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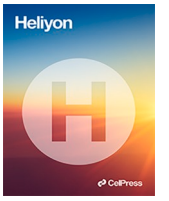

High CTCF expression mediated by FGD5-AS1/miR-19a-3p axis is associated with immunosuppression and pancreatic cancer progression

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ABSTRACT

The most common reason for cancer-related death globally is predicted to be pancreatic cancer (PC), one of the deadliest cancers. The CCCTC-binding factor (CTCF) regulates the three-dimensional structure of chromatin, was reported to be highly regulated in various malignancies. However, the underlying biological functions and possible pathways via which CTCF promotes PC progression remain unclear. Herein, we examined the CTCF function in PC and discovered that CTCF expression in PC tissues was significantly raised compared to neighboring healthy tissues. Additionally, Kaplan-Meier survival analysis demonstrated a strong connection between elevated CTCF expression and poor patient prognosis. A study of the ROC curve (receiver operating characteristic) revealed an AUC value for CTCF of 0.968. Subsequent correlation analysis exhibited a strong relationship between immunosuppression and CTCF expression in PC. CTCF knockdown significantly inhibited the malignant biological process of PC in vitro and in vivo, suggesting that CTCF may be a potential PC treatment target. We also identified the FGD5 antisense RNA 1 (FGD5-AS1)/miR-19a-3p axis as a possible upstream mechanism for CTCF overexpression. In conclusion, our data suggest that ceRNA-mediated CTCF overexpression contributes to the suppression of anti-tumor immune responses in PC and could be a predictive biomarker and potential PC treatment target.

1. Introduction

Pancreatic cancer (PC) is a highly lethal malignancy characterized by a grim 5-year relative survival rate of approximately 10 % in the United States [1] and 7.3 % in China [1]. By 2030, it is projected to become the second leading cause of cancer-related deaths in the

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USA [2]. The formidable prognosis of PC can be attributed to challenges in early diagnosis, a high rate of metastasis, and frequent development of chemotherapy resistance [3]. In the initial stages, PC patients often lack diagnostic symptoms, and the currently available screening biomarkers lack the required sensitivity and specificity for accurate PC diagnosis [2,4]. Surgical resection and cytotoxic chemotherapy are the primary treatment modalities for PC patients [5–7]. However, due to the systemic metastasis that typically accompanies PC progression, the majority (80 %) of patients are diagnosed with unresectable metastatic PC. Although chemotherapy regimens such as gemcitabine, gemcitabine/capecitabine, FOLFIRINOX, and nab-paclitaxel are commonly employed [8–10], patients frequently develop drug resistance and experience disease progression [11]. Consequently, there is an urgent need to gain a deeper understanding of the underlying molecular mechanisms driving PC malignancy and chemotherapy resistance, as well as to identify effective prognostic markers and therapeutic targets.

CCCTC-binding factor (CTCF) is a critical DNA-binding protein that plays a pivotal role in regulating chromatin looping through its interaction with cohesion [12–14]. This protein has been shown to maintain chromatin topology [15,16], including the topologically associated domain (TAD) formation mediated by CTCF, which regulates gene expression by facilitating interactions between promoters and enhancers. Additionally, CTCF has emerged as a potent regulator of immune evasion [17]. The PD-1/PD-L1 immunosuppressive axis has been recognized as a key player in cancer cell immune escape [18,19], and therapies targeting PD-1/PD-L1 have exhibited remarkable efficacy in certain cancer types [20]. Intriguingly, the deletion of CTCF has been linked to the upregulation of PD-L1 expression on the cell surface [21]. In The Cancer Genome Atlas (TCGA) pancancer datasets, CTCF ranks as the 20th most potent tumor suppressor gene (TSG) based on the prevalent occurrence of loss-of-function mutations. Furthermore, CTCF can function as a transcription factor by binding to transcription start sites. Numerous studies have reported that promoter-proximal CTCF binding is associated with the transcriptional regulation of over 2000 genes in various adult tissues [22]. Disruption of CTCF binding and subsequent alterations in gene expression have been implicated in the pathogenesis of several diseases, including isocitrate dehydrogenase (IDH) mutant gliomas [23]. Notably, CTCF-EP300-mediated enhancers can induce chromatin remodeling and transcriptional activation of oncogenes [24]. CTCF mutations alter the polarity of endometrial glandular epithelial cells and promote tumor aggregation in endometrial cancer (EEC) [25,26]. Additionally, mutations in CTCF binding motifs diminish the binding affinity of multiple transcription factors (TFs), which is a common occurrence in gastric, liver, and colorectal cancer [27–29]. In recent years, several studies have reported significant roles of CTCF in various tumor types, including gastric cancer, colorectal cancer, liver cancer, breast cancer, and prostate cancer [30–34]. Nevertheless, there is limited research regarding the involvement of CTCF in pancreatic cancer. CTCF has been shown to influence the transcriptional activity of genes implicated in PC [35–37]. Nevertheless, the biological significance and potential mechanisms underlying CTCF-driven PC progression, as well as its suitability as a prognostic marker for PC, necessitate further investigation.

In this study, we conducted a comprehensive analysis using TCGA and GTEX datasets to investigate the expression patterns and clinical implications of CTCF across multiple cancer types. We employed Kaplan-Meier survival analysis and Receiver Operating Characteristic (ROC) analysis to assess the prognostic value of CTCF expression. Our results revealed that altered CTCF expression is associated with poor overall survival (OS) in various cancers. Additionally, we explored the relationship between CTCF expression and immune activation in pancreatic cancer (PC). We found a strong correlation between CTCF expression and immunosuppression in PC, suggesting that CTCF may play a role in immune evasion mechanisms. Furthermore, we delved into the biological function of CTCF and identified a potential lncRNA-related pathway involving the FGD5-AS1/miR-19a-3p axis in PC. This pathway may contribute to the progression and development of PC, providing insights into potential therapeutic targets. Overall, our study highlights the significance of CTCF as a factor of immune response in PC. Furthermore, CTCF shows promise as a potential therapeutic target and prognostic marker for PC. Further research is warranted to validate these findings and explore the underlying molecular mechanisms in more detail.

2. Materials and methods

2.1. Data processing

Raw counts of Bulk-RNA sequencing data and relating clinical information for 33 cancers were gotten from the TCGA and the Genotype-Tissue Expression (GTEX) databases. We also downloaded pancreatic cancer tissues and corresponding normal tissue data from PAAD. All analyses were performed with the R software v3.6.3. Spearman correlation analysis was performed to evaluate the correlations between quantitative parameters.

2.2. Kaplan-Meier Mapper analysis

The KM plotter (http://kmplot.com/analysis/) was used to explore the prognostic effect of CTCF on different cancer types and investigate the prognostic value of miRNAs and lncRNAs in PC.

2.3. Construction of the nomogram

We developed nomograms to evaluate the OS rate of PC patients, by considering the identified independent prognostic factors derived from a multivariate Cox analysis utilizing the R package RMS. We employed calibration curves and the C-index to assess the performance and accuracy of the constructed nomograms.

2.4. Functional enrichment analysis

We performed gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of CTCF-related genes in PC using Goplot.

2.5. Immune infiltration analysis

To understand the association between CTCF and immune infiltration in the tumor immune microenvironment, we used the GSVA package based on the single-sample GSEA (ssGSEA) algorithm incorporating 24 immune cells to calculate the relative enrichment fraction of all immune cells in PC. Spearman correlation analysis combined with the Wilcoxon rank-sum test was performed to observe the association between CTCF expression and infiltration of immune cells in PC.

2.6. Immunohistochemistry (IHC) staining assay

IHC staining was performed as previously described [38]. CTCF antibody was also used for the IHC staining. Sections were visualized, and images were acquired using the microscope. Proteins were assessed using the Image-Pro Plus by calculating the IOD/area.

2.7. ENCORI database analysis

The ENCORI is a web-based platform that provides computational analysis tools for exploring potential interactions between non-coding RNAs (miRNAs and lncRNAs) and transcription factors, such as CTCF. Moreover, correlations between CTCF, miRNAs and lncRNAs in PC were identified using ENCORI.

2.8. Cell culture and reagents

The PANC-1 cell line, sourced from the Cell Resource Center of the Shanghai Institute of Biotechnology, Chinese Academy of Sciences, was cultured in the DMEM medium (BioChannel Biological Technology Co., Ltd.).

2.9. Plasmids and stable cell lines

CTCF knockdown and overexpression lentivirus were purchased from Shanghai Bioegene Co. Ltd, and the siRNA of FGD5-AS1, mimics of miR-19a-3p and inhibitor of miR-19a-3p were purchased from Tsingke BiotechnologyCo. Ltd. The related sequences are listed in Supplementary Table S1.

2.10. Western blot analysis

The indicated cells were lysed using RIPA Lysis Buffer (Strong, without inhibitors) (K1120, APExBIO, Houston, USA). PAGE Gel Kits (P0105 LABLEAD Inc.) were utilized to prepare the gel for electrophoresis. The specific details of the antibodies used in this study can be found in Supplementary Table S2. The blots were visualized and analyzed using the Tanon-5200 Chemiluminescent Imaging System (Tanon, China, Shanghai).

2.11. Cell proliferation assay

Cell proliferation was evaluated by the CCK-8 assay (Cell count kit-8) (C6050, New Cell & Molecular Biotech). After 48 h of post-transfection, transfected PC cells (2×103) were obtained and plated in a 96-well plate. Subsequently, 100 μL of reagent was added at precise intervals, and the mixture was incubated for a further 2 h 2000 cells/well were plated in a 6-well plate for the colony formation assay, which was used to gauge the regenerative potential of PC cells. The colonies were manually counted after the cells had been treated with a one percent crystal violet dye solution and left at room temperature for 20 min.

2.12. Wound healing assays

The wound healing assays were performed as described previously [39]. Briefly, the cells were transfected with overexpression or knockdown plasmids for 72 h and placed onto a 6-well plate until they were grown to full confluence. Wound zones were made using a 1 mL pipette tip, incubated for 72 h in a serum-free medium, and observed and photographed every 24 h.

2.13. Cell apoptosis assays

Apoptosis was measure by detecting the expression of caspase-3 and Bcl2. And annexin V/7AAD staining (Becton, Dickinson and Company, BD) was used to detect the apoptotic levels of cells by flow cytometry.

2.14. In vivo assays

Here, 1×10^7 stable PANC-1(sh-CTCF) and control PANC-1 cells were subcutaneously injected into BALB/c nude mice. The volume and weight of the tumor were measured after three weeks.

2.15. Dual-luciferase reporter assay

HEK-293 T cells were cultured in 6-well plates at 3×10^5 cells per well for 24 h. FGD5-AS1/CTCF wild-type plasmid/mutant plasmid and miR-19-3p mimics were co-transfected into HEK-293T cells. The amount of luciferase activity was determined using the Dual-Luciferase Reporter Assay Kit DL101-01 (Vazyme Biotech Co., Ltd).

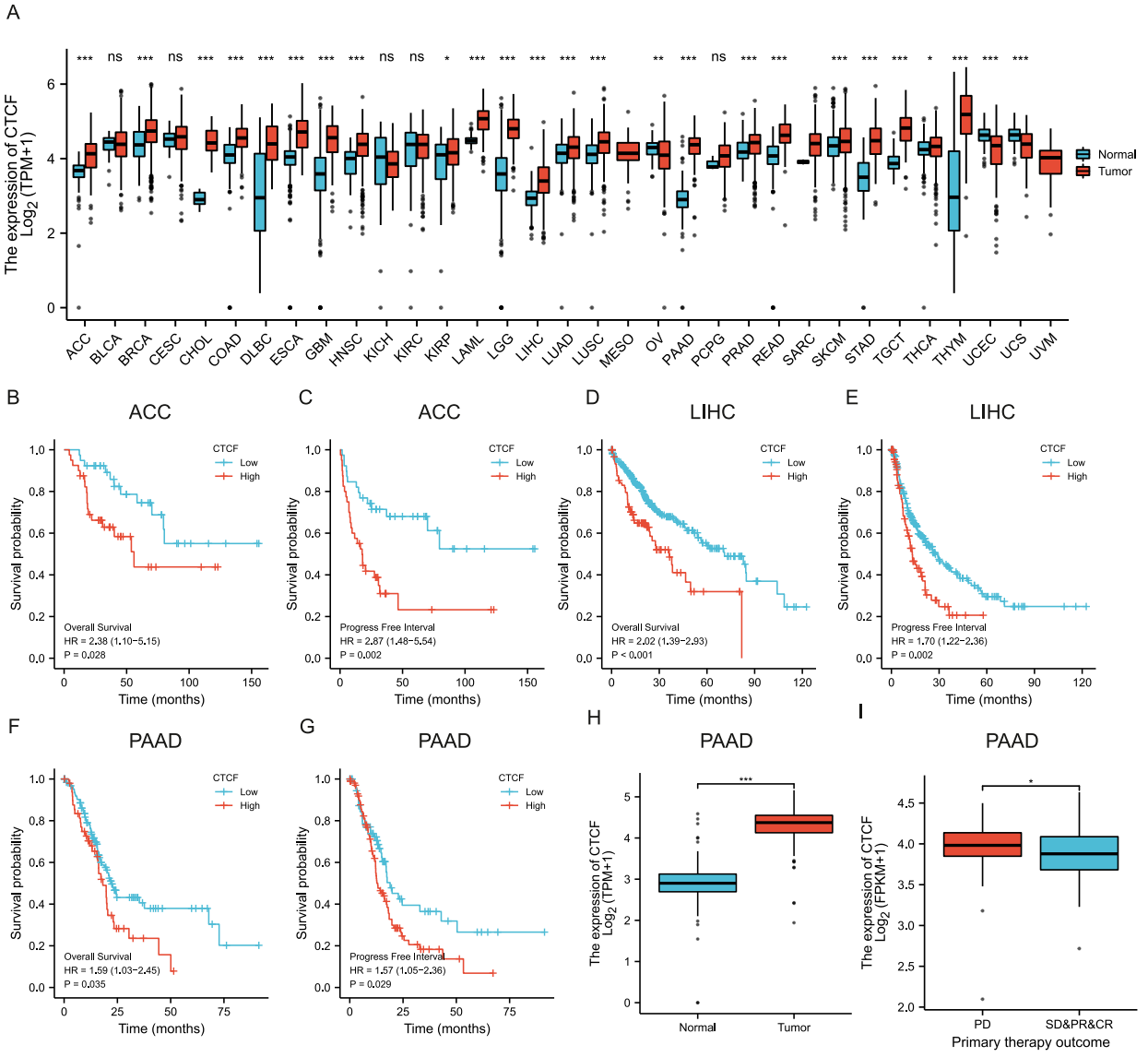

Fig. 1. Assessment of CTCF expression and prognostic in pan-Cancers. (A) CTCF expression levels in different tumor tissues and adjacent normal tissues from TCGA and GTEx databases. (B–G) Prognostic analysis of CTCF mRNA expression levels in adrenocortical cancer (ACC), liver cancer (LIHC) and pancreatic cancer (PAAD), including OS and progress free interval curves. (H) CTCF mRNA expression levels in PAAD patients and normal sample (n = 178) from TCGA and GTEx databases. (I) CTCF mRNA expression levels in PAAD progressive disease (PD) and stable disease&partial response&complete response (SD&PR&CR). NS, $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

2.16. RIP-qPCR assay

MagnaRIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) was used for RIP assay [40]. $2*10^7$ PANC-1 cells were used for RIP assays. Briefly, PANC-1 cells were lysed by RIP lysis buffer and immunoprecipitated on magnetic beads containing antibodies. In Supplementary Tables S2 and S3, the specifics of the antibodies and qPCR primers are presented.

2.17. Statistical analysis

The R platform, GraphPad Prism 8 and Zesis were applied for analysis. The experiments of this study were repeated 2–6 times, and the data are presented as mean ± SD, and the variance among the two groups was examined using Chi-square test, one-way ANOVA and the paired two-tailed Student's t-test, as appropriate.

3. Results

3.1. CTCF expression in multiple cancers and was highly expressed in PC

To observe the expression of CTCF in multi-cancer level, we obtained the mRNA expression data from TCGA and found that CTCF was upregulated in 23 of 33 cancers (Fig. 1A). In addition, KM plots indicated that CTCF was significantly associated with poorer prognosis in ACC (adrenocortical carcinoma) (Fig. 1B and C), LIHC (liver cancer) (Fig. 1D and E) and PAAD (pancreatic adenocarcinoma) (Fig. 1F and G). As shown in Fig. 1 H, I and Fig. S1, CTCF was highly expressed in progressive PC. Then, we performed IHC staining of CTCF using pancreatic ductal adenocarcinoma (PDAC) tissue microarrays from 110 pancreatic cancer patients at the Ruijin

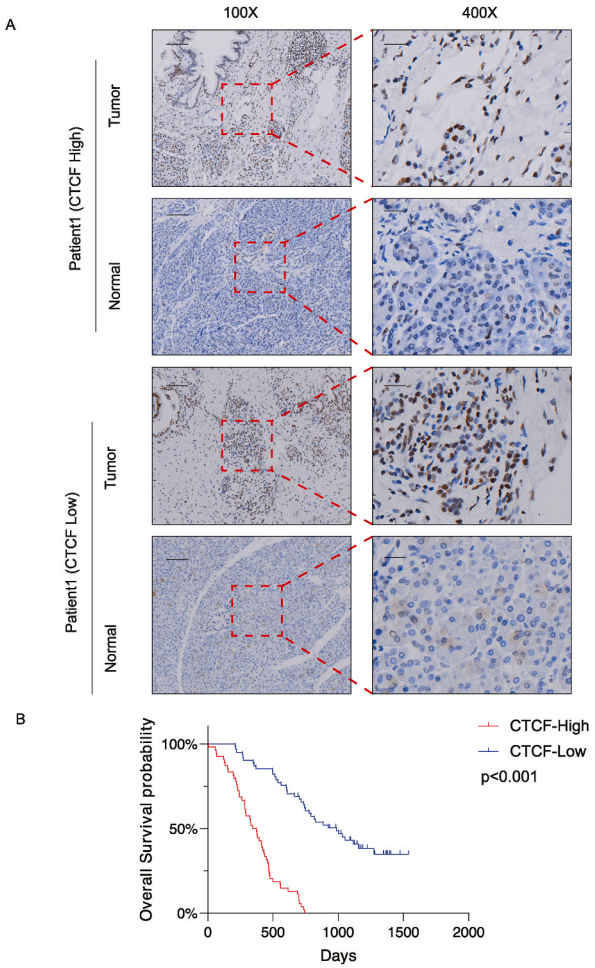

Fig. 2. CTCF was highly expressed in Pancreatic Cancer. (A) Immunohistochemical staining of CTCF expression in pancreatic cancer tissues and their matched paracancerous tissues. Original magnifications 400 × and 100 × (inset panels). (B) Kaplan Meier survival curve presenting the OS of 110 PDAC patients and matched adjacent normal samples, grouped according to the expression level of CTCF.

Hospital to demonstrate the findings (Fig. 2A and B). Considering all the above analyses, our results confirmed the expression and prognostic significance of CTCF in pan-cancer level.

3.2. Clinical diagnosis and prognostic value of CTCF in PC

The correlation between the CTCF and survival outcomes of PAAD patients from the TCGA database was assessed by the conduction of KM plot and a validation dataset (GSE21501) (Fig. 1F and G and Fig. 3A and B), and the findings indicated that CTCF contributed to poorer patient prognosis in PC (Fig. 1F and G and Fig. 3A and B). Similar results were obtained by performing Kaplan-Meier survival analysis after integrating the TCGA database and validation dataset (Fig. S2). Additionally, the ROC curve demonstrated that CTCF expression had a favorable predictive ability for distinguishing PC from normal tissues, with AUC of 0.968 (95 % confidence interval CI, 0.946–0.990) (Fig. 3C). Based on the time-dependent ROC, CTCF expression levels could be used as a better indicator for predicting the 3-year (AUC, 0.606), 4-year (AUC, 0.629) and 5-year OS (AUC, 0.733), 3-year (AUC, 0.613), 4-year (AUC, 0.619) and 5-year DSS (AUC, 0.721); and 1-year (AUC, 0.686), 3-year (AUC, 0.692) and 5-year PFI (AUC, 0.789) in PC patients (Fig. 3D–F). Similar results were observed in the analysis of GEO datasets (GSE21501) (Fig. 3G). In addition, we determined that CTCF could be a predictor of pancreatic cancer patients' outcomes, independent of clinicopathological characteristics, via univariate analysis and multivariate

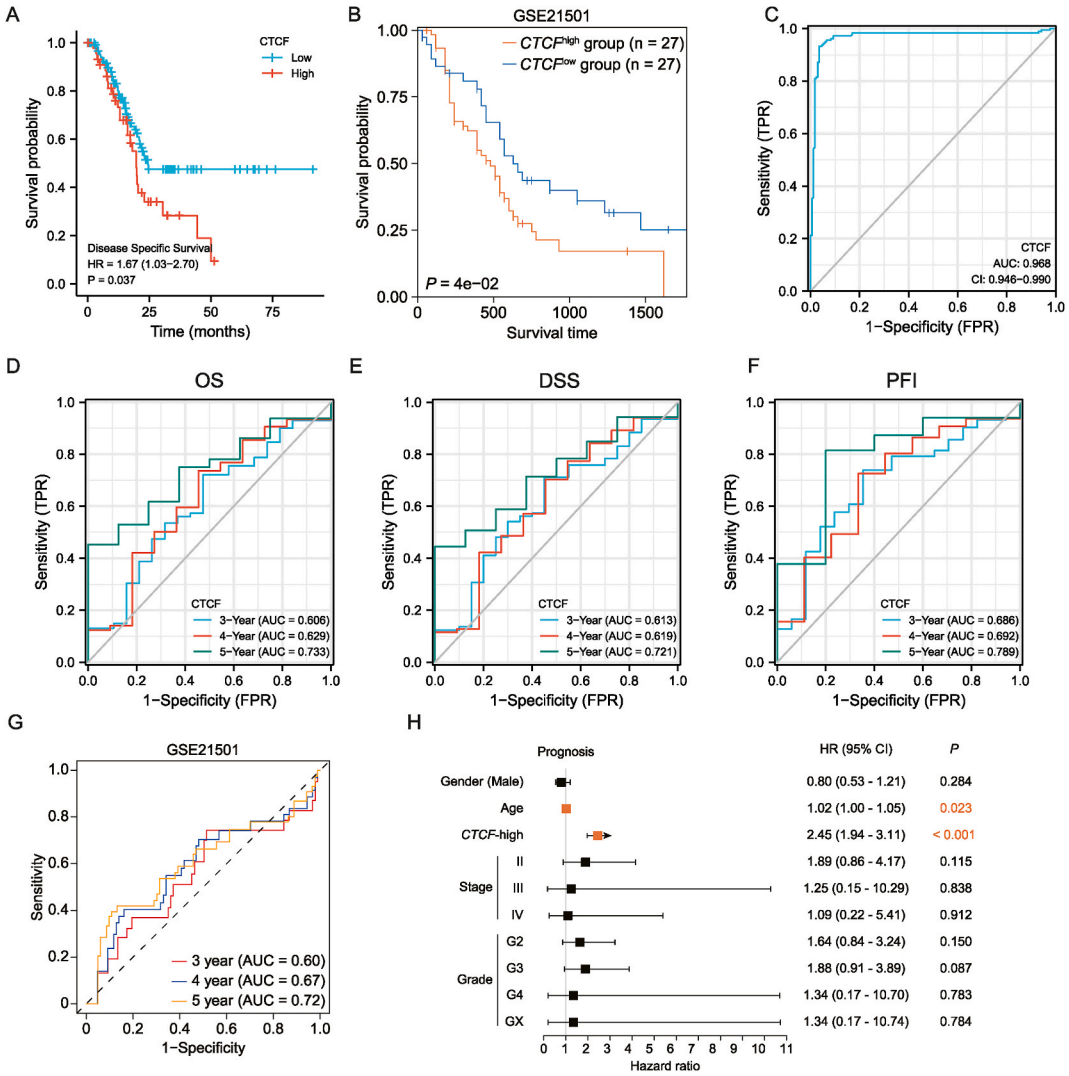

Fig. 3. Clinical diagnosis and prognostic value of CTCF in PC. (A, B) Kaplan Meier survival curve presenting the disease specific survival (DSS) of PDAC patients by the TCGA-PDAC dataset and GEO dataset (GSE21501). (C) Receiver operating characteristic (ROC) curves for classifying pancreatic cancer tissues versus normal tissues in the TCGA database. (D–F) Time-dependent ROC curves were used to determine the diagnostic value of CTCF in pancreatic cancer in the TCGA database. (G)Time-dependent ROC curves were used to determine the diagnostic value of CTCF in pancreatic cancer in the GEO database (GSE21501). Forest plot of multivariate Cox analysis for overall survival, displaying hazard ratios (HR) and corresponding confidence intervals (CI).

analysis (Fig. 3H and Table S4).

3.3. Function annotation of CTCF-related genes from GO and KEGG analyses in PC

Using LinkedOmics, we gained significant genes that had positive correlations with CTCF, and the correlations were displayed in the heatmap (Fig. 4A–C). Functional annotation (Biological Process, BP) revealed that CTCF-related genes participate in histone modification, cell cycle regulation, cytokinesis and protein-DNA complex assembly (Fig. 4D). Variations in Cell Component (CC) (Fig. 4E) and molecular functions (MF) of CTCF-related genes were associated with nuclear chromatin, nuclear speck, ribonucleoprotein granule, methyltransferase complex, transferase activity, transcription coactivator activity, hormone receptor binding

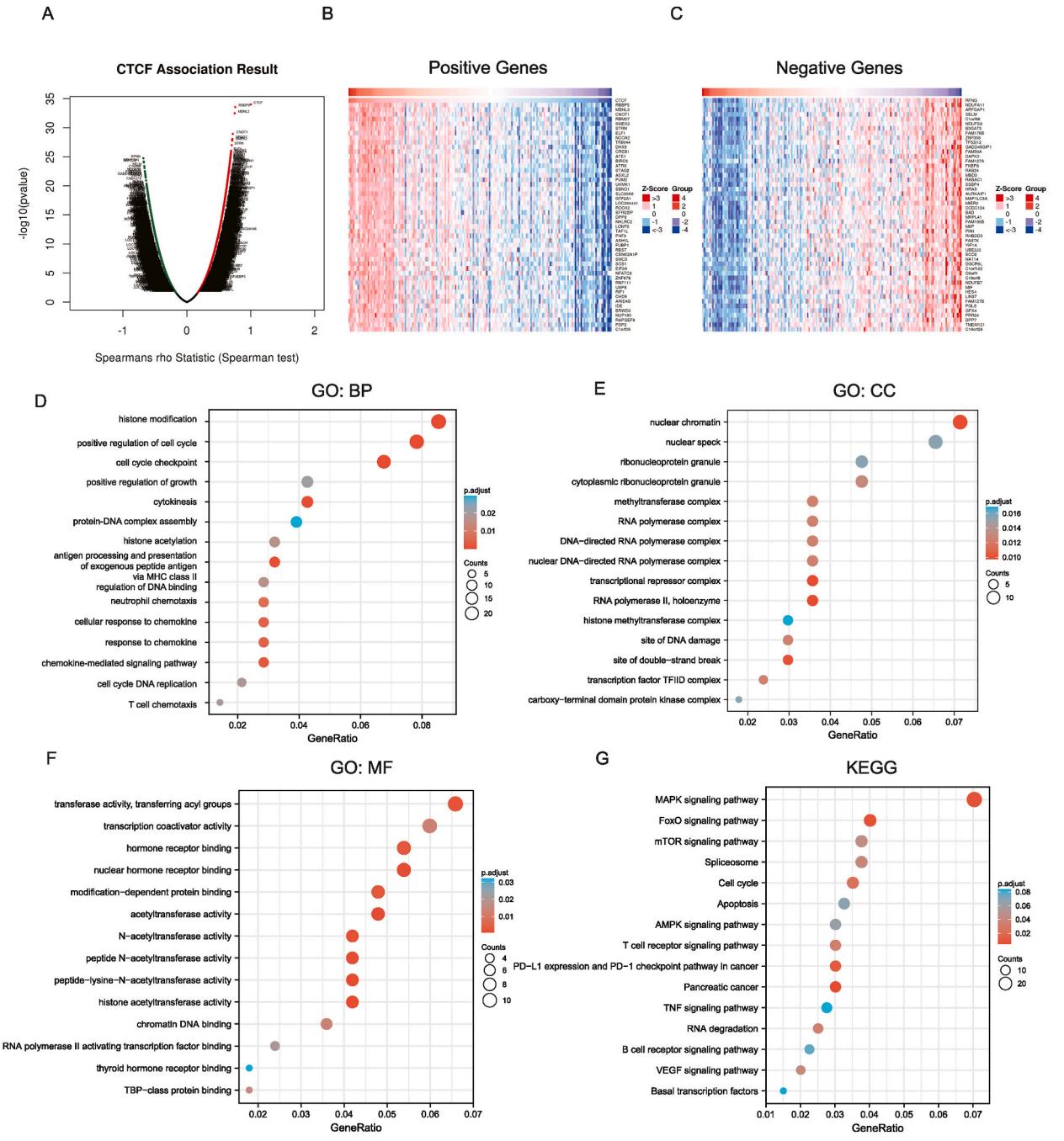

Fig. 4. Functional enrichment analysis of CTCF in PC.(A–C) The correlation analysis of CTCF expression and its top 50 co-expressed gene network. (D–G) GO and KEGG enrichment analysis of co-expressed genes.

acetyltransferase activity (Fig. 4F). As observed in Fig. 4G, the identified genes were related to signaling pathways of MAPK, FoxO, mTOR, spliceosome, cell cycle, and apoptosis by KEGG analysis.

3.4. Predictive value of the CTCF expression based on clinicopathologic variables

To clarify the clinical prognostic value of CTCF in PC, we revealed the correlation between CTCF expression and OS/PFI in diverse sub-groups by a variety of clinical features. These findings unveiled that CTCF was the shorter survival had in PC patients in the subgroups of race (specifically white race) (Fig. 5A, E), residual tumor (R0 resection, Fig. 5B, F), age (≤65 years, Fig. 5C, G), smoking (non-smoker, Fig. 5D, H), gender (male, Fig. 5I and J), primary therapy outcome (CR, Fig. 5K) and anatomic neoplasm subdivision (head of the pancreas, Fig. 5L).

3.5. Relevance of tumor immune microenvironment and CTCF expression in PC

Here, we examined the association between tumor microenvironment (TME) and CTCF expression in PC. First, we assessed the affiliation between CTCF levels and immune cell infiltration in PC using the TCGA database. As demonstrated in Fig. 6A, there was a positive association observed between the expression of CTCF and the infiltration of T helper cells (Th cells) (Cor = 0.497, P < 0.001) (Fig. 6B), central Memory T cell (Tcm) (Cor = 0.472) (Fig. 6C), Th2 cells (Cor = 0.297) (Fig. 6D), T cells (Cor = 0.284) (Fig. 6E), eosinophils (Cor = 0.284) (Fig. 6F), B cells (Cor = 0.203) (Fig. 6G), aDC (Cor = 0.195) (Fig. 6H) and Dendritic Cells (DC) (Cor = 0.184)

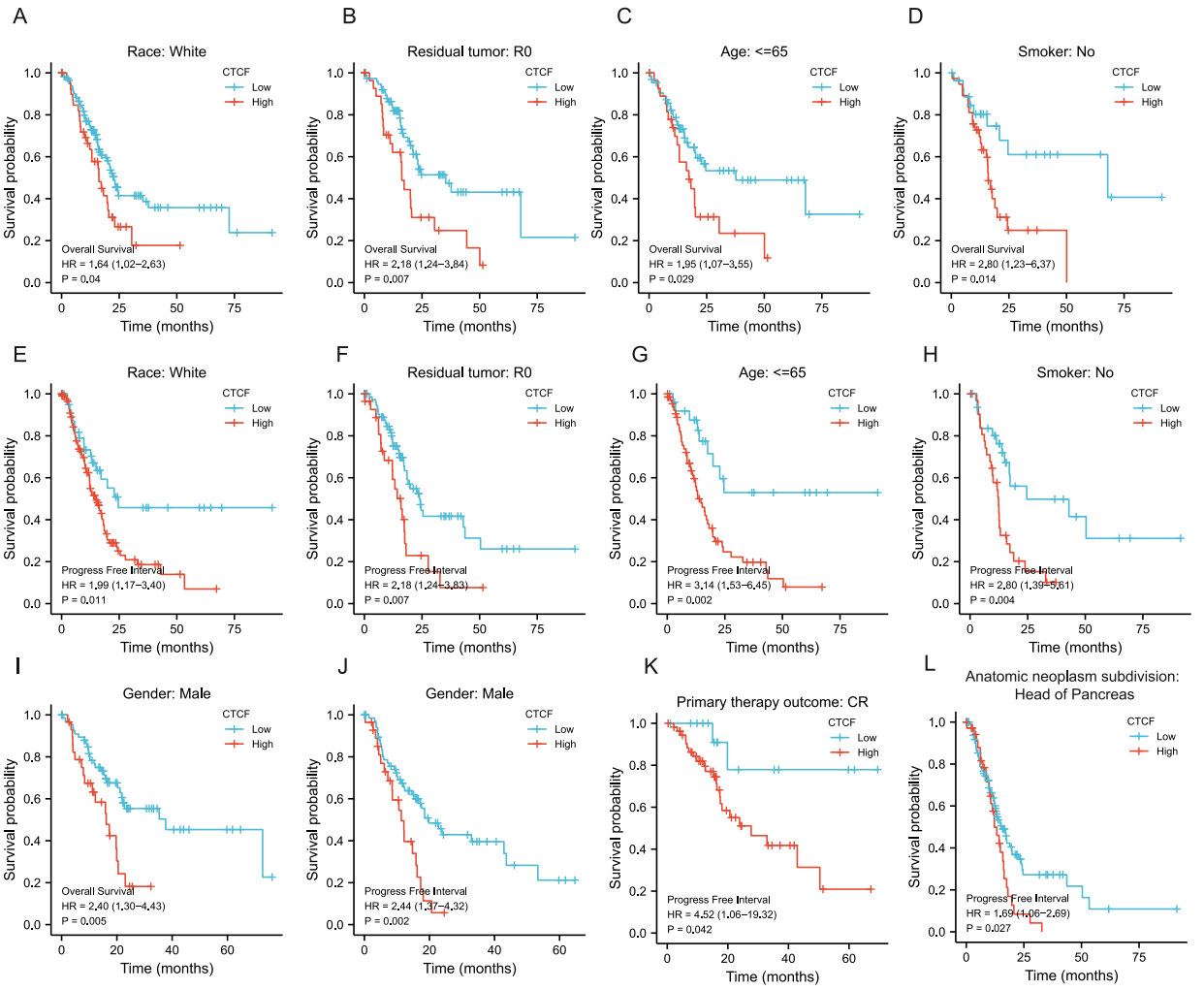

Fig. 5. Prognostic values of CTCF expression in patients with PC evaluated by the Kaplan-Meier method in different subgroups.
(A–D) OS survival curves of subgroups of race: white, residual tumor: R0, age:≤65, smoker: no patients with PC. (E–H) PFI survival curves of subgroups of race: white, residual tumor: R0, age:≤65, smoker: no patients with PC. (I) OS survival curves of male patients with PC. (J–L) PFI survival curves of primary therapy outcome: CR and anatomic neoplasm subdivision: head of pancreas. OS, overall survival; PFI, Progress free interval; CR, Complete response.

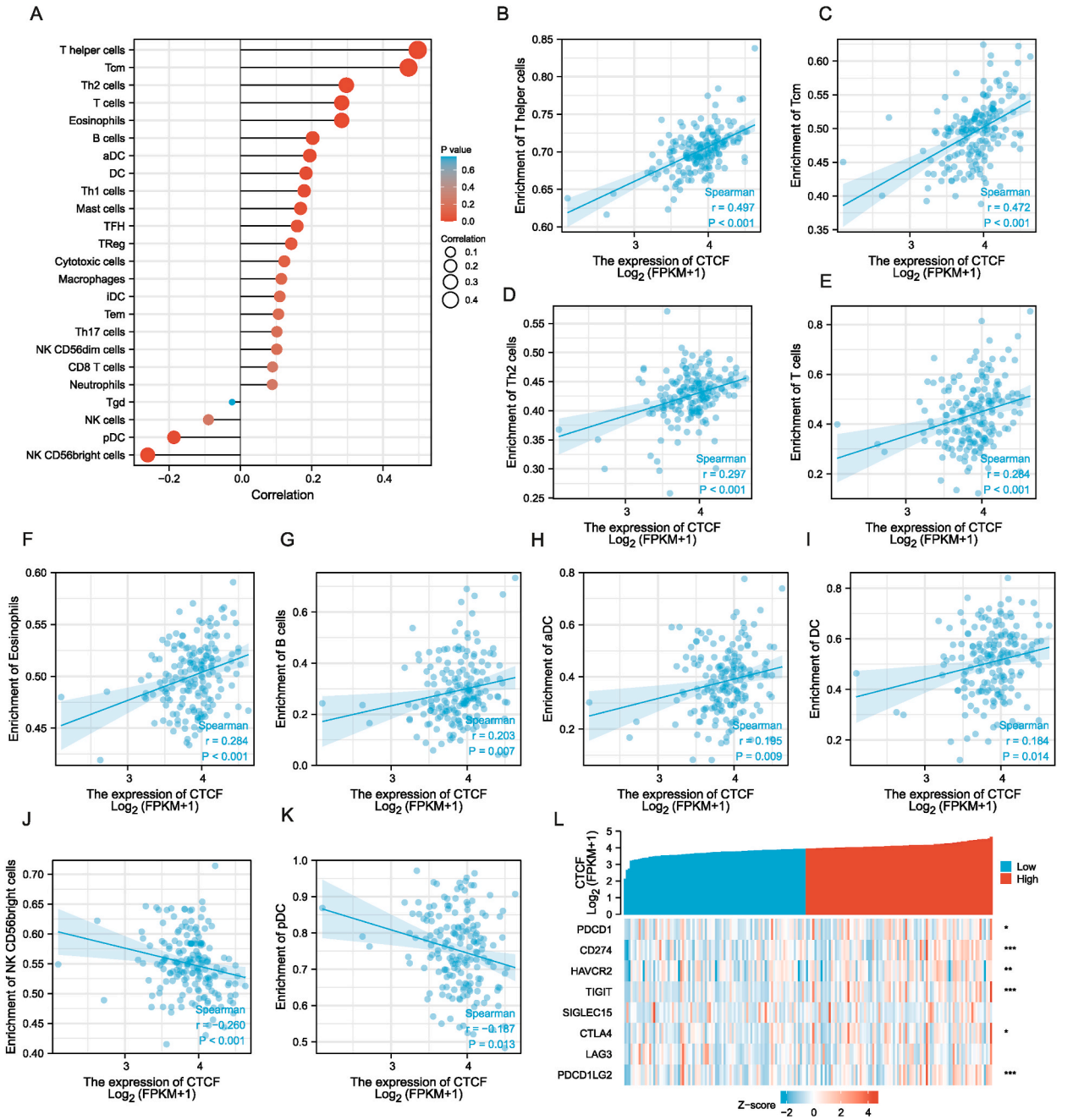

Fig. 6. Correlation analysis of CTCF expression and infiltration levels of immune cells in PC. (A–K) The correlation between CTCF expression and infiltration levels of immune cells. (L) Correlation analysis of CTCF expression and immune checkpoint-related genes in PC in the TCGA database.

(Fig. 6I) and negatively correlated with natural killer cell (NK) CD56 bright cells (Cor = −0.26) (Fig. 6J) and pDC (Cor = −0.187) (Fig. 6K) in PC.

PC patients, expressing high CTCF had a significant increase in immune cell infiltration, such as T cells, aDC, eosinophils, mast cells, T helper cells (Th), Tcm, Th1 cells and Th2 cells (Fig. S3A). Additionally, the amount of pDC infiltrates was significantly diminished. We further used the ESTIMATE algorithm to measure the distinction in microenvironmental scores between the two groups (CTCF-High group and CTCF-Low group), and the results indicated that the high CTCF expression group accompanied by higher immune scores on the basis of the ImmuneScore and ESTIMATEScore ($p < 0.05$) (Fig. S3B).

Next, spearman correlation analysis of the TCGA dataset was utilized to evaluate the relationship between CTCF and checkpoint member expression in PC-induced immune responses. As indicated in Fig. 6L and Fig. S3C, CTCF was strongly concordant with

TNFSF15 (Cor = 0.415, P < 0.001), NRP1(Cor = 0.409, P < 0.001), CD28 (Cor = 0.361, P < 0.001) and CD200R1(Cor = 0.357, P < 0.001). Further, we noticed a positive correlation between the expression of CTCF and checkpoint-associated genes in PAAD (Fig. 6L). Collectively, it is suggested that anti-tumor immunity could be engaged in the CTCF-regulated oncogenesis of PAAD.

3.6. Prognostic value of immune cell-based CTCF expression in PC

It is demonstrated that CTCF was involved in the immune infiltration of PC and that CTCF upregulation led to poor survival outcomes in PC patients. Hence, we hypothesized that CTCF might partially impact the prognosis of PC patients via immune infiltration. KM curves were utilized to examine the correlation between CTCF expression and PAAD patient prognosis following decreased $CD4^+$ T cells (Fig. 7A), enriched $CD8^+$ T cells (Fig. 7B), decreased $CD8^+$ T cells (Fig. 7C), enriched eosinophils cells (Fig. 7D), enriched eosinophils cells (Fig. 7E), enriched macrophages cells (Fig. 7F) and enriched macrophages cells (Fig. 7G), enriched NKT cells (Fig. 7H) and decreased NKT cells (Fig. 7I). The findings indicated that immune infiltration might partially contribute to the prognosis of PAAD patients with high CTCF expression.

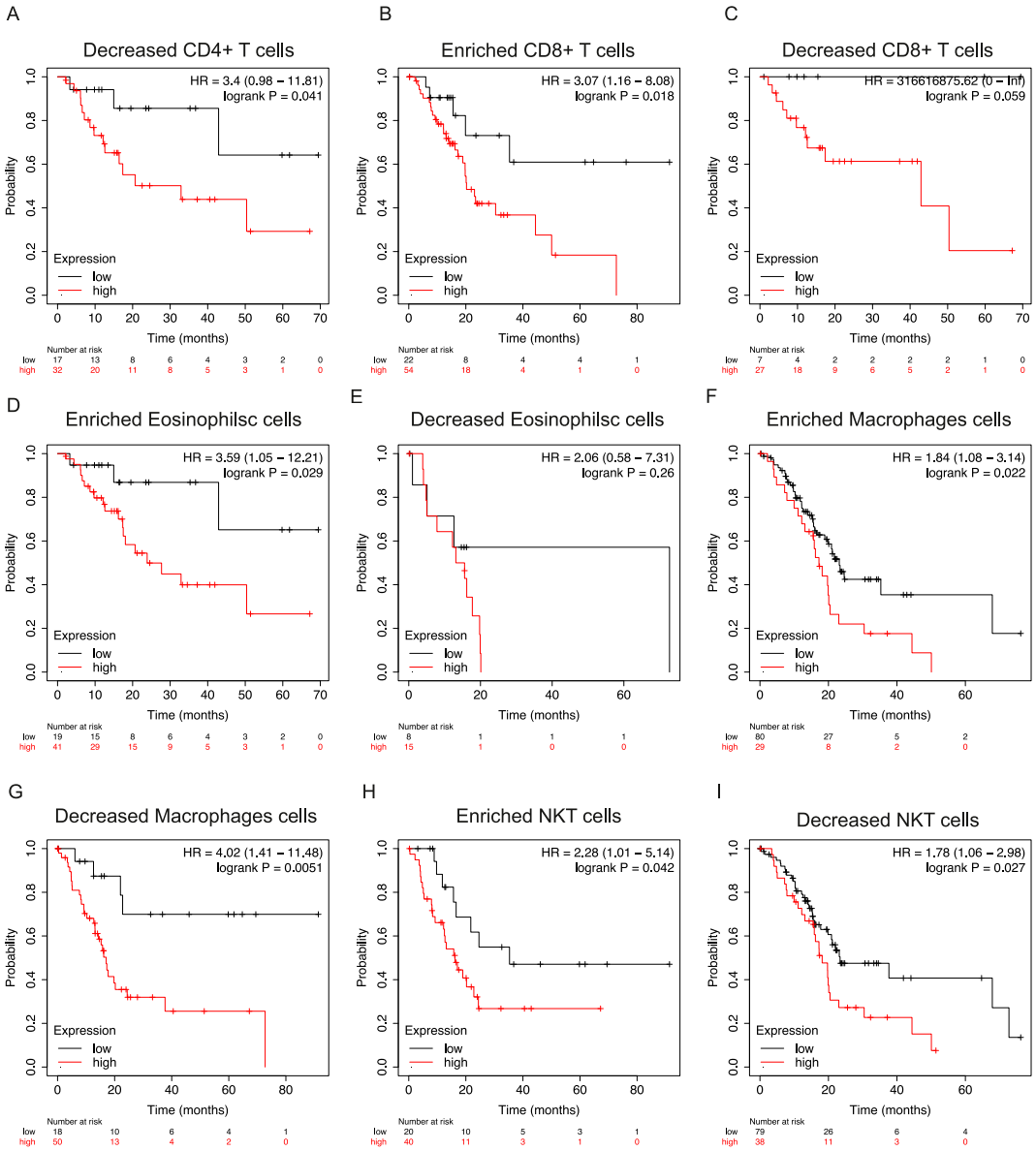

Fig. 7. Kaplan–Meier survival curves according to the high and low expression of CTCF in immune cell subgroups in PC.(A–I) Correlations between CTCF expression and overall survival in different immune cell subgroups in PC patients were determined by Kaplan–Meier plotter.

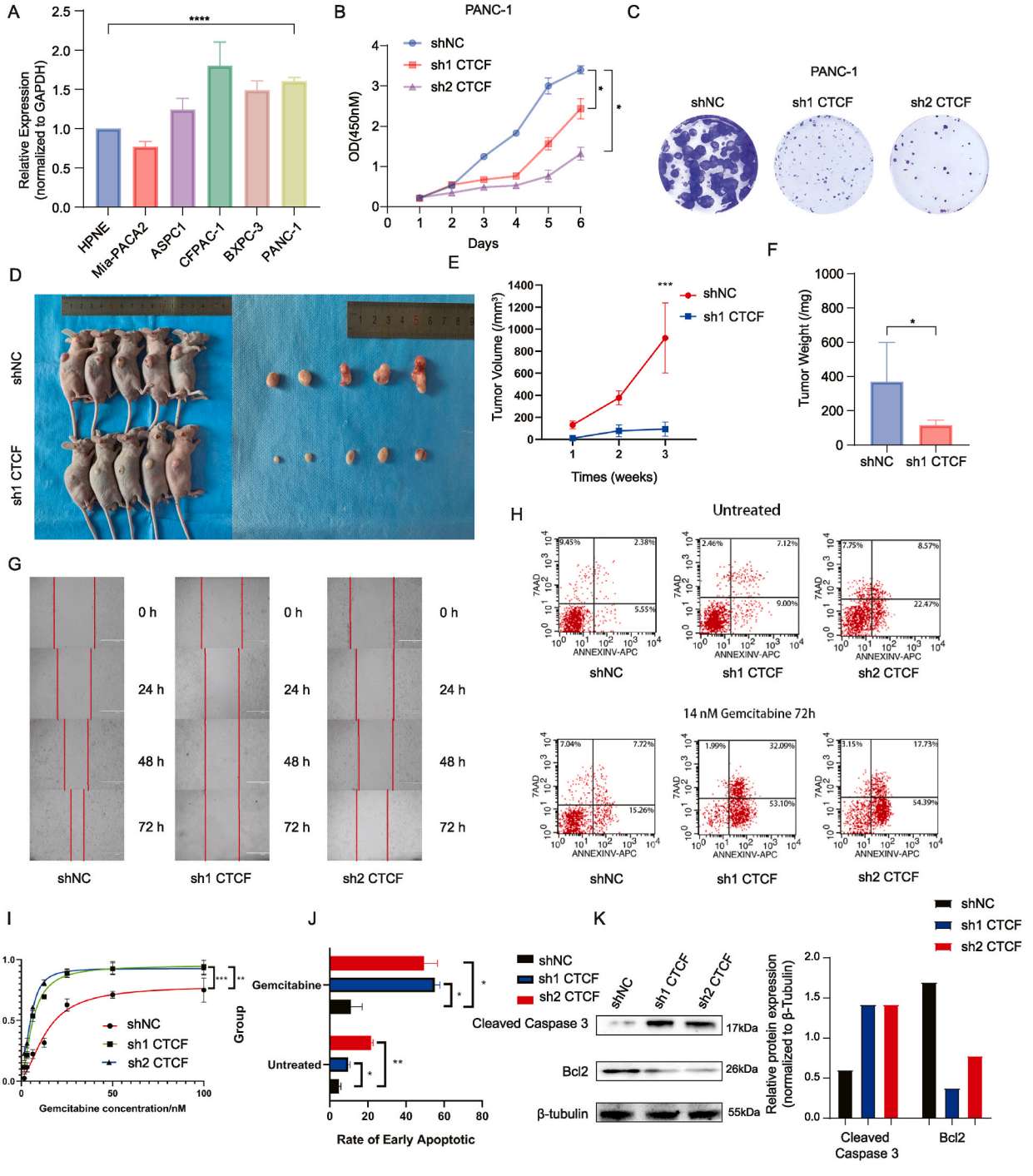

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Fig. 8. CTCF regulates PC cell proliferation, cell migration, and cell invasion. (A) qPCR was conducted to detect expression levels of CTCF in pancreatic cancer cell lines. The relative expression of CTCF mRNA was shown using GAPDH as an endogenous control. (B, C) CCK-8 and colony formation results show the proliferation rate status of negative control and CTCF knocked down in PANC-1 cells. OD value at 450 nm was measured. (D) Representative images of xenograft tumors derived from CTCF knocked down and negative control PANC-1 cells that were subcutaneously injected into Balb/c athymic nude mice. (E, F) Tumor weights and tumor volumes of xenograft tumors derived from the CTCF knocked down and negative control in PANC-1 are shown. Tumor volumes were calculated as volume = length × $(\text{width})^2/2$. The data are represented as mean ± SD. (G) Wound-healing assay to detect the migration ability of PANC-1 cells with or without sh CTCF. Photos were taken at 0, 24h, 48h and 72 h. (H, J) Flow cytometry was used to analyze the apoptosis rate in PANC-1 cells transfected with sh CTCF and negative control with or without gemcitabine treatment. (I) Depletion of CTCF sensitized the cellular response to gemcitabine treatment. (K) Left: Western blot analysis showed the relative expression levels of cleaved Caspase 3 and Bcl2 regulated by sh CTCF and NC in PANC-1 cells. β-tubulin were used as the internal control. Right: Relative expression cleaved Caspase 3 and Bcl2 (normalized to β-tubulin). The data are presented as the mean ± SD of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

3.7. Construction of a nomogram model based on CTCF

We constructed a nomogram for predicting the survival of PC patients based on independent prognostic markers of OS. According to the calculated total score, the patients were divided into a high-risk group and a low-risk group that associated with a worse prognosis (Fig. S4A). Moreover, the corrected C-index of 0.62 (95 % CI = 0.6112–0.629) via calibration curve analysis (Figs. S4B–F) implied that our nomogram demonstrates a strong ability to accurately forecast the outcome of patients with PC.

3.8. CTCF acts as a carcinogenic factor in facilitating the proliferation, migration and apoptosis of PC cells

Assessment of CTCF levels in PC cell lines showed its high expression in PANC-1 cells compared to other PC cells (Fig. 8A). Colony formation assays and CCK8 revealed that PC cell proliferation decreased after CTCF knockdown in PANC-1 cells (Fig. 8B and C). In nude mice subcutaneous models, CTCF knockdown resulted in a decline in both weight and tumor volume (Fig. 8D–F). In addition, we found that the knockdown of CTCF in PANC-1 cells led to slower wound healing than compared to vector-transfected PANC-1 cells (Fig. 8G). PC stable cell lines with CTCF knockdown led to an increase in apoptosis (Fig. 8H). Additionally, we also found that the knockdown of CTCF resulted in increased sensitivity to gemcitabine (Fig. 8H–J) and promoted apoptosis (Fig. 8K). In sum, it is suggested that CTCF exhibits significant expression levels in PC and exerts a noticeable influence on its cellular proliferation, migration, and apoptosis.

3.9. CTCF was downstream of FGD5-AS1/miR-19a-3p axis

Increasing evidence confirms that the competing endogenous RNAs (ceRNAs) mechanism is important in tumor development. To elucidate whether ncRNAs regulate CTCF, we anticipated miRNAs that could regulate CTCF and identified 144 candidate miRNAs (Table S5). Due to the negative regulatory effects of upstream miRNAs on CTCF expression at the post-transcriptional level, we predicted a negative relationship between CTCF and upstream miRNAs. Through the analysis of TCGA-PAAD database, it was observed that the expression of CTCF was inversely proportional to hsa-miR-19a-3p (Fig. 9A), hsa-miR-19b-3p (Fig. 9B), hsa-miR-33a-5p (Fig. 9C), hsa-miR-30c-5p (Fig. 9D), hsa-miR-219a-5p (Fig. 9E), hsa-miR-30b-5p (Fig. 9F), hsa-miR-141-3p (Fig. 9G), hsa-miR-590-5p (Fig. 9H), hsa-miR-500b-5p, hsa-miR-27b-3p and hsa-miR-362-3p among the 144 candidate miRNAs, while there was no statistical relationship between CTCF and other miRNAs in PAAD (Fig. S5A). Subsequently, hsa-miR-19a-3p had a lower expression than others in the normal tissues following the detection of RNA levels among the candidate miRNAs (Fig. 9I) in the TCGA-PAAD database (Fig. S5B). In addition, we further investigated the association between hsa-miR-19a-3p and PAAD patients' survival outcomes (OS/DSS/PFI) in the TCGA database (Fig. 9J-L and Figs. S6A–J) and found that the higher expression of hsa-miR-19a-3p significantly contributed to a better prognosis of PC. Next, we predicted prospective lncRNAs cooperated with miR-19a-3p in ENCORI database. CeRNA hypothesis assumed that lncRNAs release target mRNAs downstream of miRNAs by sponging miRNAs. In the ceRNA network, lncRNAs and the target mRNAs had a positive connection, while lncRNAs and the target miRNAs showed a negative association. FGD5-AS1 and AC104447.1 were found to be inversely proportional to hsa-miR-19a-3p (Fig. 9M−O) and positively correlated with CTCF (Fig. 9P, Q and Fig. S6K) after we investigated the connection between the expression of miR-19a-3p/CTCF and 107 LncRNAs using the TCGA-PAAD database (Table S6). We further found that FGD5-AS1 and AC104447.1 were increased in PAAD (fig. 9R and S). Next, the prognostic significance of FGD5-AS1 and AC104447.1 was assessed in the TCGA-PAAD database, and it was indicated that a higher expression of FGD5-AS1 obviously implied a poor clinical prognosis in PAAD patients (Fig. 9T, Fig. S6L). Thus, the above finding identified that the FGD5-AS1/miR-19a-3p axis was one of the most potential upstream ceRNAs of the CTCF in PC.

3.10. miR-19a-3p suppressed PC progression by targeting CTCF

To elucidate the relationship between CTCF and miR-19a-3p, dual-luciferase reporter assays were performed, and it was validated the physical binding of CTCF and miR-19a-3p (Fig. 10A). Additionally, RNA immunoprecipitation (RIP) assays better demonstrated the interaction of CTCF and miR-19a-3p, and AGO2 could act as a scaffold to modulate the binding of miR-19a-3p and CTCF (Fig. 10B). As shown in Fig. 10C and D, miR-19a-3p could negatively regulate CTCF expression in mRNA and protein levels. To investigate the role of miR-19a-3p in targeting CTCF, we further performed the ability of miR-19a-3p in proliferation, migration, and apoptosis in PC.

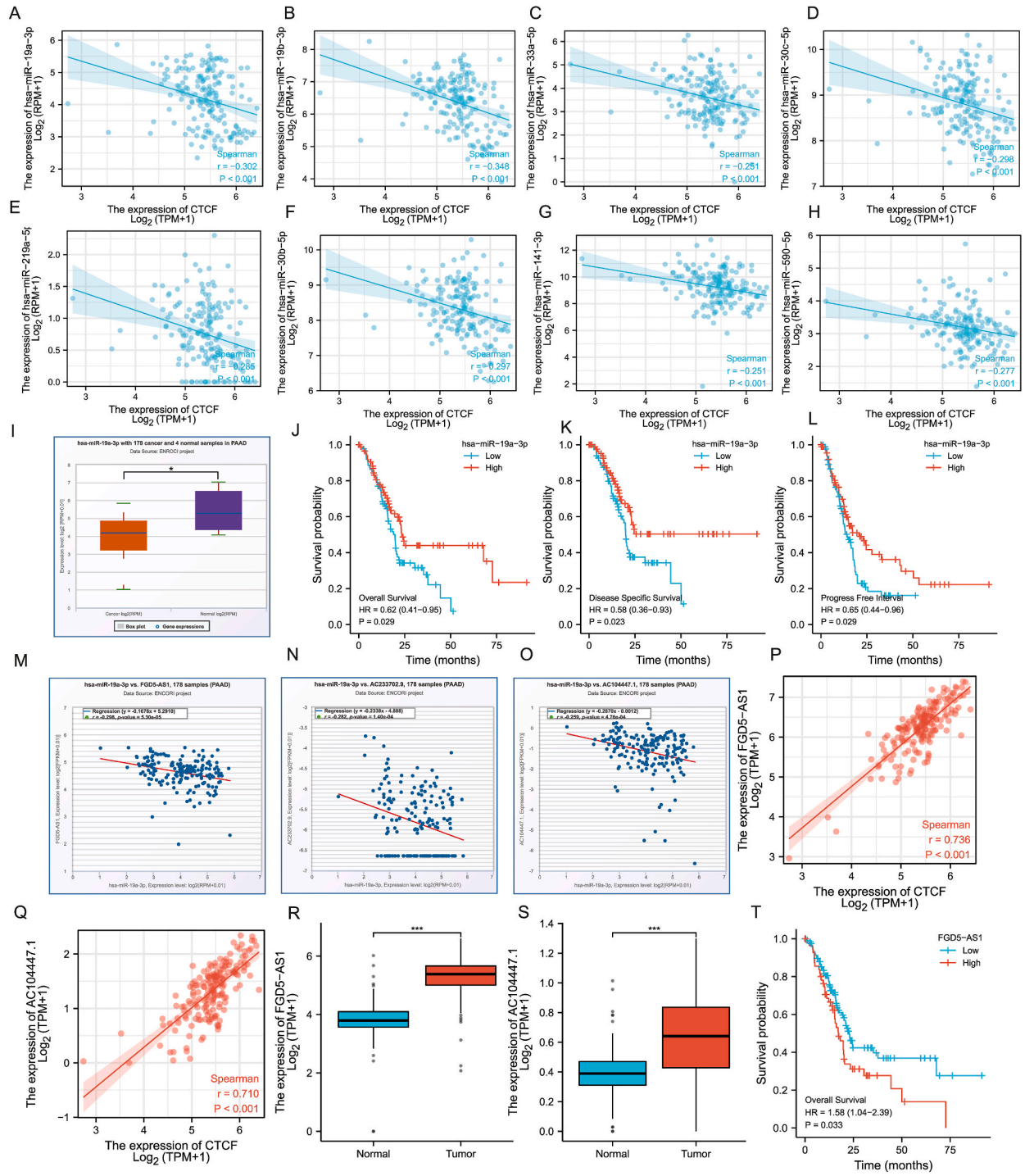

Fig. 9. FGD5-AS1/hsa-miR-19a-3p/CTCF regulatory network. (A–H) Correlation between CTCF and upstream miRNAs (miR-19a-3p, miR-19b-3p, miR-33a-5p, miR-30c-5p, miR-219a-5p, miR-30b-5p, miR-141-3p and miR-590-5p) in TCGA-PAAD. (I) The ENROCI database was used to analyze the expression of has-miR-19a-3p with cancer (n = 178) and normal samples (n = 4) of PAAD. (J–L) Kaplan–Meier survival curves showed that PAAD patients with high has-miR-19a-3p expression exhibited poor overall survival, disease-specific survival, and progression-free survival of has-miR-19a-3 in PAAD determined by the TCGA-PAAD dataset. (M−O) Correlation between has-miR-19a-3p and predicted prospective lncRNAs (FDG5-AS1, AC233702.9 and AC104447.1). (P, Q) Correlation between CTCF and predicted prospective lncRNAs: FGD5-AS1 and AC104447.1. (R, S) FGD5-AS1 and AC104447.1 expression level was also significantly increased in PAAD compared with normal tissues. (T) Kaplan–Meier survival curves showed that PAAD patients with low FGD5-AS1 expression exhibited poor overall survival. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

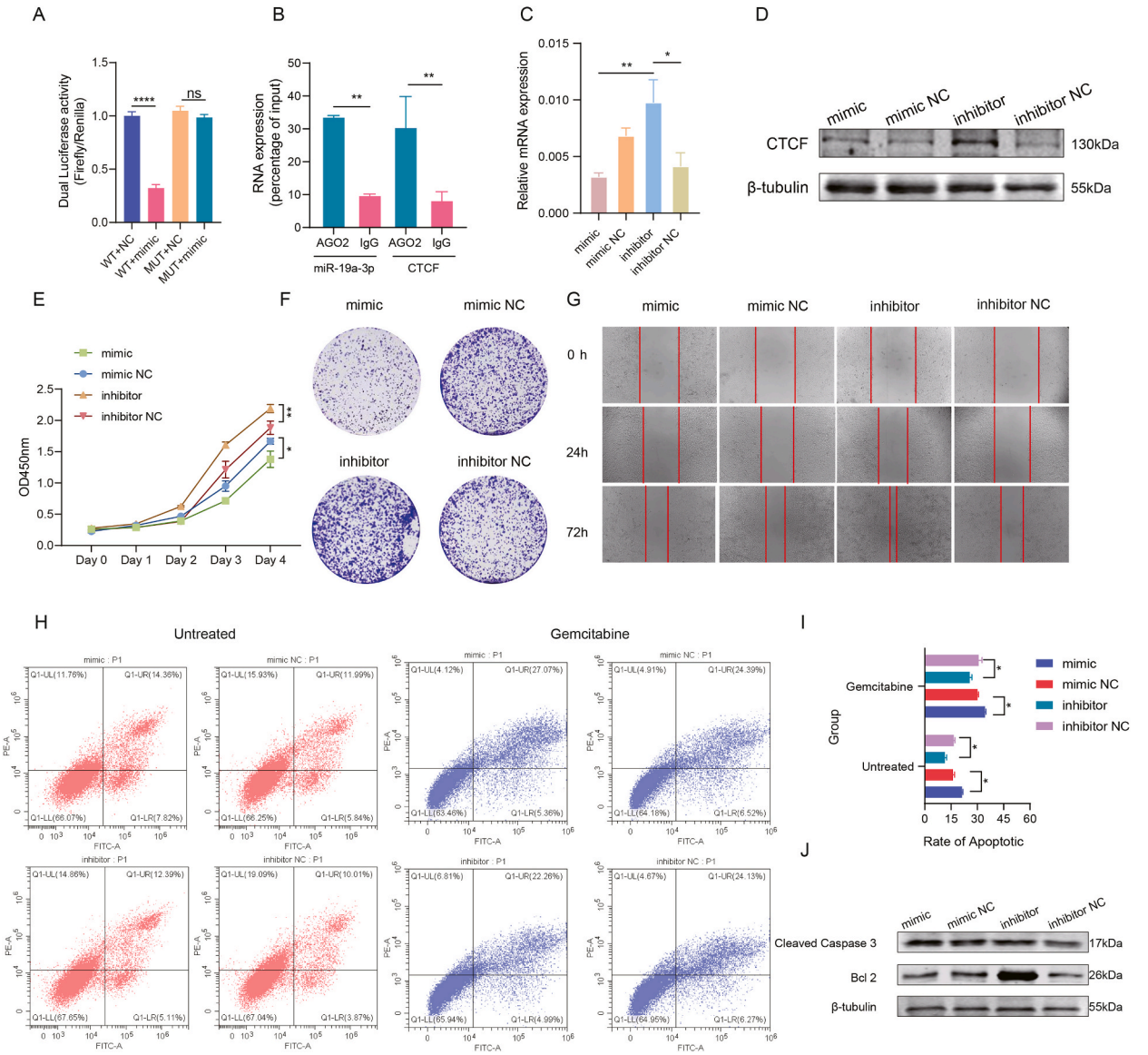

Fig. 10. miR-19a-3p targeted CTCF to suppress the proliferation, migration and apoptosis of pancreatic cancer cells.
(A) Dual luciferase activity in HEK-293T cells co-transfected with CTCF wild-type or mutant sequence and miR-19-3p mimics. (B) RIP assay was performed using rabbit AGO2 and IgG antibodies in PANC-1 cells. Relative expression levels of CTCF and miR-19a-3p were determined by qPCR. (C, D) RNA level (C) or protein level (D) of CTCF in PANC-1 cells after transfected miR-19a-3p mimics or miR-19a-3p inhibitor. (E, F) CCK-8 and colony formation results show the proliferation rate status of negative control, miR-19a-3p mimics and miR-19a-3p inhibitor in PANC-1 cells. OD value at 450 nm was measured. (G) Wound-healing assay to detect the migration ability of PANC-1 cells transfected with miR-19a-3p mimics or miR-19a-3p inhibitor. Photos were taken at 0, 24h and 72 h. (H) Flow cytometry was used to analyze the apoptosis rate in PANC-1 cells transfected with miR-19a-3p mimics or miR-19a-3p inhibitor and treated with or without gemcitabine treatment. (I) Depletion of miR-19a-3p sensitized the cellular response to gemcitabine treatment. (J) Western blot analysis showed the relative expression levels of cleaved Caspase 3 and Bcl2 regulated by miR-19a-3p in PANC-1 cells. β-tubulin were used as the internal control. The data are presented as the mean ± SD of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

CCK-8 and colony formation assays were conducted, and the results revealed that miR-19a-3p could inhibit the proliferation ability of PC cells (Fig. 10E and F). Consistently, it was observed similar results in wound healing assays (Fig. 10G). Furthermore, western blotting assays and flow cytometry experiments were performed and the miR-19a-3p could remarkably promote the apoptosis of PC cells (Fig. 10H–J). Additionally, when the PANC-1 cells were treated with gemcitabine, the apoptotic rate

of the miR-19a-3p inhibitor group was lower than that in the control group and it was seen opposite result in the miR-19a-3p mimics group (Fig. 10H and I).

3.11. FGD5-AS1 acted as a ceRNA to regulate CTCF expression in PC

Aiming to investigate the role of FGD5-AS1 as a ceRNA in pancreatic cancer and its interaction with miR-19a-3p, the dual-luciferase reporter assays and RIP assays were used and the interaction of FGD5-AS1 to miR-19a-3p was better demonstrated. (Fig. 11A and B).

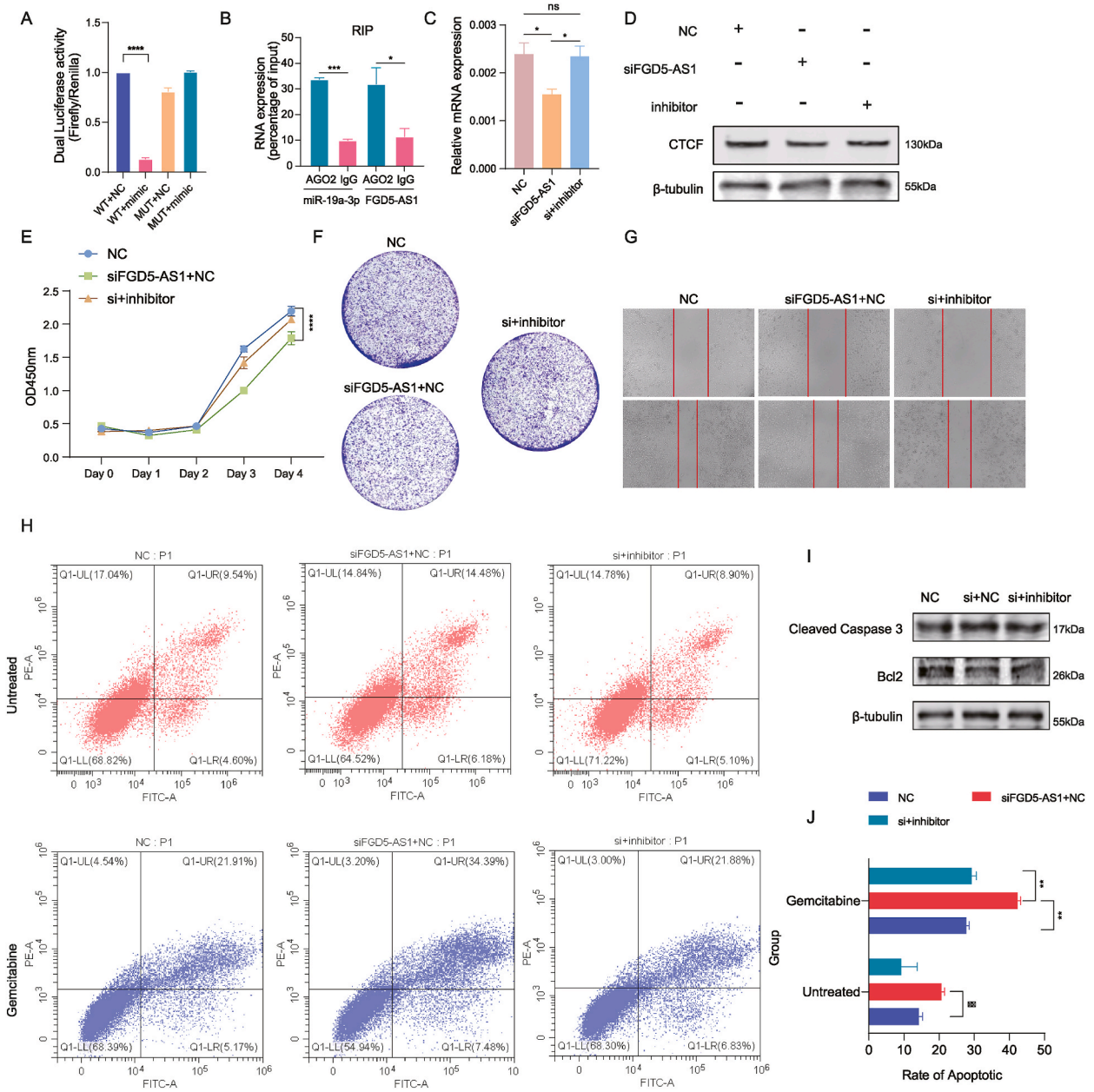

Fig. 11. FGD5-AS1 acted as a ceRNA to regulate CTCF expression in pancreatic cancer cells. (A) Dual luciferase activity in HEK-293T cells co-transfected with FGD5-AS1 wild-type or mutant sequence and miR-19-3p mimics. (B) RIP assay was performed using rabbit AGO2 and IgG antibodies in PANC-1 cells. Relative expression levels of FGD5-AS1 and miR-19a-3p were determined by qPCR. (C, D) RNA level (C) or protein level (D) of CTCF in PANC-1 cells after transfected with siFGD5-AS1 and miR-19a-3p inhibitor. (E, F) CCK-8 and colony formation results show the proliferation rate status of negative control, siFGD5-AS1 and siFGD5-AS1+miR-19a-3p inhibitor in PANC-1 cells. OD value at 450 nm was measured. (G) Wound-healing assay to detect the migration ability of PANC-1 cells transfected with siFGD5-AS1 and miR-19a-3p inhibitor. Photos were taken at 0, 24h, 48h and 72 h. (H)Flow cytometry was used to analyze the apoptosis rate in PANC-1 cells transfected with siFGD5-AS1 and miR-19a-3p inhibitor and treated with or without gemcitabine treatment. (I) Depletion of FGD5-AS1 sensitized the cellular response to gemcitabine treatment. (J) Western blot analysis showed the relative expression levels of cleaved Caspase 3 and Bcl2 regulated by FGD5-AS1 in PANC-1 cells. β-tubulin were used as the internal control. The data are presented as the mean ± SD of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Next, we utilized qPCR and western blotting assays to assess the effects of FGD5-AS1 knockdown on CTCF expression and found that CTCF was down-regulated in si-FGD5-AS1 group, and this decrease was rescued by miR-19a-3p inhibitor (Fig. 11C and D). To assess the impact of FGD5-AS1 on PC cell, phenotype assays were performed (Fig. 11E–G). The knockdown of FGD5-AS1 could notably inhibit the viability of proliferation and migration in PC cell. Likewise, FGD5-AS1 suppressed apoptosis significantly in PC cells through WB and flow cytometry assays. Additionally, FGD5-AS1 enhanced the chemosensitivity of PANC-1 cells to gemcitabine, as indicated by the increased apoptotic response. These findings, as shown in Fig. 11H–J, suggested that FGD5-AS1 played a role in attenuating apoptosis and improving the chemosensitivity of PANC-1 cells to gemcitabine. We demonstrated that FGD5-AS1 could facilate the proliferation, migration, and chemosensitivity of PC cells, while suppressing apoptosis. Notably, the reversal of these effects by miR-19a-3p implied that FGD5-AS1 could function as a miR-19a-3p sponge, thereby exerting its pro-tumor function in PC (Fig. 11A–J). To clarify the importance of FGD5-AS1 in PDAC, we found a significantly lower tumor weight in the si-FGD5-AS1 group by constructing a subcutaneous tumor model (Figs. S7A–C). It might imply that the CTCF-related ceRNA axis plays an important role in PDAC progression.

4. Discussion

The elevated expression of genes involved in tumor progression is closely associated with a decreased survival rate among cancer patients [41]. The diagnostic efficacy of markers like CA199, CA125, and cfDNA in pancreatic cancer (PC) is constrained by the inherent heterogeneity of the disease [42,43]. Therefore, the identification of novel biomarkers is of utmost importance in predicting prognosis and developing personalized therapies for pancreatic cancer [44,45]. This study showed that CTCF exhibited significantly higher expression levels in multiple cancers compared to adjacent normal tissues, as evidenced by analysis of the TCGA and GTEx databases. CTCF, being a candidate transcription factor, has been implicated in promoting tumor progression in several cancer types, as evidenced by studies reporting its elevated expression levels in these cancers compared to adjacent normal tissues [46–48]. The expression and functional implications of CTCF in cancer are not well-established due to limited research in this specific context. However, the molecular mechanism of CTCF has been extensively studied in TAMs specifically in PDAC [49,50]. In this study, our focus was on pancreatic cancer (PC), where CTCF exhibited upregulation. Importantly, we discovered a significant correlation between increased CTCF expression and unfavorable OS, DFS, and PFI prognosis across various subgroups, including race, age, smoking status, and gender. Moreover, our analysis revealed the potential of CTCF as a diagnostic biomarker to differentiate PC from normal pancreatic tissues, as evidenced by the ROC curve analysis (Fig. 12).

Prior studies have indicated the involvement of CTCF in tumor cell proliferation, migration, and apoptosis [14]. However, the underlying mechanisms of CTCF in cancer haven't been extensively investigated in those studies [13]. The findings of functional

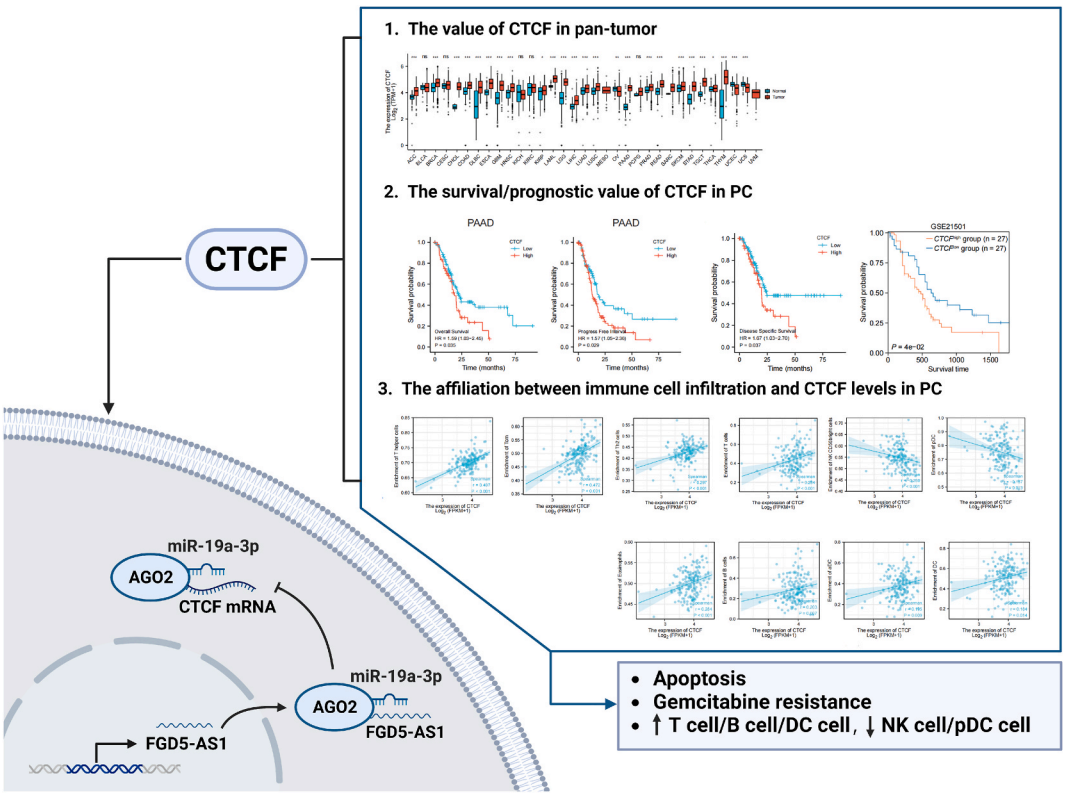

Fig. 12. Graphical summary of major findings in this study.

enrichment analysis revealed significant associations between CTCF and various biological processes and signaling pathways. These include altered histone modifications, tumor growth, transcription factor coactivation activity and many signaling pathways, cell cycle regulation, apoptosis, and T cell receptor signaling pathway. Several studies found that CTCF might facilitate the malignant progression of COAD via c-myc pathway and the p53/Hedgehog signaling axis. Nevertheless, it is important to note that these findings require additional experimental validation, as they may provide further insights into the diverse biological functions of CTCF in PC.

The study revealed a notable upregulation of CTCF in TAMs and established a correlation between elevated CTCF expression and unfavorable prognosis in PC. The results of our study indicate the potential contribution of CTCF in modulating the TME in PC. The unique TME in PC contributes to its high mortality rate. The TME of PC is characterized by an abundance of immunosuppressive cells, such as TAMs, Treg, and MDSCs. These immunosuppressive cells play pivotal roles in promoting tumor progression and conferring resistance to treatment. Our findings revealed significant associations between CTCF expression and specific tumor-associated immune cells in PC. The relationship between CTCF levels and Th2, Tcm, and aDC were identified. Conversely, CTCF expression showed a negative correlation with NK cells, CD56 bright cells, and pDC in PC. Some reports demonstrated a direct effect of Th2 cells on tumor growth and progression. In this regard, Th2 $CD4^+$ T cells were injected into host mice to treat B-cell lymphomas in vivo. It was discovered that that $CD4^+$ Th2 cells inhibited the growth of lung metastases arising from B16 melanoma. Additionally, our own data analysis exhibited a significant correlation between CTCF and T cell receptor activity, T cell chemotaxis, and PD-1 related pathways in functional enrichment analyses. In our upcoming research, our focus will be to investigate mechanism of CTCF in T cells. We hypothesize that CTCF promotes the PC progression by inhibiting the anti-tumor immune response.

To investigate the potential upstream mechanisms underlying CTCF up-regulation in PC, we made a comprehensive analysis of miRNA-CTCF interactions. Through this approach, we identified hsa-miR-19a-3p as a potential miRNA that targets CTCF in PC. Our findings revealed a significant negative correlation between CTCF expression and hsa-miR-19a-3p levels. Moreover, we found a downregulation of hsa-miR-19a-3p in PC tissues. In our survival analysis using the Kaplan-Meier method, we found a strong association between decreased hsa-miR-19a-3p expression and unfavorable prognosis in PAAD. Based on ceRNA hypothesis, we postulated that the related miRNA would exhibit a negative correlation with CTCF, suggesting a potential negative post-transcriptional regulatory mechanism of the miRNA on CTCF. Through a comprehensive analysis that incorporated the ceRNA hypothesis, correlation analysis, expression analysis, and survival analysis, we determined that hsa-miR-19a-3p is the most promising regulatory miRNA for CTCF in pancreatic adenocarcinoma (PAAD). Furthermore, our findings suggest a negative regulation of hsa-miR-19a-3p by CTCF in PC, indicating the presence of a potential regulatory feedback loop between CTCF and hsa-miR-19a-3p.

To clarify upstream lncRNAs of hsa-miR-19a-3p, we finalized FGD5-AS1 and AC104447.1 as two oncogenic lncRNAs of PAAD from 107 alternative lncRNAs based on the ceRNA hypothesis, which were significantly and negatively correlated with hsa-miR-19a-3p and positively associated with CTCF through ENCORI database. Based on correlation, we identified FGD5-AS1 as the most promising candidate related lncRNA for CTCF in PAAD. Emerging research has shed light on FGD5-AS1 which was found highly expressed in multiple cancer types. Notably, recent studies have associated increased FGD5-AS1 expression with heightened tumor cell progression, suggesting its potential involvement in facilitating cancer progression. In contrast, there is little research on PC. Hence, we remain to find the mechanism of its role in PC. In summary, we have identified the CTCF related ceRNA axis as a important pathway in PAAD, as depicted in Fig. 12.

This study depicted the clinical relevance, TME characteristics, biological functions and molecular mechanisms of CTCF in PC. CTCF could as a promising prognostic factor and a potential target for PADC treatment.

Ethics approval and consent to participate

Sample collections were approved by Ethnics Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine. The informed consents were obtained from patients or their guardians, as appropriate.

Data Availability

Data associated with this study has been deposited into a publicly available repository- Mendeley Data DOI: 10.17632/8dmssf8w8k.1.

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Consent for publication

Not applicable.

CRediT authorship contribution statement

Yihao Liu: Writing – original draft, Conceptualization. **Wenxin Qi:** Formal analysis, Data curation. **Jingxin Yin:** Methodology, Investigation. **Xirui He:** Visualization, Validation. **Songqi Duan:** Visualization, Formal analysis. **Haili Bao:** Project administration,

Methodology. **Chen Li:** Investigation, Funding acquisition. **Minmin Shi:** Validation, Data curation. **Jiao Wang:** Funding acquisition, Conceptualization. **Shaohua Song:** 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.

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Abbreviations

CTCF	CCCTC-binding factor
PDAC	pancreatic ductal adenocarcinoma
TME	tumor microenvironment
FGD5-AS1	FGD5 antisense RNA 1
ceRNA	competing endogenous RNAs
TAMs	tumor associated macrophages
RIP RNA	immunoprecipitation
ACC	adrenocortical carcinoma
LIHC	liver cancer
Tcm	central Memory T cell
Th	helper T cell
DC	Dendritic Cells
NK	natural killer cell

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22584.

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