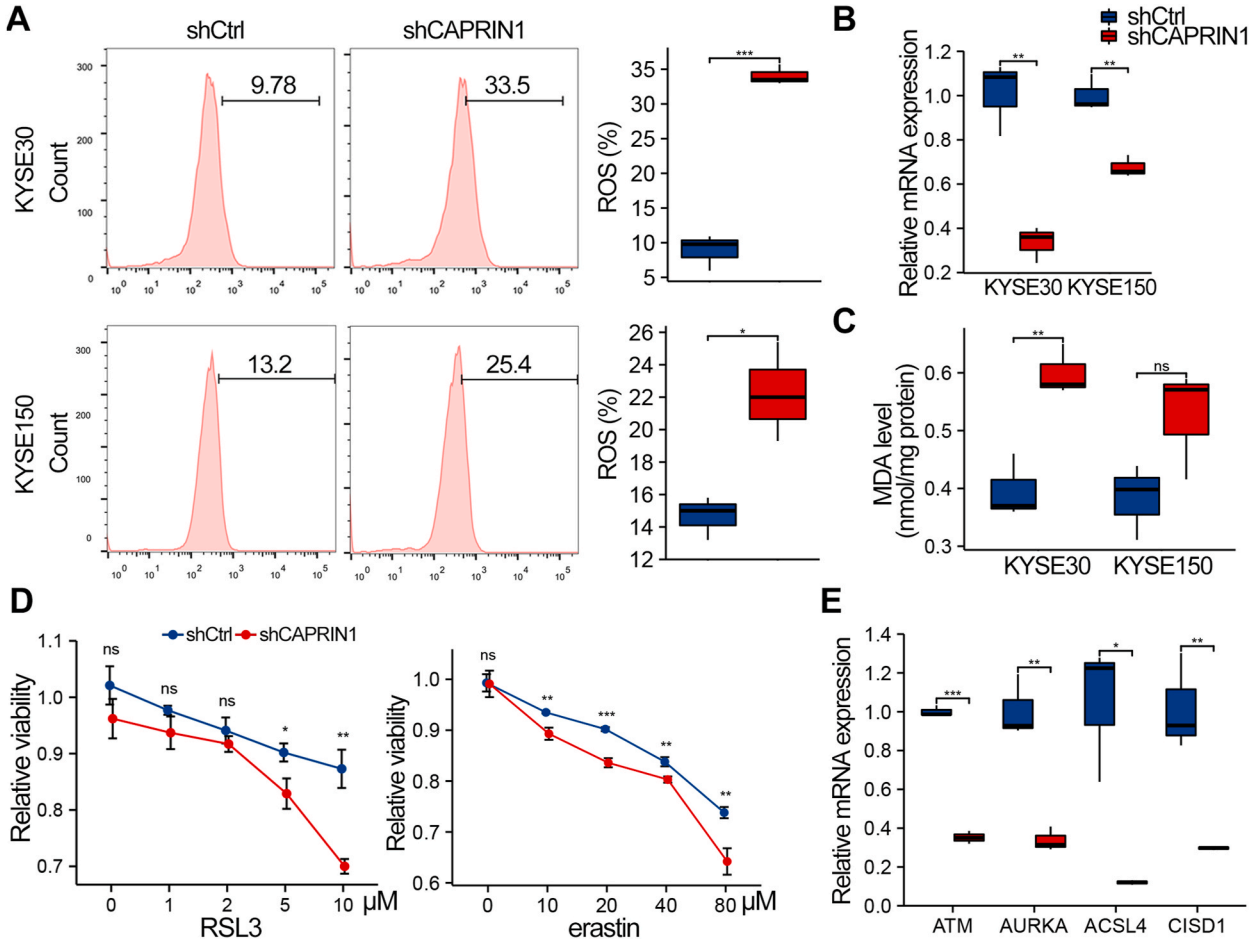

Fig. 7. Silencing CAPRIN1 enhances ferroptotic cell death. (A) Levels of cellular ROS determined by flow cytometry using H2DCFDA. (B) qRT-PCR was performed to validate effective shRNA-mediated suppression of CAPRIN1 in the human ESCA cell lines. (C) Levels of malondialdehyde (MDA) with CAPRIN1 knockdown. (D) Effects of CAPRIN1 on KYSE30 cell proliferation 48 h after treatment with RSL3 or erastin at the indicated concentrations. (E) Expression profiles of FRG mRNAs 48 h after CAPRIN1 knockdown in KYSE30 cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

COAD, and LIHC, respectively (Fig. 6B). We observed a significant positive correlation between CAPRIN1 mRNA expression with ATM ($r = 0.380$, $P < 0.001$) and HMGB1 ($r = 0.310$, $P < 0.001$) in ESCA, with SCP2 ($r = 0.400$, $P < 0.001$) and MAPK9 ($r = 0.480$, $P < 0.001$) in COAD. A similar trend observed on PIK3CA ($r = 0.760$, $P < 0.001$) and ATG16L1 ($r = 0.710$, $P < 0.001$) in LIHC (Fig. 6C). Notably, the hub genes were significantly associated with CAPRIN1 expression and prognosis of ESCA, COAD, and LIHC, either positively or negatively ($P < 0.05$, Fig. 6D). shRNA targeting CAPRIN1 in the ESAC cell line was used to further validate the role of CAPRIN1 in ferroptosis. Knocking down CAPRIN1 significantly decreased the CAPRIN1 mRNA expression (Fig. 7B) and mediated an apparent increase in both ROS levels (Fig. 7A) and MDA production (Fig. 7 C) in the ESCA cell line. We evaluated whether knocking down CAPRIN1 affects ferroptosis under ferroptotic stress, and found that its downregulation promoted the death of KYSE30 cells induced by erastin or RSL3 in a dose-independent manner (Fig. 7D). Furthermore, CAPRIN1 knockdown significantly downregulated the expression of ATM, AURKA, ACSL4, and CISD1 mRNAs (Fig. 7E). Collectively, these results show that CAPRIN1 potentially regulates ferroptosis in gastrointestinal cancer.

4. Discussion

Numerous studies have not only demonstrated a functional link between CAPRIN1 expression and the occurrence of diseases, especially tumors [44,45], but also CAPRIN1's ability to predict poor survival of patients with lung and liver cancers [26,46]. In this study, we searched multiple public databases to investigate expression patterns, alteration frequencies, activated pathways, and prognostic potential of CAPRIN1 in different tumor types; this is geared towards clarifying its role in tumorigenesis and the development of gastrointestinal cancer. Our findings confirmed that CAPRIN1 is a prognostic biomarker for seven types of gastrointestinal cancer, as demonstrated by a high diagnostic accuracy (AUC >0.8), thereby affirming its clinical relevance. So far, studies describing the potential prognostic role of CAPRIN1 and its involvement in the pathogenesis of different gastrointestinal tumors have not reached maturity.

Also, the molecular mechanisms underlying its action remain unknown. Several recent studies have reported G3BP-Caprin1-USP10 complexes could mediate stress granule condensation [23,25,47]. The PPI results suggested that G3BP1, NAT10, and G3BP2 were the genes with the most outstanding connectivity with CAPRIN1. CAPRIN1 has been implicated in mRNA localization/stability and translational regulation [48]. CAPRIN1 has previously been identified as a RNA binding regulator and is responsible for mRNA transport [49]. In the present study, GO and KEGG terms and pathway enrichment results showed that CAPRIN1 was positively or negatively involved in RNA transport, Cell cycle, innate immune, matrisome, and the neuronal system in different cancer types, all of which are strongly implicated in the initiation and progression of gastrointestinal cancer [50–52]. There is increasing evidence that suppression of expression of human Caprin-1 resulted in slowing of the proliferation rate due to prolongation of the G1 phase of the cell cycle [53]. We previously conducted animal experiments to show that Caprin-1 knockdown affect ESCA tumor growth [28]. The core matrisome mainly comprises proteins that constitute a major component of the extracellular matrix [54]. An earlier study identified specific matrisome genes to predict HBV-related LIHC and PAAD progression and prognosis using bioinformatics analysis [51,55]. Previous studies have demonstrated that CAPRIN1 haploinsufficiency causes a neurodevelopmental disorder [56], CAPRIN1/RNG105 deletion impaired synaptic strength and plasticity and long-term memory formation [57]. Abundant evidence implicates the interactions between gastrointestinal cancers and elements of the central nervous systems (such as neurotrophin, muscarinic, and β-adrenergic receptors) [50]. Understanding the role of nervous systems in tumor progression may lead to novel therapeutic targets. Whether CAPRIN1 plays a role in the gut-brain interactions in gastrointestinal cancer needs further validation.

Several studies have reported the significance of the immune microenvironment and immunotherapy with immune checkpoint blockade in cancer progression [58–60]. As such, the identification of novel prognostic and diagnostic biomarkers or immunotherapeutic targets is critical for tumor management. Previous reports indicate that CAPRIN1 is abnormally regulated in activated T or B lymphocytes or hemopoietic progenitors, differentiated monocytic leukemia, or M1 macrophages [29]. Also, it is crucial for immune B and T cell proliferation [30]. Our GSEA results showed negative enrichment of innate immune pathways in response to CAPRIN1 upregulation in ESCA. Further analysis revealed that CAPRIN1 expression was positively correlated with B cell, macrophage, neutrophil infiltration levels, and diverse immune marker sets in several cancer types, especially in COAD and LIHC. Specifically, we found a significant association between CAPRIN1 expression with immune markers, including T cells, macrophages, monocytes, and neutrophils, with most immune cells changing dramatically after purity adjustment in ESCA. We revealed that the relationship between immune infiltration and CAPRIN1 in TIMER database was different from CIBERSORT database. Different databases may have different algorithms and get the different results. Further research is required to determine the functional mechanisms. Researchers showed that M1-derived extracellular vesicles promote immunological memory in colon cancer [61]. In addition, macrophage polarization-associated lnc-Ma301 interacts with caprin-1 to inhibit hepatocellular carcinoma metastasis through the Akt/Erk1 pathway [62]. The effect of CAPRIN1 on tumor immune infiltrations differs between different tumor types for different immunogenicity. Therefore, this provides a new perspective on the treatment options for gastrointestinal cancers.

Previous studies have demonstrated that CAPRIN1 regulates interferon (IFN)-mediated innate immunity by interacting with Stat1 [63], G3BP1, and G3BP2 [64] mRNAs. However, its regulatory roles and mechanisms in cancer progression are poorly understood. Recent studies have demonstrated that the IFN-I/STAT1 signaling pathway can regulate antitumor immune responses by modulating innate and adaptive immunity [65]. In the current study, CAPRIN1 overexpression presented a positive correlation with markers of infiltrating immune cells in COAD and LIHC. For instance, CAPRIN1 was significantly correlated with adaptive immunity, and several gene markers involved in innate immunity, including CD16 of monocytes, CCL2 of TAMs, M2 macrophages (CD206, CD163, and IL10), neutrophils (CD11b and CD15), and dendritic cells (BDCA-4 and CD11c) in ESCA. This was further supported by experimental evidence demonstrating differential expression of immune genes were expressed in different gastrointestinal cancer cell lines and ESCA

tumor tissues. In addition, the expression of CAPRIN1 and CD11b proteins was elevated in different gastrointestinal cancer cell lines and tumor tissues from ESCA patients. MHC-I is an essential protein that recognizes tumor-specific $CD8^+$ T cells and influences the efficacy of immunotherapy in multiple cancer types [66]. We found that the protein expression of MHC-1 and as well as its binding protein Shn2 was elevated in cancer cell lines, such as HepG2 (LIHC), HCT116 and SW480 (COAD), and KYSE150 and KYSE30 (ESCA) as well as ESCA tumor tissues. It was also determined that CAPRIN1 knockdown decreased the expression of MHC-1.

Furthermore, high levels of CAPRIN1 were positively correlated with the expression of several immune checkpoint genes, such as PD-L1 (CD274), CTLA4, TIM3 (HAVCR2), PD-L2 (PDCD1LG2), and SIGLEC15 in most gastrointestinal cancer. Among them, CD274, PDCD1LG2, and HAVCR2, which are commonly expressed on T-cells, were strongly correlated with CAPRIN1 expression in COAD, LIHC, PAAD, READ and STAD. Additional studies using by IHC or flow cytometry could further validate these findings. Immune checkpoint blockade (ICB) therapy has achieved remarkable clinical benefits in various types of cancers. Since patients with higher TIDE score are more likely to have a higher opportunity of antitumor immune escape [67–69]. Combined with TIDE algorithm analysis, we found that patients with high CAPRIN1 expression and higher TIDE score are more promising in responding to ICB in ESCA and LIHC. The MHC-1 expression also demonstrated a significant association with PD-L1 in advanced esophagus carcinomas [70,71]. It was reported that a ribonucleoprotein complex comprising CAPRIN1, c-Myc and HIF-1 mRNAs participates in inflammatory processes [72]. Among these components, c-Myc plays a vital role in the generation of host immune responses including expression of immune checkpoint genes CD47 and PD-L1 [73]. Western blot analysis revealed that PD-L1 protein level was not significantly different between ESCA tumor and normal tissues. The discrepancy between our results and those of prior studies [74] could be the smaller number of enrolled participants or different methods employed across populations. In addition, CAPRIN1 expression exhibited a significant correlation with IFN-gamma dominant and inflammatory, lymphocyte depletion in ESCA, COAD, READ, and STAD, which was in line with prior findings [63,64]. Taken together, our findings demonstrate that CAPRIN1 played a key role in the regulation of the tumor immune microenvironment of gastrointestinal cancer. However, the potential molecular mechanism requires further study.

RBPs have been identified as oncogenic drivers that regulate ferroptosis in cancer cells [75,76]. Additionally, previous reports have reported that CAPRIN1 could modulate the cellular oxidative stress by interacting with the G3BP1 mRNA [23]. For example, Mao et al. [77] reported that G3BP1-p53 interaction caused cell cycle arrest, apoptosis, and ferroptosis. In the present study, analysis of the PPI network showed that the G3BP1 was the most important gene associated with CAPRIN1. Correlation results demonstrated that CAPRIN1 was significantly correlated with several FRGs in gastrointestinal cancer except CHOL. Furthermore, HMGB1 was upregulated in cancer tissues and associated with CAPRIN1 and poor prognosis of ESCA patients. HMGB1 is a novel regulator of ferroptosis that is involved in various biological processes, especially in inflammatory disorders and cancer [78]. In addition, CAPRIN1 was strongly associated with PIK3CA and ATG16L1 expression in LIHC, which were over-expressed in tumor tissues and correlated with poor patient prognosis. It was also found that COAD patients with low SCP2 and MAPK9 expression exhibited worse prognosis. Results from a previous systematic analysis demonstrated that the promoter of MAPK9 was aberrantly hypermethylated in colorectal cancer [79]. Our experimental data revealed that knockdown of CAPRIN1 expression triggered ROS and MDA accumulation in ESCA cells, both of which promote ferroptosis. CAPRIN1 increases sensitivity to RSL3 and erastin-induced ferroptosis, and upregulates the expression of ATM, AURKA, ACSL4, and CISD1. A previous study reported that ATM is an essential kinase that involved in the development of ferroptosis through its effects on iron metabolism [80]. It was further showed that ATM is activated in response to DNA damage in a subset of malignant tumors [81]. Therefore, it is necessary to investigate the relationship between CAPRIN1 expression and ferroptosis in gastrointestinal cancer.

Moreover, tumor infiltration, ferroptosis, and the interaction among them influence tumor progression. Ferroptosis signatures could predict clinical diagnosis, prognosis and define immune microenvironment in cancers [82–84]. Therefore, CAPRIN1 may promote the progression of gastrointestinal cancer by regulating immune infiltration and ferroptosis. Our study has certain limitations, the focus of the study was on the bioinformatic characterization of CAPRIN1 in gastrointestinal cancer types. Therefore, it was difficult to explain the mechanism of each cancer type with precision. We were unable to determine whether CAPRIN1 may affect patient survival across biological processes, such as immune infiltration and ferroptosis. The functions and in-depth mechanisms of CAPRIN1 were only explored *in vitro*. Further investigations are needed to confirm the CAPRIN1's function and underlying mechanism in the growth and progression of different types of cancers.

This study found that CAPRIN1 was highly expressed in cancer tissues and influenced the survival of all gastrointestinal cancer types. Interestingly, our results indicate that CAPRIN1 also fine-tunes the innate and antigen-dependent immune response. CAPRIN1 may associate with ferroptosis, suggesting its potential therapeutic and prognostic value.

Ethical approval and consent to participate

The study was reviewed and approved by the Ethics Committee of Taihe Hospital (No.2022KS010), and conducted in accordance with the principles of the Helsinki Declaration.

Data availability statement

The datasets presented in this study can be found in online repositories. The original contributions presented in the study are included in the article/Supplementary Material online. Further inquiries can be directed to the corresponding author.

CRediT authorship contribution statement

Yan Gao: Writing – original draft, Data curation, Conceptualization. **Ruimin Wu:** Methodology, Data curation. **Zhijun Pei:** Methodology, Funding acquisition. **Changbin Ke:** Methodology, Funding acquisition. **Daobing Zeng:** Software, Methodology. **Xiaohui Li:** Supervision, Formal analysis. **Yanmin Zhang:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by Shaanxi Province Science Fund for Distinguished Young Scholars (2023-JC-JQ-59), the Shaanxi Province Science and Technology Development Plan Project (2022ZDLSF05-05), the Hubei province's Outstanding Medical Academic Leader program, the Foundation for Innovative Research Team (No.T2020025) of Hubei Provincial Department of Education, and the Key Discipline Project of Hubei University of Medicine. We would like to thank HOME for Researchers (https://www.home-for-researchers.com/static/index.html#/) for English language editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28794.

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