Overexpression of RNF180 Inhibits Progression and Metastasis by Epithelial Mesenchymal Transformation and Immune Infiltration in Renal Cancer

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Research Article

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Abstract

Background: Molecular biomarkers combined with histopathological examination are of critical importance in the diagnosis and treatment of clear cell renal cell carcinoma (ccRCC). The occurrence and development of ccRCC is closely related to abnormal E3 ubiquitin enzyme. Although recent studies have revealed that Ring Finger Protein 180 (RNF180)known as an E3 ubiquitination enzyme, could regulate the cell proliferation, and differentiation in different cancers, the precise role of RNF180 in renal cancer remains to be elucidated.

Methods: Western-blot, qPCR and CCK-8 assay were performed to initially explore and further validate the function of RNF180. The effect of RNF180 on cell migration and invasion was verified by Transwell assay and EdU assay. The morphology of the tissue slides was evaluated by HE staining and immunohistochemical assay. Cell line-derived xenograft models in mice further validated the effect of RNF180 in vivo. Bioinformatics tools based on public databases were used to assess patient survival and immune infiltration.

Results: RNF180 is significantly down-regulated in multiple cancer-related databases and ccRCC cell lines. By using immunohistochemical analysis, we found that the expression level of RNF180 was also downregulated in ccRCC and the lower expression level of the RNF180 was associated with poorer clinical outcomes in renal cancer patients. Overexpression of RNF180 inhibits proliferation, migration and invasion of ccRCCs in vitro. In vivo, RNF180 could also modulate renal cancer progression in murine subcutaneous xenografts and liver metastasis. Strikingly, we further investigated the underlying mechanism and found that overexpression of RNF180 inhibited the progression of ccRCC by reducing epithelial-mesenchymal transformation (EMT) and may related to immune infiltration.

Conclusion: These results indicated that RNF180 can be used as a new diagnostic marker and prognostic factor, and can be served as a therapeutic target for ccRCC.

Background

Renal cell carcinomas (RCCs) are primary malignancies originating in the renal tubular epithelium and account for 90-95% of renal tumors [1]. In China, about 30% of patients with ccRCC are in high-grade or advanced stages at the time of diagnosis. Nevertheless, about 10% to 20% of patients diagnosed early in ccRCC were still at risk for recurrence and metastasis [2]. About 15 years ago, cytokines such as IL-2 and α-IFN were the only drugs available for advanced RCC, but the treatment of kidney cancer was limited due to its severe side effects and low efficiency[3]. But with the advent of targeted therapies, tyrosine kinase inhibitors such as axitinib and sunitinib, and mTOR inhibitors such as enviroximes have become standard of care in advanced patients [4]. In recent years, immunotherapy has emerged as a promising option, especially PD-1/PD-L1 inhibitors [5]. Although multiple approaches are available to treat metastatic ccRCC, treatment remains complex and challenging [6]. While these interventions may improve overall survival, complete remission of metastatic ccRCC is still difficult to achieve [7]. Therefore,

the postoperative systemic treatment of advanced ccRCC patients' needs to be further explored. It is particularly important to find new therapeutic targets for ccRCC and elucidate its therapeutic mechanism.

RNF180, also known as Rines, was first identified by Miyuki et al in 2008 as an E3 ubiquitin ligase, mainly distributed in the cytoplasm of the endoplasmic reticulum [8]. Recent studies on RNF180 mainly focus on its anti-cancer effect. For example, in gastric cancer, RNF180 is significantly down-regulated in gastric cancer compared with adjacent normal tissues, and its low expression indicates a significantly poor overall survival rate in patients [9]. Similarly, similar results have been found in non-small cell lung cancer, where low RNF180 expression levels predict poor biological behavior in NSCLC, and T staging is significantly correlated with RNF180 expression [10]. Previous studies found that RNF180 inhibited lymphatic metastasis of gastric cancer cells by inhibiting the activation of HGF [11]. Another study showed that in gastric cancer, RNF180 inhibited the progression of gastric cancer through ubiquitination and degradation of RHOC, which also provided a new explanation for the mechanism of RNF180 inhibition of gastric cancer [12]. However, in colorectal cancer, RNF180 has been found to interact with WISP1, ubiquitinating WISP1, and in mouse models, low expression of RNF180 ultimately leads to tumor growth inhibition [13]. In gliomas, RNF180 binds to and promotes PLK2 ubiquitination, thereby inhibiting the oncogenic ability of PLK2 [14]. However, the panoramic view of RNF180 remains to be unveiled. the distinct role of RNF180 in ccRCCs needs further investigation.

Epithelial-mesenchymal transition (EMT) is a biological process, and its most common biomarkers are E-cadherin, alpha SMA and alpha MMP [15]. EMT is an important marker associated with cancer progression and metastasis, and it is involved in many signaling pathways, such as TGF-β [16]. When TGF-β is activated, Smad2 and Smad3 are phosphorylated and bind to Smad4, thereby regulating the EMT process [17, 18]. In contrast, the Smad pathway usually acts as a node for crosstalk with other major signaling pathways, such as the MAPK pathway [19]. In a pancreatic cancer study, it has been demonstrated that activation of the MAPK pathway induces a decrease in E-CAD, Zo-1, and β-catenin, while increased expression of fibronectin, MMP9, Vimentin, and a-SMA is involved in the EMT process [20]. Another breast cancer study showed that MAPK activation down-regulated E-cadherin expression and significantly increased EMT [21]. TGF-β and MAPK and their regulatory relationship to EMT have also been observed in different cell lines, such as lung cancer cell A549 and human proximal tubule cell HK-2 [22, 23] . Membrane receptor phosphorylation and GSK3β inactivation in the Wnt pathway, followed by the release of β-catenin, activate target genes closely related to the EMT process [24]. β-catenin and GSK3β are key components of the Wnt pathway and major downstream targets of the TGF-β pathway [25]. At the same time, snails are the positive regulator of β-catenin, which then forms the positive feedback of the Wnt pathway and enhances the EMT process [26]. Studies on the relationship between the Wnt pathway and EMT in NSCLC showed that inhibition of β-catenin antagonized the Wnt pathway and eliminated the EMT process [27]. In breast cancer, when Wnt is activated, GSK3β kinase activity is inhibited, leading to the initiation of EMT programming [28]. In ovarian cancer, a nontraditional cyclin G2 inhibits EMT by inhibiting the Wnt/β-catenin signaling pathway and down-regulating key components of Wnt [29]. In conclusion, TGF-β, MAPK and Wnt pathways are closely related to the EMT process. However, more details remain to be investigated.

EMT is closely associated with the tumor microenvironment (TME). In cancer, TME consists of various mesenchymal cells and cytokines that support the tumor parenchyma, among which important components include lymphocytes, cancer-associated fibroblasts (CAF), tumor-associated macrophages (TAM), etc [30]. The cells in TME promote tumor progression by autocrine or paracrine of various cytokines, such as TGF-β and IL-6. And the tumorigenic mechanism involved in the above cytokines has a close relationship with EMT [31]. In prostate cancer, CAFs promote the EMT process by secreting TGF-β and upregulating ZEB1 expression in prostate cancer cells [32]. In renal clear cell carcinoma, TME, especially CD4+ T cells in TME, mediates the activation of Akt/GSK-3β/β-catenin signaling pathway to promote EMT by secreting IL-6, which marks the poor prognosis of renal clear cell carcinoma [33]. Thus, EMT is considered an important mechanism of tumor microenvironment pro-cancer, however, more detailed mechanisms need to be further explored.

In this study, we found that the expression of RNF180 was decreased and low expression of RNF180 was associated with poor prognosis by analyzing multiple cancer-related database. In clinical samples and cell lines, lower expression of RNF180 was associated with poorer clinical outcomes in renal cancer patients. Both in vitro and in vivo experiments have shown that overexpression of RNF180 inhibited the proliferation, invasion and metastasis of renal carcinoma. The mechanism showed that overexpression of RNF180 inhibited the progression of ccRCC by reducing epithelial-mesenchymal transformation (EMT). As of all, our study reveals the potential mechanism by which RNF180 regulates renal cancer progression, which may contribute to the development of a new diagnostic and prognostic marker.

Methods

Cell culture

786-O and A498 cells were obtained from the American Type Culture Collection (ATCC). 786-O cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ ml streptomycin and incubated in a 5% CO2 humidified incubator at 37°C. A498 cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ ml streptomycin and incubated in a 5% CO2 humidified incubator at 37°C.

Western blot

Cells were first lysed using cell lysis solution to obtain proteins, which were separated by electrophoresis. The proteins were transferred to nitrocellulose membranes (GE Healthcare). The membranes were blocked with Tris-buffered saline (TBS, pH 7.4) containing 5% skim milk and 0.1% Tween-20, then washed twice with TBS containing 0.1% Tween-20, incubated with primary antibody for 2 hours and secondary antibody for 1 hour at room temperature. Finally, the proteins of interest were visualized using an ECL chemiluminescence system (Santa Cruz).

Quantitative RT-PCR

Total RNA was isolated from transiently transfected cells using TRIzol reagent (Tiangen). cDNA was generated by reverse transcription using the Superscript RT kit (TOYOBO) according to the manufacturer's instructions. PCR amplification was performed using the SYBR Green PCR master mix Kit (TOYOBO). All quantifications were conducted at the level of the endogenous control GAPDH. Primer sequences are listed in Table 1.

CCK-8 assay

Cell proliferation rate was determined using Cell Counting Kit-8 (CCK-8). Control or siRNA-transfected cells were seeded onto 96-well plates at a density of 1000 cells per well. A 7-day incubation period was set, during which 10 μl of CCK-8 solution was added to the cell culture medium each day and incubated for 1 h. The resulting color was measured at 450 nm using a microplate absorbance meter (Bio-Rad). Each assay was repeated three times.

Cell migration and invasion assay

Cell migration was evaluated using 24-well transfer cells with polycarbonate membranes (pore size 8 μm) (Corning) according to the manufacturer's protocol. The membranes were coated with Matrigel basement membrane matrix (1 μg/μl) (BD Bioscience). Cells (0.5- 2.5×104) were then inoculated into the upper chamber in serum-free medium. The lower chamber was filled with medium containing 10% FBS. After 24 hours of incubation, the cells were removed from the upper chamber, fixed with 4% paraformaldehyde, and stained with crystal violet for 20 minutes. After washing three times with water, digital images were obtained from the membranes. Cell regions were selected using the Scan Scope CS system (Aperio Technologies). Migrating cells were quantified for five randomly selected regions on each membrane, and the average was defined as the migration or invasion index on three independent membranes. For invasion, the membranes used were Matrigel-coated invasion chambers (BD Biosciences), pre-hydrated in serum-free medium.

EdU assay

The EdU assay was performed using the EdU Apo llo 567 Cell Tracking Kit (Rib-bio). Cells from the test and control groups (5 x 103/well) were inoculated onto 96-well plates and incubated with 5-ethynyl-20-deoxyuridine (EdU; 200 μM) for 2 hours at 37°C. Cells were fixed with 4% paraformaldehyde for 20 min, treated with 0.5% TritonX-100 for 10 min, washed three times with PBS, and then incubated with 100 μL of Apollo reagent for 30 min. Cell nuclei were labelled with Hoechst 33342. The percentage of EdU-positive cells was calculated using image J software. The data reported represent the mean of three independent experiments.

In vivo tumorigenesis and metastasis assay

Cells stably overexpressing RNF180 (2×10^6) or control cells were subcutaneously injected into the right flanks of BALB/c (nu/nu) mice. Tumor size was measured once a week. After 3 weeks, the mice were

sacrificed for tumor burden analysis. The tumor volume was calculated as volume = (length × width^2)/2. For intrahepatic metastasis assays, cells (3 × 10^6/mouse) were injected into the livers of nude mice. For intrahepatic metastasis assays, cells (3 × 10^6/mouse) were injected into the livers of nude mice. Tumor nodules formed on the Liver surfaces were macroscopically determined and counted. The Livers were excised and embedded in paraffin. Further, the tissue sections (5 μm) were stained with H&E to visualize the structure. The Institutional Animal Care and Use Committee (IACUC) is from Ethics Committee of General Hospital of Shanghai Jiao Tong University.

Tissue sample collection and follow-up

We collected tumor and adjacent non-tumor tissues from 72 patients with ccRCC who underwent radical nephrectomy between January 2007 and September 2017 at Shanghai General Hospital of Shanghai Jiao Tong University School of Medicine (Shanghai, China). After surgery for cure, all patients received standard followed up according to the guideline. The median follow-up for the entire cohort was 45 months (range: 2–95 months). The follow-up of all patients included in this study was completed in December 2020. Patient consents were obtained for the use of the tissue samples. The study protocol and permission for the use of the clinical data were given by the Institutional Research Ethics Committee of Shanghai General Hospital (Shanghai, China).

Immunohistochemistry

Tissue microarrays (TMAs) were made at Otto Biotechnology Shanghai (Shanghai, China) using the 72 paired tissues described above, including tumor and adjacent normal tissues. Immunohistochemistry (IHC) was performed using the streptavidin peroxidase method (Zymed Laboratories Inc., San Francisco, CA, USA). RNF180 antibody was purchased from NOVUS (NBP1-86127, USA) and diluted 1:2000. IHC scores were determined by the estimated proportion of positive tumor cells in percentage. Assess the average degree of staining within a tumor, multiple regions were analyzed, and at least 100 tumor cells were assessed. The H-score system assessed the cytoplasmic or nucleus expression. The H-score formula is Histoscore = $\sum$(I × Pi), where I = intensity of staining and Pi = percentage of stained tumor cells, producing a cytoplasmic score ranging from 0 to 200. The scoring was independently assessed by two assessors (AWHC and JHMT) who were unaware of the clinical outcomes.

Statistical analysis

Data are presented as mean±SEM. All experiments were conducted with three or more replicates. Statistical analyses for most studies were performed by Student's t-test and were performed using GraphPad Prism v7.0 software and R studio. $p<0.05$ was statistically significant.

Bioinformatic analysis

Oncomine (http://www.oncomine.org), Gepia (http://gepia.cancer-pku.cn/),Timer (http://timer.cistrome.org/), EPIC (http://epic.gfellerlab.org) , TCGA website (https://tcga-

data.nci.nih.gov/tcga/) , GSE46699 and GSE105261 were used to acquire RNF180 RNA expression in cancer database. All data were downloaded following approval of this project by the consortium.

Results

Expression levels of RNF180 in different types of cancer

The cancer-related database, GEPIA and Oncomine were used to analyze the expression of RNF180 mRNA in ccRCC. The results showed that RNF180 was usually overexpressed (green) in tumors compared with downregulated (red) in normal tissues based on GEPIA database (Fig. 1A). In addition, the expression profiles of RNF180 in various cancer types and corresponding normal controls were investigated in the Oncomine database. We found that RNF180 expression was significantly higher only in brain and CNS cancer. In addition, our results indicated that RNF180 mRNA expression levels in breast, colorectal, esophageal, gastric, head and neck, Liver Cancer, Lung cancer, pancreatic cancers were significantly under expressed compared to the corresponding normal tissues in some data sets (Fig. 1B). To further evaluate RNF180 expression in various cancer types, we investigated RNF180 expression using the RNA-seq data of multiple malignancies in TCGA by TIMER. The results indicated that the expression levels of RNF180 were significantly increased in GBM (glioblastoma multiforme) and PCPG (Pheochromocytoma and Paraganglioma) compared to adjacent control samples (Fig. 1C).

However, RNF180 expression was significantly lower in BLCA (Bladder Urothelial Carcinoma), BRCA (breast invasive carcinoma), CESC (Cervical squamous cell carcinoma and endocervical adenocarcinoma), COAD (Colon adenocarcinoma), ESCA (esophageal carcinoma), HNSC (Head and Neck squamous cell carcinoma), KICH (Kidney Chromophobe), KIRC (kidney renal clear cell carcinoma), LIHC (liver hepatocellular carcinoma), LUAD (lung adenocarcinoma) , LUSC (lung squamous cell carcinoma), STAD (stomach adenocarcinoma), THCA (Thyroid carcinoma), UCEC (Uterine Corpus Endometrial Carcinoma) than in normal controls (Fig. 1b). Therefore, RNF180 showed the same expression trend in renal cancers in both the microarray and RNA-seq data.

RNF180 is a potential biomarker for ccRCC by TCGA

By reviewing the TCGA database, we found that RNF180 could serve as a potential biomarker for ccRCC. We first used the TCGA database to obtain the mRNA expression of RNF180 and its relationship with the prognosis of ccRCC. As shown in Figure 2A, the transcript level of RNF180 was significantly reduced in ccRCC compared with normal tissues ($p < 0.001$). Next, we further evaluated its relationship with clinicopathological features. RNF180 showed correlation with tumor grade, and its transcript level was well distinguished in G1 versus G3 and G4 (Figure 2B). We also observed that mRNA transcript levels of RNF180 were significantly correlated with the clinical stage of patients (Figure 2C). In terms of pathological samples, mRNA transcript levels of RNF180 were significantly distinguished in different TNM stages (Figure 2 D-F). In summary, the above evidences support our results that RNF180 may be a potential biomarker for patients with ccRCC.

Low expression of RNF180 predicts poor prognosis of ccRCC patients

The above results lead us to question whether the expression level of RNF180 could be a prognostic biomarker of ccRCC. To verify this hypothesis, TCGA database and GSE22541 dataset were used to analyze the overall survival between different expression level of RNF180 by Kaplan–Meier analysis (Figure 3 A-B). We found that low expression of RNF180 was correlated to low OS. To further validate the findings, IHC was performed to evaluate the RNF180 expression in clinical samples from 72 patients (Figure 3C). The patient characteristics and clinicopathological factors by RNF180 expression were displayed in Table 2. The results indicated that the low expression of RNF180 was significantly correlated to primary T stage (p=0.027), TNM stage (p=0.049) and Fuhrman grade (p=0.05). Despite age (p=0.380), gender (p=0.318) and tumor size (p=0.408) showed little relevant to expression of RNF180. IHC scores also supported the findings. The scores between tumor and adjacent normal tissues were significantly distinguished (p= 0.0424, Figure 3D), and such results could also be found between G1+G2 and G3+G4 (p=0.0491, Figure 3E) and between stage I+II and stage III+IV (p=0.0275, Figure 3F). Overall survival of patients with ccRCC was also reported by Kaplan–Meier curves (Figure 3G) and the results confirmed our previous hypothesis that low expression of RNF180 indicated low OS. We concluded that the expression level of RNF180 was a prognostic predictor for ccRCC patients

RNF180 is downregulated in ccRCCs clinical samples and cell lines

To further confirm the results of the bioinformatics analysis, we then evaluated the mRNA and protein expression of RNF180 in clinical samples and cell lines of ccRCCs. As shown in Figure 4A, in clinical samples of ccRCC, the results of Western Blot showed that protein levels of RNF180 were downregulated. The results of quantitative real-time PCR (qRT-PCR) corroborated the findings that the expression of RNF180 mRNA was relatively lower in tumors than in normal tissues (Figure 4B). Additionally, Western Blot and qRT-PCR were performed in ccRCCs cell lines. Similarly, the results in ccRCCs cell lines showed that both mRNA and protein levels of RNF180 were downregulated (Figure 4C-D). As expected, RNF180 expression was significantly decreased at both the cellular level and histological level in ccRCCs.

Overexpression of RNF180 inhibits proliferation, migration and invasion of ccRCCs in vitro

Altered expression levels of RNF180 in ccRCC may have a potential impact on the progression of ccRCC. To confirm this hypothesis, we constructed 786-O and A498 cell lines overexpressing RNF180 by transfection with overexpression plasmids and performed Western-Blot assays and qRT-PCR to verify this (Figure 5A). Next, CCK-8 assays were performed to assess the proliferation of the cells. The results showed that overexpression of RNF180 significantly reduced the proliferation rate of ccRCCs cells (Figure 5B). Similarly, the results of the EdU assays suggested that overexpression of RNF180 inhibited the proliferation of ccRCCs cells (Figure 5C). Migration and invasion are also hallmarks of tumor progression. We performed transwell assays to assess the migratory and invasive abilities of the cells. Our results showed that migration and invasion of ccRCCs cells were inhibited after RNF180 overexpression (Figure 5D). In summary, overexpression of RNF180 inhibited the proliferation, migration and invasion of ccRCCs cells in vitro.

Effects of RNF180 on tumor growth *in vivo*

To confirm the effect of RNF180 protein expression on tumor growth in vivo, we established a tumor transplantation model in nude mice by inoculating 4×10^6 786-O cells into nude mice and transfecting them with RNF180 expression vectors or the corresponding negative control vectors. After 8 weeks, the transplanted tumor area was smaller in the RNF180 overexpression group than in the control group (Figure 6A). Similarly, IHC staining showed that RNF180 was highly expressed in xenografts consisting of flag-RNF180 treated cells. The overexpressed RNF180 group had lower Ki67 levels than the control group (Figure 6B). In addition, the mean tumor volume in the group with increased RNF180 expression was significantly smaller than that in the group with decreased RNF180 expression (Figure 6C). Similar results were found for the tumor mass measurement (Figure 6D).

According to the biological effects of RNF180 mentioned above, the role of RNF180 overexpression on metastasis in vivo needs to be further proved. Cells with RNF180 overexpression or empty vector transfection were subcutaneously injected into nude mice to inhibit cell liver metastasis. Compared with the control group, less nodules of liver metastasis were observed in mice injected with RNF180 overexpression cells (Figure 6E). Histopathological studies confirmed that liver disease was caused by ccRCC cell invasion and subsequent tumor growth (Figure 6F). Therefore, we believe that RNF180 overexpression can inhibit the growth and metastasis of renal carcinoma.

These results were consistent with the results of the CCK8 and EdU assays described above. Therefore, we concluded that RNF180 was closely associated with the progression of renal cancer.

Overexpression of RNF180 significantly reduces EMT in ccRCC cells.

To investigate how RNF180 is involved in tumorigenesis and development in ccRCCs, a gene set enrichment analysis (GSEA) was performed to explore the biological pathways regulated by RNF180 based on the TCGA database. The results showed that RNF180 was highly correlated with the signatures of MAPK signalling, TGF-β signalling and WNT-β signalling (Figure 7A-C). EMT represents cancer progression and metastasis. TGF-β signaling, WNT-β signaling and MAPK signaling are involved in the EMT process by regulating many transcription factors, such as Vimentin, Snail1 [18,21,27]. Altered expression levels of E-cadherin and N-cadherin are also the biomarkers of EMT process [17]. Therefore, we hypothesized that RNF180 influences the EMT process. To test this hypothesis, we assessed the alteration of several EMT markers in RNF180 overexpressing cells. The results indicated that in RNF180 overexpression plasmid-transfected 786-O and A498 cells, E-cadherin expression was increased at the protein and mRNA levels, while Vimentin, Snail1 and N-cadherin expression was decreased (Figure 7D-F). Accordingly, it can be concluded that overexpression of RNF180 is able to inhibit EMT of ccRCCs in vitro.

Correlations of RNF180 expression with immune infiltration level in ccRCC

Somatic copy number alterations (sCNA) of RNF180 have significant correlations with infiltrating levels of neutrophils (Figure 8A). Immune cells in the TME can affect patient survival, and the above findings

support a prognostic role of RNF180 in ccRCC. Hence, it would be meaningful to explore the association between immune infiltration and RNF180 expression. We determined whether RNF180 expression was correlated with the immune infiltration level by calculating the coefficient of RNF180 expression and immune infiltration level in ccRCC in TIMER. The results indicated that RNF180 expression was also significantly correlated with the infiltration levels of CD8+ T cells, CD4+ T cells, macrophages and neutrophils in ccRCC, while showed no significant correlation of infiltration levels of B cells and dendritic cells (Figure 8B).

Immune infiltration is associated with survival in ccRCC patients with low RNF180 expression

By mining the EPIC database, we were able to explore the correlation between immune infiltration and survival of ccRCC patients. In general, immune infiltration had a significant effect on survival of ccRCC patients in patients with low RNF180 expression, while no significant effect was seen in patients with high RNF180 expression. Specifically, we found that among lymphocytes, high infiltration of B cells (Figure 9A), CD8+ T (Figure 9B) cells and CD4+ T (Figure 9C) cells significantly increased the overall survival time of patients, while high infiltration of NK cells (Figure 9D) decreased the overall survival time of patients. In contrast, among other components of the tumor microenvironment (TME), we found that high levels of cancer-associated fibroblasts (CAF) decreased the overall survival (OS) of patients (Figure 9E), while high levels of vascular endothelial cells significantly increased the OS of patients (Figure 9F). With the above findings, it is obvious to indicate that the type and level of immune infiltration has a significant impact on the prognosis of patients with ccRCC with low expression of RNF180.

Discussion

Currently, studies have shown that E3 ubiquitin ligase plays a regulatory role in many cancers by regulating the degradation of tumor promoters or inhibitors [34]. VHL disease, first identified as a tumor suppressor gene, is an inherited cancer syndrome with high frequency mutations associated with neuroblastoma, pheochromocytoma, and ccRCC [35]. VHL protein (PVHL), a product encoded by the VHL gene, is a key component of the PVHL E3 ubiquitin ligase complex, which has been found to act as a regulator of many substrates, including HIF-beta, ZHX2, NDRG3, and APKC [36]. Recent studies have found other E3 ligases related to ccRCC, such as SMURF1 [37], KLHL2 [38], TRIM13 [39], UHRF1 [40], which act as tumor suppressive factors through various mechanisms. However, although much has been written about the role of E3 ligases in ccRCC, furthermore studies are still needed to examine the role of E3 ligases in ccRCC from more perspectives. In our study, RNF180 is the E3 ligases that is related to progression of ccRCC.

Tumor development usually involves alterations in many normal physiological pathways, and ccRCC are no exception. Currently, we are yet to fully understand what the pathways associated with ccRCC are. In this study, we first queried the expression level of RNF180 in ccRCC appeared to be decreased through the TCGA database, to consider whether the expression level of RNF180 could be causally related to the progression of ccRCC. Subsequently, through survival analysis, we found that the downregulation of

RNF180 levels was indeed associated with poor prognosis in patients with ccRCC. Following in vitro experiments confirmed that overexpression of RNF180 significantly inhibited the proliferation, invasion and metastasis of ccRCC cells.

EMT is a hallmark of malignancy and is often associated with the progression and metastasis of cancer. EMT involves many signaling pathways, including the TGF-β pathway, Wnt/β-cat pathway and MAPK pathway, etc. The above pathways regulate the activity of their corresponding transcription factors and modulate the content of EMT-related proteins, thus mediating the EMT process. For example, in TGF-β pathway, EMT is induced by TGF-β/SMAD axis, with downstream transcription factors activated like Snail, which leads to EMT process [17]. For the Wnt pathway, TCF/LEF complex is a key transcription factor that regulates the EMT process by enhancing the expression of vimentin, c-myc, MMP [24]. Our experiments established a link between EMT and RNF180 in ccRCC, and proved that RNF180 may mediate EMT through the pathways above by TCGA database and in vitro experiments. We also demonstrated that the onco-suppressive ability of RNF180 was mainly achieved through the inhibition of EMT. Therefore, we suggest that RNF180 inhibits the progression of ccRCC by suppressing the EMT ability of cells, indicating that RNF180 could be a new target for future targeted therapy.

TME is closely related to tumor progression. various types of lymphocytes in TME and CAF, as the main components in TME, are involved in tumor progression and metastasis [30]. Our experiments first revealed a significant association between the degree of neutrophil infiltration in TME and sCNV of RNF180. As an important form of cellular aneuploidy mutation, sCNA is usually very common in tumors and is an important cause of tumorigenesis and progression and is often associated with poor patient prognosis [41]. sCNA may be associated with tumor immunity as T. Davoli et al. found that high levels of sCNA were associated with low response rates and poorer prognosis in patients treated with melanoma immunotherapy, suggesting a possible association of sCNA with tumor immunity [42]. We next explored the association between the level of immune infiltration and the level of RNF180 expression. We found that the level of infiltration of two types of T cells (CD4+ and CD8+), macrophages, and neutrophils, which are important components of TME, had a significant positive correlation with the expression of RNF180. Based on the results we have derived that RNF180 inhibits tumor proliferation, invasion, and metastasis, we speculated whether the elevated level of immune infiltration might be associated with patient survival. Therefore, we further performed a survival analysis of RNF180 expression and immune infiltration levels with OS based on the TCGA database. The results revealed that immune infiltration was significantly associated with patient prognosis in the lower RNF180 patient group. Taken together, we suggest that RNF180, as a potential tumor suppressor, may have an impact on patient prognosis by participating in the regulation of immune infiltration in the tumor microenvironment of ccRCC, which in turn affects tumor proliferation, invasion and metastasis. Thus, the results show the association of RNF180 with immune infiltration and provide a new insight into the mechanism and optional targets for immunotherapy of bladder cancer. However, we still need further experimental validation to confirm the association.

Previous studies on RNF180 have revealed its cancer inhibitory ability in various solid tumors [9, 10]. The mechanism may be related to the ubiquitination of substrates associated with cancer progression, which has been demonstrated in cancers such as gastric cancer, colorectal cancers and glioma [12-14]. In the present study, we confirmed that the onco-suppressive effect of RNF180 in ccRCC was associated with EMT and identified changes in EMT-related protein content and established a preliminary association between the two. We also showed a link between RNF180 and immune infiltration, revealing that the tumor suppressive function of RNF180 is associated with immune infiltration. In the next step of our study, we will further validate this association and try to probe at the molecular level whether RNF180, through its ubiquitination function, mediates changes in the levels of these proteins that lead to EMT, immune infiltration and cancer progression.

Conclusion

We demonstrated that the tumor suppressive effect of RNF180 in ccRCC was associated with EMT, and we also revealed a link between RNF180 and immune infiltration, and this finding suggests that the tumor suppressive function of RNF180 is associated with immune infiltration.

List Of Abbreviations

RNF180: Ring Finger Protein 180; ccRCC: clear cell renal cell carcinoma; EMT: Epithelial-mesenchymal transition; TME: tumor microenvironment; CAF: cancer-associated fibroblasts; TAM: tumor-associated macrophages; IHC: Immunohistochemistry; TCGA: The Cancer Genome Atlas; GSEA: Gene Set Enrichment Analysis; GEO: Gene Expression Omnibus; sCNA: somatic copy number alterations

Declarations

Ethics approval and consent to participate

The studies involving human participants were reviewed and approved by Medical Ethical Committee of Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine.

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analysed during the current study are available in TCGA and GEO databases. (TCGA: https://tcga.xenahubs.net; GEO: https://www.ncbi.nlm.nih.gov/geo/). The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declared that no competing interest exists.

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Authors' contributions

EZ and XW were the main conceivers and leaders of this article. EZ, HT performed main experiments and wrote the manuscript. HT and YG made great data analysis. CF, ZY, TG, YQ, BS, HL and YW collected clinical samples. MT and YF revised the manuscript. All authors contributed to the article and approved the submitted version.

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Not applicable

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Tables

Table 1. Sequence of primers

Gene name	Forward (5’-3’)	Reverse (5’-3’)
RNF180	5'-AGT TAC AAG AAG GCA GTT CC-3'	5'-AAT CCA ATG ACC CAG TTC AC-3'
GAPDH	5'-AAT CCC ATC ACC ATC TTC-3'	5'-AGG CTG TTG TCA TAC TTC-3'

Table 2 is not available with this version

Figures

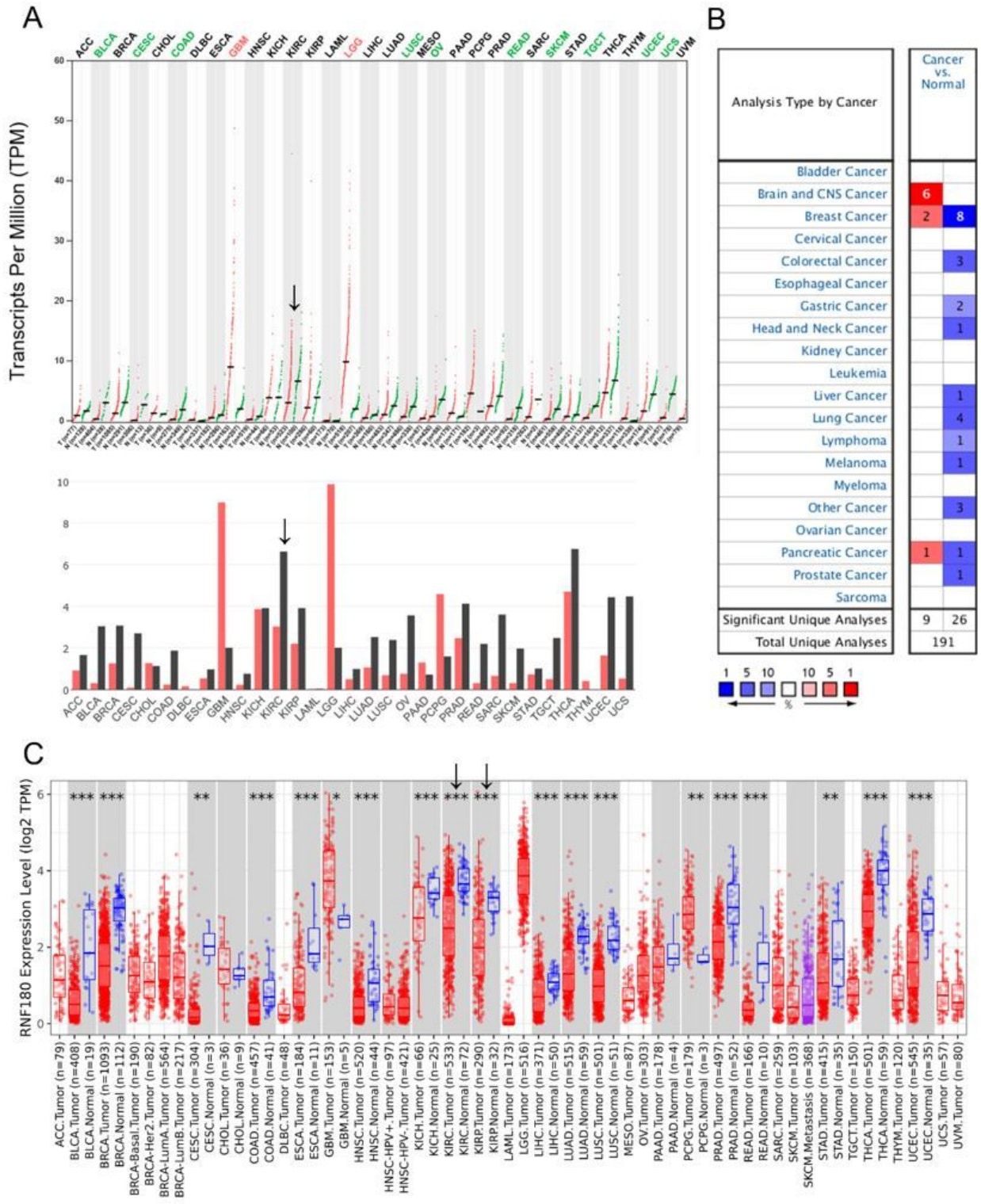

Figure 1

RNF180 expression levels in different types of human cancers. (A) RNF180 RNA expression in different cancer on GEPIA. (B) Increased or decreased level of RNF180 in datasets of different cancers compared with normal tissues in the Oncomine database. (C) Human RNF180 expression levels in different tumor types from TCGA were determined by TIMER (*P < 0.05, **P < 0.01, ***P < 0.001)

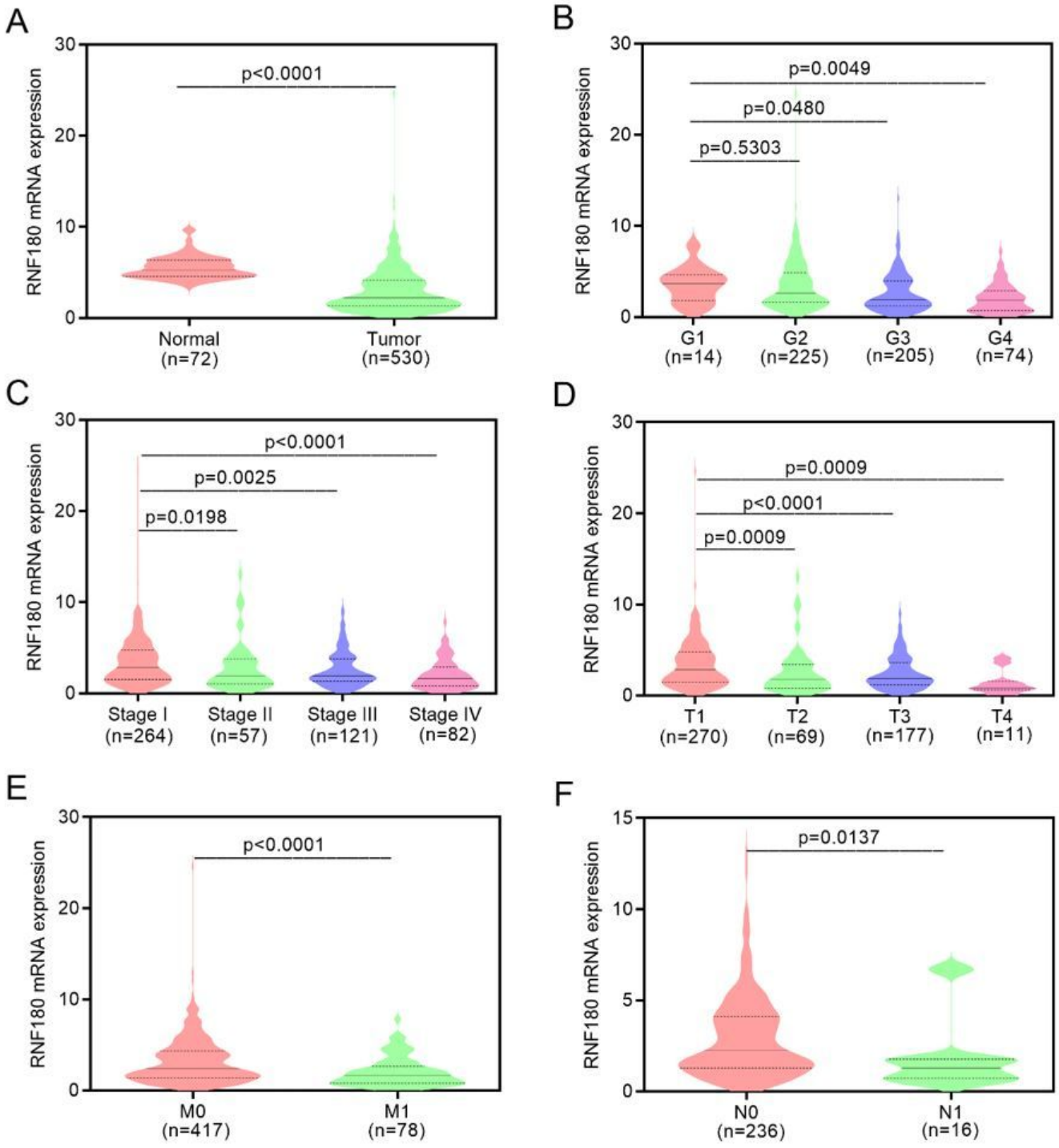

Figure 2

RNF180 is a potential biomarker for clear cell RCCs (ccRCC) assessed by the Cancer Genome Atlas (TCGA). (A) Compared with normal tissues, the transcript level of RNF180 in ccRCCs tumor tissues was decreased. (B) In tumor grading, the lower level of transcription of RNF180 was correlated with higher grades. (C) In tumor stages, the transcription of RNF180 in patients with higher stages of ccRCC decreased significantly. (D) There was significant difference in the transcription of RNF180 among the T1~T4 phases of ccRCC patients. (E) As ccRCC became metastatic, the transcription of RNF180 significantly decreased. (F) RNF180 transcription wan significantly associated with lymph node invasion.

(Tumor staging is based on AJCC standards. T: tumor, representing the extent of the primary tumor; N: lymph node, representing whether there is regional lymph node metastasis; M : Metastasis, which represents whether there is distant metastasis).

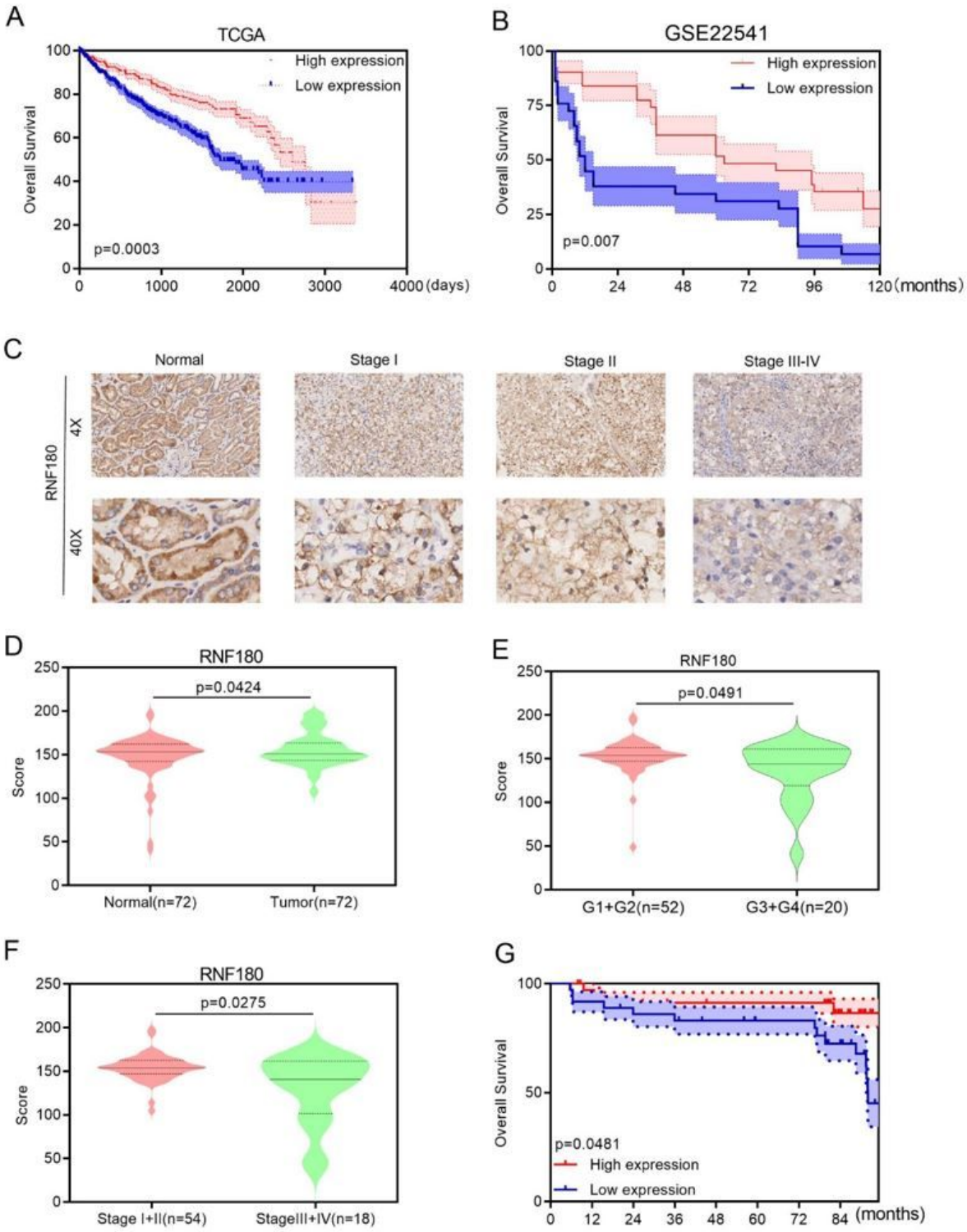

Figure 3

RNF180 is associated with prognosis in ccRCC patients. (A) Overall survival (OS) curve of ccRCC patients from TCGA database by Kaplan–Meier analysis. (B) Overall survival (OS) curve of ccRCC patients from GSE22541 database by Kaplan–Meier analysis. (C) Immunohistochemistry (IHC) of clinical samples from normal tissues to stage IV tumor tissues. 72 in total samples were evaluated by IHC. The IHC score of RNF180 was calculated as the staining intensity (0, 1, 2, or 3) × the staining extent (0–100%). (D) The IHC scores between tumor tissues and adjacent normal tissues. (E) The IHC scores between G1+G2 tissues and G3+G4 tissues. (F) The IHC scores between early stage (I+II) tissues and late stage (III+IV) tissues. (G) Overall survival (OS) curve of ccRCC patients from our clinical patients by Kaplan–Meier analysis.

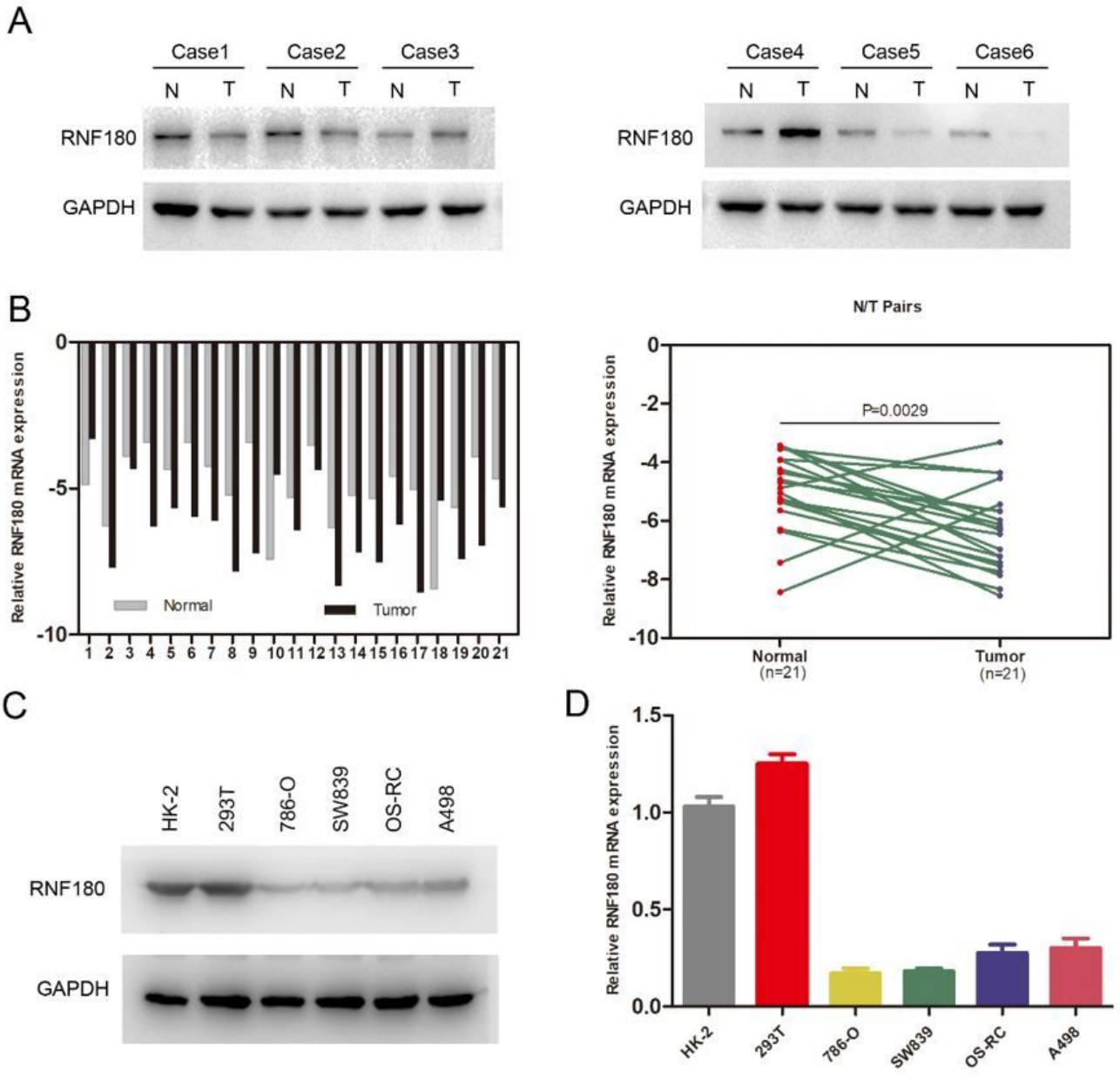

Figure 4

The RNF180 expression is downregulated both in clinical ccRCC samples and in renal cancer cell lines. We conducted (A) Western Blot and (B) qRT-PCR in clinical ccRCC samples and in matched adjacent normal tissues to evaluate the expression level of RNF180. (C) Western Blot was also conducted to investigate RNF180 expression in cell lines HK-2, 293T, 786-O, SW839, OS-RC and A498. GAPDH was used as loading control in Western blot. (D) qRT-PCR was conducted in cell lines HK-2, 293T, 786-O, SW839, OS-RC and A498 to probe RNF180 mRNA levels.

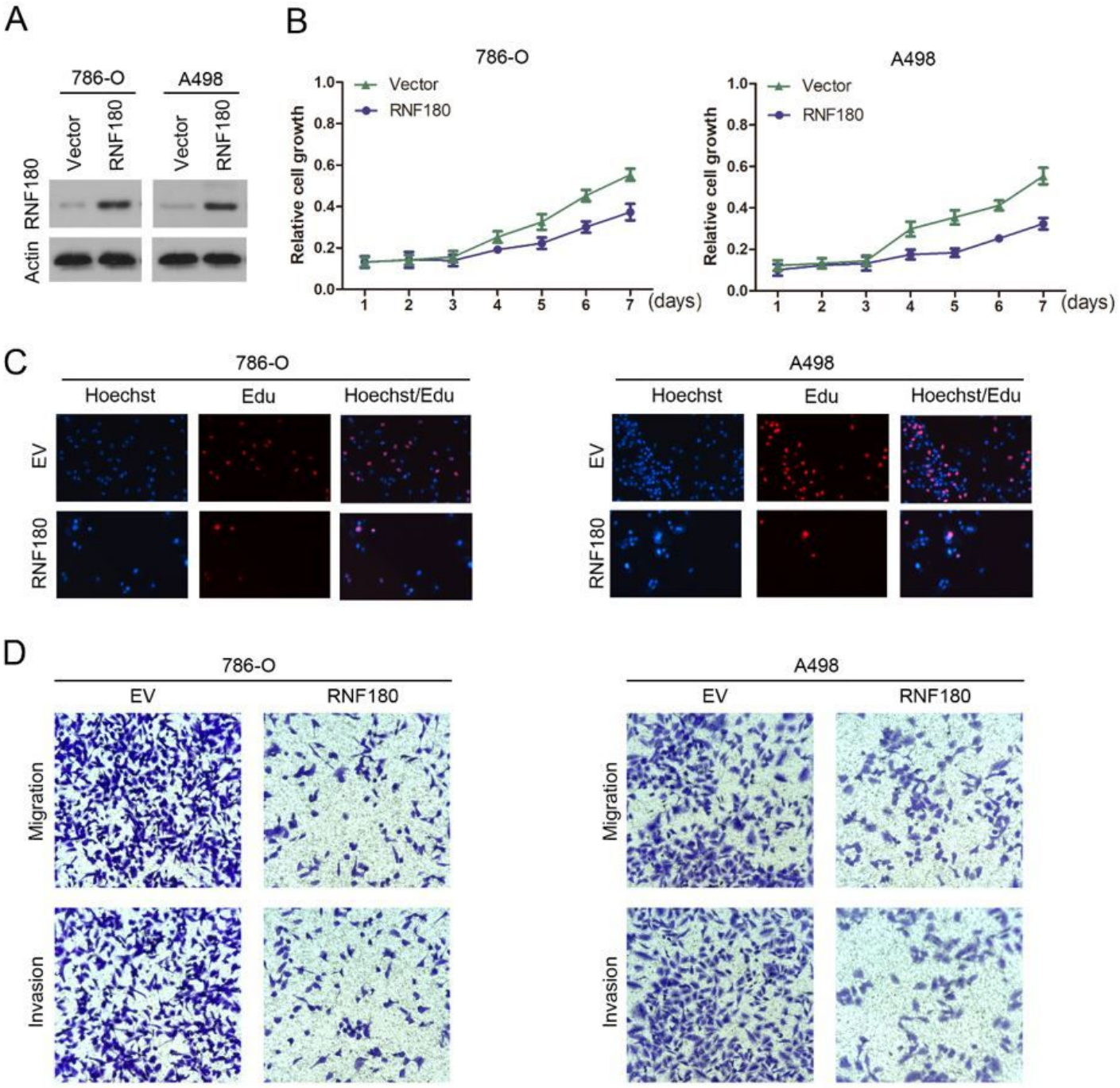

Figure 5

Overexpression of RNF180 inhibits cell proliferation, migration and invasion in vitro. (A) The efficiency of RNF180 overexpression in cell lines 786-O and A498 transfected with expression vectors of RNF180 was verified with Western Blot, actin was used as loading control. (A) CCK-8 assays revealed cell growth curves of 786-O and A498 cells. (B) EdU assays of 786-O and A498 indicated cell proliferation impacted by RNF180. EdU (red), nuclear (Hoechest, blue). Representative photographs were taken at ×200 magnification. (C) Migration and invasion assays for ccRCC cell lines 786-O and A498, representative photographs were taken at ×200 magnification.

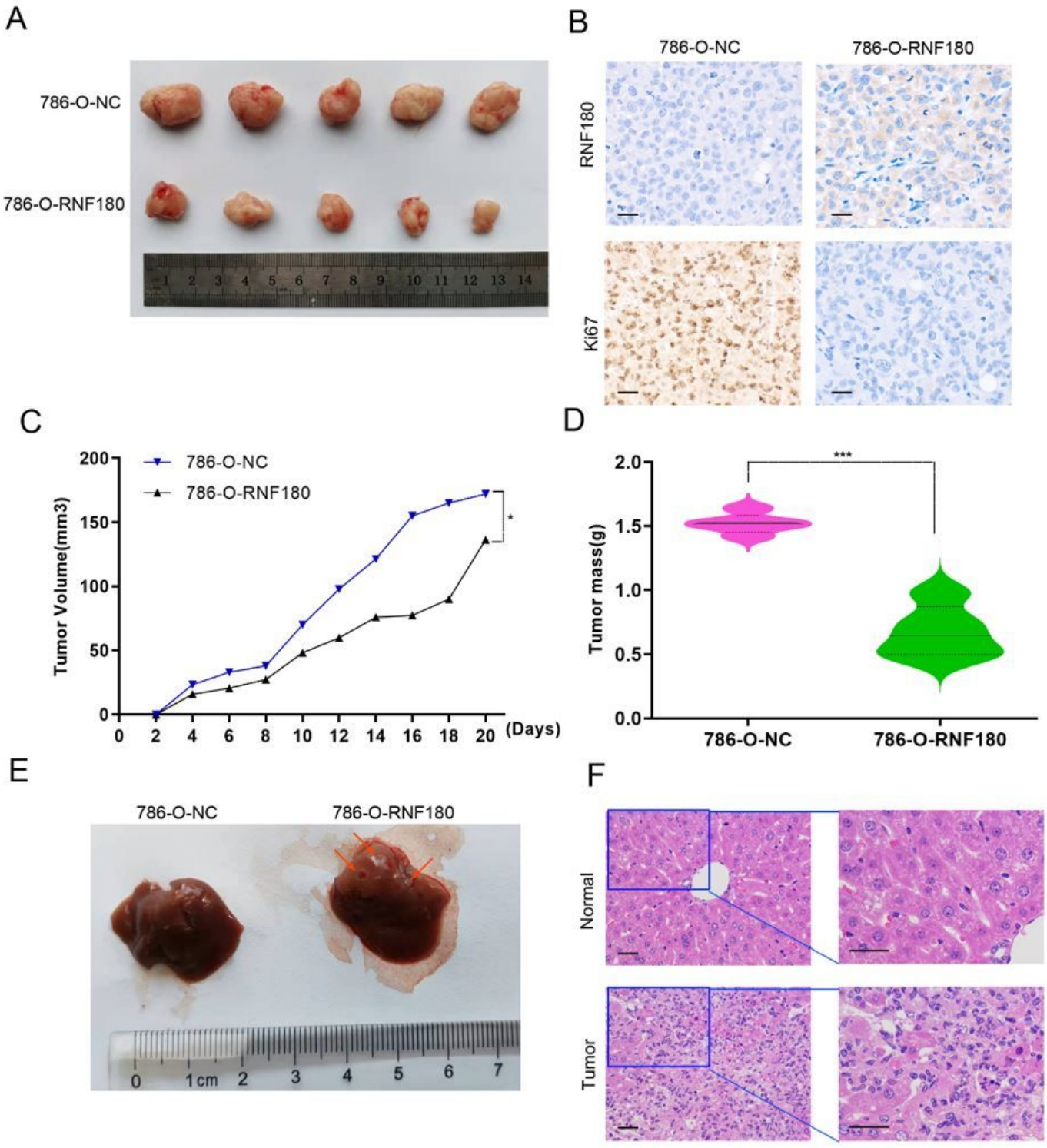

Figure 6

RNF180 inhibits tumor growth in vivo. (A) The size of the xenograft tumors was smaller in the RNF180 overexpression group compared to the negative control group. (B) IHC staining of RNF180 and Ki-67 expression in 3 groups. (C) Tumor growth curves. Tumor volumes were calculated as V (mm3) = length × width × width/2. (D) xenografts of 786-O-RNF180-overexpression stable cells in nude mice. Images showing intrahepatic metastasis (E) and morphological characters by HE staining (F), **P < 0.01. All assays were conducted in triplicate.

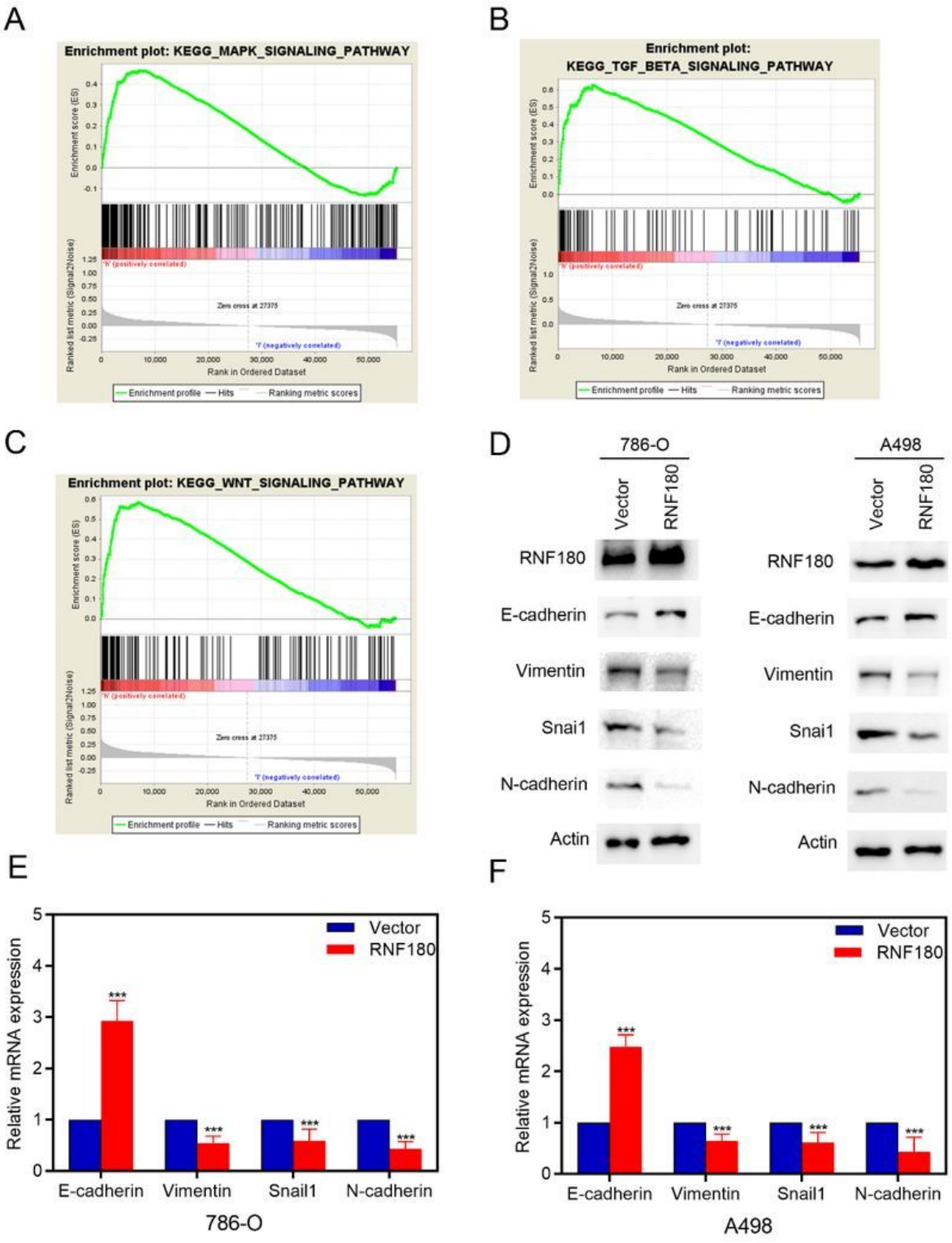

Figure 7

Decreased expression of PPT2 facilitates EMT in ccRCC in vitro. (A-C) GSEA was performed to investigate the biological pathways involved in RNF180 regulation on the basis of TCGA database. (D) The alterations of EMT markers (E-cadherin, Vimentin, Snail1 and N-cadherin) were tested by Western Blot in cell lines 786-O and A498. Actin was used as loading control. (E-F) The mRNA level of EMT markers(E-

cadherin, Vimentin, Snail1 and N-cadherin) between normal and RNF180 overexpressed cells(786-O and A498).

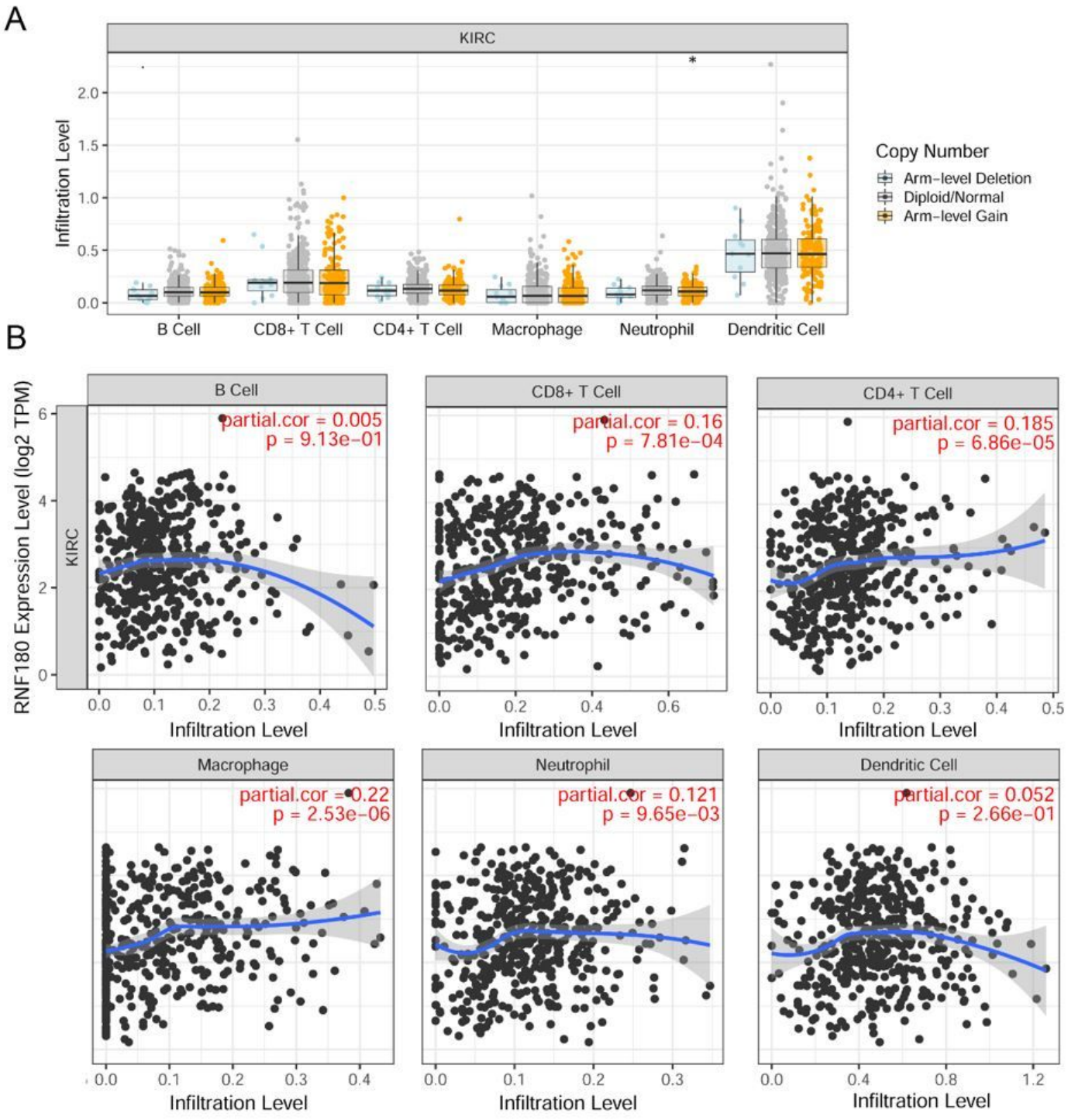

Figure 8

Correlations of RNF180 expression with immune infiltration level in ccRCC. (A) RNF180 sCNA affects the infiltrating levels of B cells, CD8+ T cells, macrophages, neutrophils, and dendritic cells in ccRCC. (B) RNF180 expression has significant positive correlation with infiltrating levels of CD8+ T cell, CD4+ T cell,

macrophage and neutrophil and no relation with infiltrating levels of B cells, dendritic cells and other types of cells in ccRCC. P < 0.05 is considered as significant.

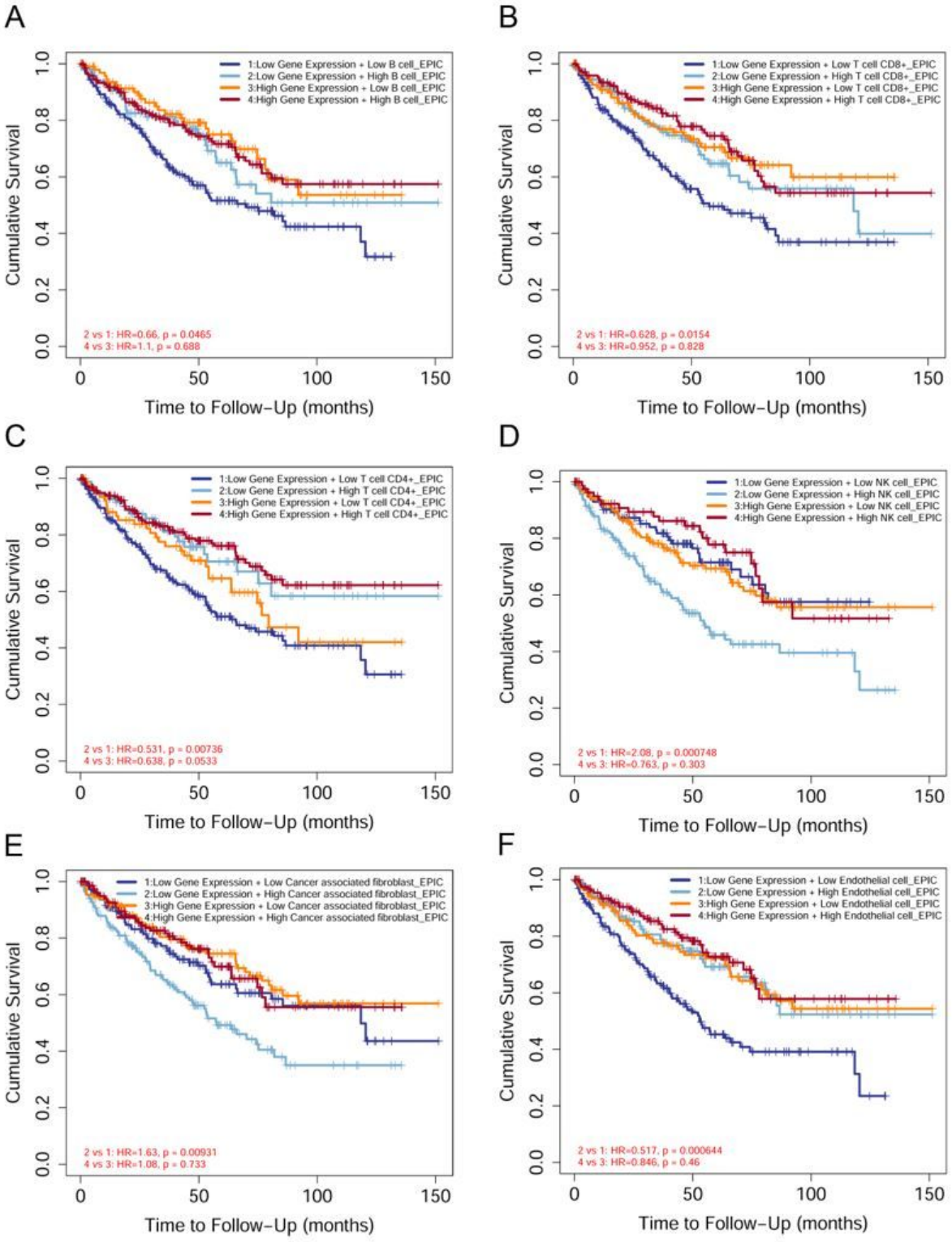

Figure 9

Survival analysis of high and low expression of RNF180 and level of immune infiltration by EPIC platform. (A) B cells, (B) CD8+ T cells, (C) CD4+ T cells, (D) NK cells, (E) CAFs, (F) Endothelial cells. P <

0.05 is considered as significant.