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Revealing PAM as a Prognostic Biomarker and Therapeutic Target in Clear Cell Renal Cell Carcinoma

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Abstract

Background: Accumulating evidence suggests that peptidylglycine α-amidating monooxygenase (PAM) is involved in vital physiological and pathological processes, including the development and progression of cancer. Nevertheless, the precise contributions of PAM-mediated pathways in clear cell renal cell carcinoma (ccRCC) remain poorly understood. Clarifying the role of PAM in ccRCC could yield novel insights into the disease's pathogenesis and offer potential therapeutic strategies.Methods: Using genome-wide association study (GWAS) data from the UK Biobank and whole-blood eQTL data, we screened for ccRCC-related genes and identified PAM as a potential oncogene. Bioinformatics analyses, including differential expression, prognostic, genomic, and methylation analyses,

were conducted to characterize the role of PAM in ccRCC. In addition, functional pathways of PAM were explored using gene set enrichment analysis. The association between PAM expression, immune cell infiltration, and immunotherapy response was also evaluated. Subsequently, in vitro tumor phenotype experiments, such as cell viability, wound healing and modified Boyden chamber assays, were conducted to validate the bioinformatics predictions.
Results: Our findings indicated that PAM expression was elevated in ccRCC tissues compared to adjacent normal ones, and is associated with unfavorable disease-free survival in ccRCC patients. Genomic alterations such as gene amplifications were detected in ccRCC, with PAM expression linked to multiple cancer pathways. Furthermore, PAM expression was positively correlated with immune cell infiltration and negatively with immune cell function in ccRCC. In vitro functional assays revealed that PAM downregulation reduced the proliferative and migratory capacity of ccRCC cells.
Conclusions: Our studies reveal that PAM serves as a potential prognostic biomarker and therapeutic target in ccRCC. Further researches are warranted to validate its clinical utility and investigate its potential for guiding personalized treatment strategies in ccRCC patients. Understanding the role of PAM in ccRCC progression may provide novel insights for the development of targeted therapies and biomarker-based approaches for ccRCC management.

1 Introduction

Renal cell carcinoma (RCC), commonly known as kidney cancer, arises from the epithelial cells of the renal tubules [1]. Internationally, RCC is the 14th most prevalent malignancy in adults, with over 400,000 new cases each year, accounting for 2.2% of all cancer diagnoses. Annually, RCC causes more than 150,000 deaths, representing 1.8% of all cancer-related mortalities [2]. It is the second most frequent malignancy of the urinary system [3]. Studies have established a correlation between the Human Development Index (HDI) and RCC mortality, with higher rates in more developed areas [4]. With the global rise in HDI, there is a growing need to address RCC-related mortality.
RCC is characterized by substantial heterogeneity at the molecular, genomic/epigenomic, morphological, and clinical levels [5]. Clear cell RCC (ccRCC), the most common RCC subtype, comprises 70–80% of cases and has a mortality rate of approximately 40%. Advances in medical imaging and improved screening have led to the early detection of asymptomatic tumors in over 60% of cases, often treated successfully with radical nephrectomy or nephron-sparing surgery, resulting in favorable outcomes. However, some patients present with advanced disease or experience progression. Post-surgery, approximately 30% of patients develop distant metastases, commonly in the lungs, liver, and brain [6].
Radical nephrectomy remains the primary treatment for ccRCC, particularly effective for tumors confined to the perirenal fascia (Gerota's fascia). However, treatment options for advanced ccRCC or recurrence/metastasis after surgery are limited [7, 8]. These factors significantly impact therapeutic outcomes and reduce overall survival rates [9]. Prognostic assessment of ccRCC relies mainly on pathological staging and grading, with a dearth of comprehensive biomarkers. Consequently, there is an urgent need for novel biomarkers to predict ccRCC progression and outcomes.
The pathogenesis of ccRCC is not fully understood. Previous research has implicated various factors in the initiation and progression of ccRC

C, including oncogene activation, tumor suppressor gene inactivation, and dysregulated growth factor expression [10, 11]. Mutations or inactivation of the tumor suppressor gene VHL are pivotal in sporadic ccRCC. In approximately 70–80% of ccRCC cases, VHL undergoes mutation, deletion, or methylation, leading to the loss of VHL protein function. This disrupts the degradation of hypoxia-inducible factor (HIF), causing its accumulation and the activation of vascular endothelial growth factor (VEGF). VEGF binding to VEGFR on endothelial cells activates protein tyrosine kinases (PTKs) and downstream signaling pathways, such as Ras, initiating the Raf/MEK/ERK and PI3K/Akt/mTOR cascades that drive angiogenesis, lymphangiogenesis, tumor growth, and metastasis. Growth factors, cytokines, and hormones can also indirectly regulate VEGF expression through pathways like PI3K/Akt and MAPK [12, 13].

The pathogenic process may involve the enzyme-encoding gene peptidylglycine α-amidating monooxygenase (PAM). Located on chromosome 5q15, the PAM gene spans over 160 kb with 25 exons [14]. PAM is a bifunctional enzyme with 2 catalytic subunits that possess distinct activities: peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL) [15, 16]. These domains utilize oxygen, ascorbate, and
copper ions to convert peptide hormone precursors into active α-amidated forms, enhancing their stability, activity, and receptor-binding capabilities, thus enabling their physiological functions [17, 18]. PAM, which is vital for life, is the sole known enzyme capable of catalyzing C-terminal alpha-amidation [16]. It is widely expressed in mammalian cells, with peak activity in the pituitary gland and hypothalamus [19].

PAM activity regulation in humans has been linked to various diseases [16, 20]. Increased α-amidation activity has been observed in medullary thyroid carcinoma, neuroendocrine tumors, and pancreatic endocrine tumors, as well as in conditions such as multiple sclerosis and post-polio syndrome [21–24]. Timothy M. et al. suggested that PAM staining intensity in primary neuroendocrine tumors could serve as a prognostic biomarker [25]. However, no studies have investigated PAM expression in ccRCC or its prognostic significance.

2 Materials and methods

2.1 Tissue specimens

Ten pairs of ccRCC tissues and adjacent non-tumor tissues were collected from patients who underwent surgical treatment at Zhongnan Hospital of Wuhan University between June and December 2023. Cases were selected based on the following criteria: (1) confirmation of ccRCC by postoperative pathological diagnosis; (2) intact tissue specimens, with non-tumor tissues situated at least 3 cm from the tumor margins. Tissues were stored in the hospital's biobank using liquid nitrogen. All patients provided written informed consent, and the study was granted ethical approval by the Clinical Research Ethics Committee of Zhongnan Hospital of Wuhan University, Hubei Province (Ethics Approval Number: 2023110K).

2.2 Cell culture and lentiviral transduction

ACHN and OS-RC-2 cells were purchased from the Chinese Type Culture Collection Center (Wuhan, China) and were tested to be mycoplasma-free. The cells were maintained in DMEM or 1640 medium (Gibco, USA) containing 10% fetal bovine serum and cultured in incubator (Thermo Fisher, USA) containing 5% CO_2, at 37 °C with appropriate humidity. To generate stable cell lines with PAM deficiency, according to the manufacturer's instructions, related lentiviral vectors along with the psPAX.2 and pMD2.G packaging systems were transfected into HEK293T cells using Lipo3000 reagent (Invitrogen, USA). Seventy-two hours later, the viral particles were collected and filtered. Then, HK-2 cells were infected and selected with 1 μg/mL puromycin (Beyotime, China) to obtain stable cell lines.

2.3 Immunohistochemistry (IHC)

For IHC staining, renal sections were incubated with anti-PAM antibody (1:200, #26972, Proteintech, China) overnight at 4 °C. Images of renal tissue was obtained using a microscope (Olympus, Japan), and the relative expression of these proteins was quantified using ImageJ software.

2.4 Quantitative PCR (qPCR)

Total RNA was extracted from ACHN and OS-RC-2 cells using TRIzol reagent (Invitrogen, USA) and reverse transcribed into cDNA using HiScript III Reverse Transcriptase (Vazyme, China). Following the manufacturer's protocol, Taq Pro Universal SYBR qPCR Master Mix (Vazyme, China) was used to perform qPCR.

2.5 Cell viability assay

ACHN and OS-RC-2 cells were seeded in 96-well plates. The medium was replaced with 10% CCK8 reagent (MCE, USA), and then incubated for 1 hour. The absorbance was measured at 450 nm using a microplate reader (Thermo Fisher, USA).

2.6 Modified Boyden chamber assay

Cells were seeded into the chambers or chambers containing Matrigel solution (Corning, USA). The cells were then incubated at 37 °C for 48 hours, after which a wet cotton swab was used to remove non-migratory cells from the upper surface of the chamber. The cells were fixed with 4% formaldehyde solution for 15 minutes, followed by staining with 0.1% crystal violet (Google Biotech, USA) for 15 minutes. Finally, photographs were taken using a microscope (Olympus, Japan).

2.7 eQTL, GWAS, and bioinformatics data

In the Summary-data-based Mendelian Randomization (SMR) analysis, cis-eQTL genetic variants were used as instrumental variables (IV) for gene expression. The analysis utilized eQTL data from blood, as blood may reflect hormonal or metabolic traits associated with RCC. The eQTL data were sourced from the V7 version of the GTEx aggregation dataset. Detailed information on sample collection and treatment can be found in other articles [26]. The aggregate data included 338 blood subjects [27]. The eQTL data can be downloaded from https://cnsgenomics.com/data/SMR/#eQTLsummarydata.

The GWAS aggregate data for kidney cancer were provided by the UKB database (http://www.nealelab.is/uk-biobank), encompassing a total of 1,114 kidney cancer cases and 461,896 controls. The GWAS aggregate data can be downloaded from https://gwas.mrcieu.ac.uk/datasets/ukb-b-1316/.

Pan-cancer RNA-Seq data (FPKM values) and corresponding The Cancer Genome Atlas (TCGA) survival information [28] were extracted from the UCSC Xena Browser (https://xena.ucsc.edu/)[29]. Next, data for 105 TCGA-KICH, 950 TCGA-KIRC, and 352 TCGA-KIRP cohort patients (FPKM and Counts values), along with corresponding phenotype and DNA methylation data, were downloaded. Copy number variations (CNV) in TCGA-STAD were collected and processed using the GISTIC 2.0 algorithm [30], and somatic mutation spectra (Varscan) were obtained as mutation annotation format (MAF) [31] using the R package "maftools."

Gene expression profiles and clinical information from the Gene Expression Omnibus (GEO) [32] were downloaded for GSE167573, GSE29609, GSE22541, GSE111360, GSE121636, GSE139555, GSE145281, GSE159115, and GSE171306. CPTAC-CCRCC can be downloaded from TCIA (https://www.cancerimagingarchive.net/collection/cptac-ccrcc/), ICGC-EU from ICGC (https://dcc.icgc.org/), and E_MTAB_1980 from BioStudies (https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-1980). For all acquired cohorts, normalization was performed using the "normalizeBetweenArrays" function in the R package

2.8 SMR analysis

In SMR analysis, cis-eQTLs are used as IV, gene expression is the exposure, and renal cancer is the outcome. The analysis is performed using t he method implemented in the SMR software. SMR applies the Mendelian Randomization (MR) principle to jointly analyze GWAS and eQTL agg regate statistics, testing for pleiotropic associat ions between gene expression and traits, which are due to shared and potentially causal variant s at the locus. Detailed information about the S MR method has been reported in previous publi cations [34]. An IV heterogeneity (HEIDI) test [3 4] is conducted to assess whether there is linka ge in the observed associations. Rejecting the n ull hypothesis () suggests that the observed ass ociations may be due to two different genetic v ariants in high linkage disequilibrium that are i mbalance with each other. The default settings in SMR are used (for example, , minor allele freq uency [MAF] > 0.01, removing SNPs with very st rong linkage disequilibrium [LD, r^2 >0.9] with t he top-associated eQTL, and removing SNPs w ith low LD or not in LD [r^2 < 0.05] with the top -associated eQTL), and the false discovery rate (FDR) is used to adjust for multiple testing.

2.9 Bioinformatics analysis

We explored the mRNA and protein expression levels of PAM in normal or tumor tissues. The re lationship between PAM expression and clinical outcomes, including overall survival (OS), progr ession-free interval (PFI), disease-free interval (DFI), and disease-specific survival (DSS), was a nalyzed and visualized. Univariate Cox proporti onal hazards analysis was performed based on PAM expression.

We utilized cBioPortal (https://www.cbioportal. org/) to depict the pan-cancer genome landsca pe of PAM from the perspectives of CNV and si ngle nucleotide polymorphisms (SNPs) [35]. Th e correlation between PAM and RNA modificati on factors, as well as immunomodulatory factor s, was analyzed at the pan-cancer transcriptom ic level. The correlation between PAM and imm une cell infiltration was calculated using algorith ms including CIBERSORT, CIBERSORT-ABS, Q UANTISEQ, MCPCOUNTER, XCELL, and EPIC. The Tumor Immune Dysfunction and Exclusion (TIDE) database was used to evaluate the impa ct of PAM on immune cell function [36].

Gene set enrichment analysis (GSEA) was perf ormed based on PAM expression (top 30% an d bottom 30%) to predict potential cancer pat hways associated with PAM [37, 38], including KEGG, GO, and Hallmark pathways [39-42].

2.10 Statistical analysis

The Mann-Whitney U test was used to assess di fferences between groups. The correlation betw een variables was analyzed using Spearman's c orrelation analysis. A P-value of less than 0.05 w as considered statistically significant for intergr oup comparisons. Data processing and statistic al analysis were performed using R (version 4.1. 3). In addition, data visualization was achieved with the assistance of Sangerbox [43], BEST [4 4], and cBioportal.

3 Results

3.1 Genes Associated with Renal Cancer Occurrence as Determined by SMR

GWAS aggregate data were based on GWAS a nalysis of 463,010 subjects (including 1,114 ren al cancer cases and 461,896 controls) from the UKB database. After checking the allele freque ncies in the dataset and performing LD prunin g, the final SMR analysis included approximatel y 9.85 million eligible SNPs. The sample size of eQTL data from whole blood was 338, with 4,4 90 eligible probes. Detailed information is sho wn in Table 1.

Table 2 shows the genes that exhibit pleiotropi c associations with renal cancer after multiple t esting corrections using whole blood eQTL dat a. Specifically, apart from PPIP5K2, HIST1H4H did not pass the HEIDI test, and RP11-448G15. 3, CTD-3064M3.1, RP4-673D20.1 are non-co ding genes. A total of eight genes, RERE, CASP 9, PLEKHM2, PPIG, HTRA3, PAM, CDCA7L, and IQSEC3, were identified as significantly associa ted with renal cancer.

3.2 Screening of ccRCC-Related Biomarkers through mRNA, Protein Expression, and Prognostic Analysis

Figure 1

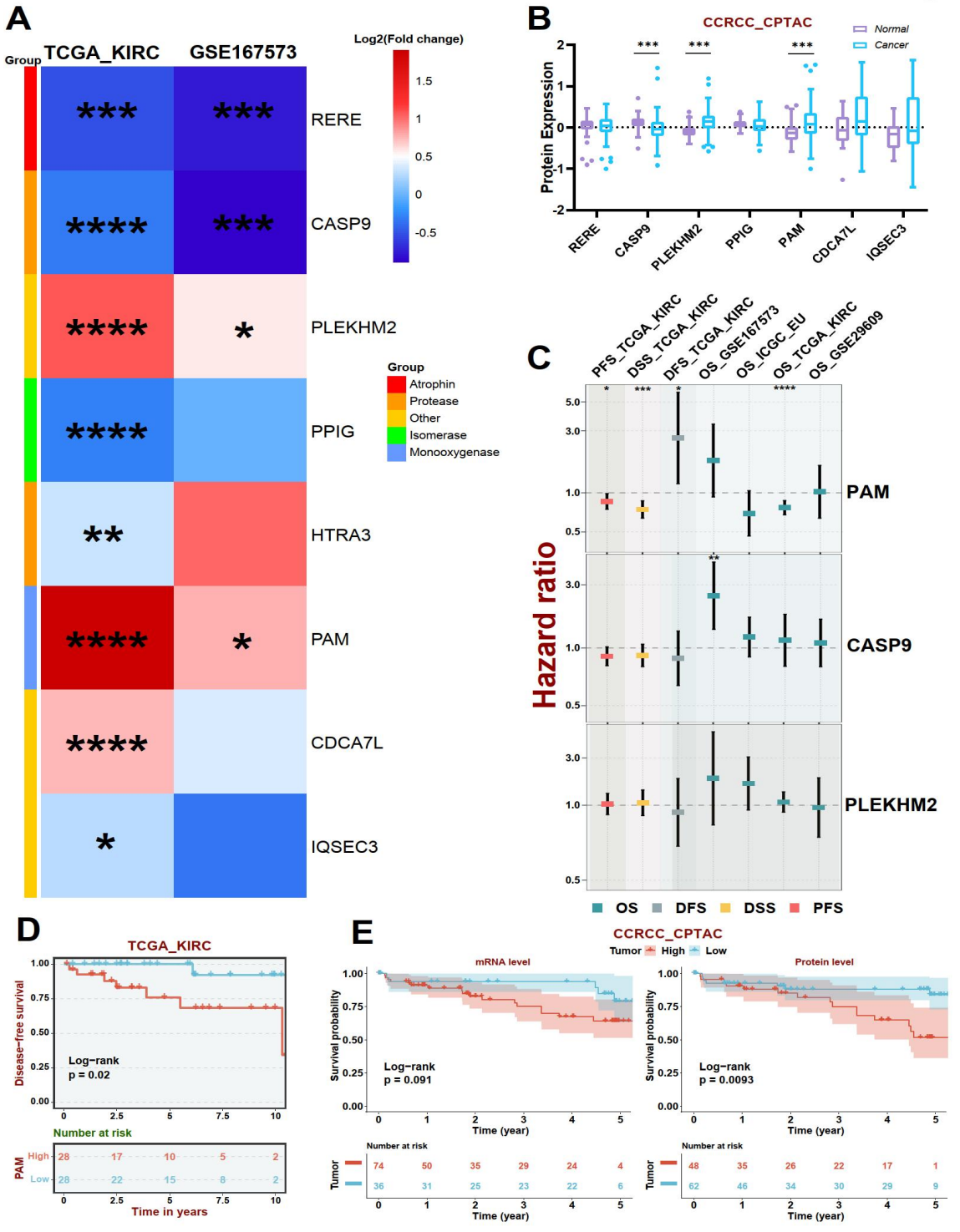

Figure 1. mRNA, Protein Expression, and Prognostic Analysis in ccRCC. **(A)** Differential mRNA expression analysis between tumor and normal tissues in the TCGA-KIRC and GSE167573 cohorts. **(B)** Differential protein expression analysis between tumor and normal tissues in the CPTAC-CCRCC cohort. **(C)** Cox regression analysis in ccRCC cohorts. **(D)** Kaplan-Meier curves for PAM high and low expression groups in the TCGA-KIRC cohort. **(E)** Kaplan-Meier curves for PAM high and low expression groups in the CPTAC-CCRCC cohort. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; NS, not significant.

To further screen for biomarkers associated with ccRCC, we conducted mRNA, protein expression, and prognostic analyses of the aforementioned eight genes. In both the TCGA-KIRC and GSE167573 cohorts, the genes with differential mRNA expression between tumor and normal tissues included RERE, CASP9, PLEKHM2, and PAM (Figure 1A). In the CPTAC-CCRCC cohort, the genes with differential protein expression between tumor and normal tissues included CASP9, PLEKHM2, and PAM (Figure 1B). Furthermore, we analyzed the relationship between these three genes and prognostic indicators in multiple ccRCC cohorts and found that only PAM was associated with the prognosis of ccRCC (Figure 1C). The KM curve showed that high expression of PAM was indicative of a poorer DFS in ccRCC patients (Figure 1D). In the CPTAC database, high protein expression of PAM also predicted a poorer survival in ccRCC patients, although at the mRNA level, the significance hypothesis was not met, but there was still a certain predictive effect (Figure 1E).

3.3 Methylation Modification and Genomic Pattern of PAM in ccRCC

To investigate how PAM affects tumor heterogeneity and cell stemness in RCC, we explored the genomic characteristics of PAM and the methylation modifications it undergoes. In RCC, PAM is widely positively correlated with the mRNA expression of genes related to RNA methylation (including M1A, M5C, M6A)-including writers, readers, and erasers (Figure 2A). The level of DNA methylation of PAM in ccRCC and renal papillary cell carcinoma is higher than that in renal chromophobe cell carcinoma (Figure 2B), and the mRNA expression of PAM is negatively correlated with the level of DNA methylation (Figure 2C). In ccRCC, PAM undergoes more gene amplifications and fewer gene mutations, while in renal papillary cell carcinoma, it mainly undergoes gene mutations, and no genomic changes were found in renal chromophobe cell carcinoma (Figure 2D). Subsequently, we analyzed the correlation between PAM expression and stemness scores, tumor heterogeneity markers such as RNAss (RNA expression-based), EREG.EXPss (epigenetically regulated RNA expression-based), DNAss (DNA methylation-based), EREG-METHss (epigenetically regulated DNA methylation-based), DMPss (differentially methylated probes-based), ENHss (enhancer Elements/DNA methylation-based), TMB (tumor mutational burden), mutant-allele tumor heterogeneity (MATH), MSI (microsatellite instability), purity, ploidy, homologous recombination deficiency (HRD), loss of heterozygosity (LOH), and neoantigen (NEO). The results showed that in ccRCC, PAM is negatively correlated with MATH, LOH, and DMPss, and in renal papillary cell carcinoma, it is negatively correlated with RNAss, MATH, LOH, EREG.EXPss, and DMPss, and positively correlated with purity (Figure 2E). This suggests that PAM may affect the treatment response in ccRCC patients. PAM is a monooxygenase with two enzyme domains, including Cu2_monooxygen and Cu2_monoox_C, and there are mutation sites on these two domains, indicating that gene mutations can have a significant impact on the function of PAM (Figure 2F).

Figure 2

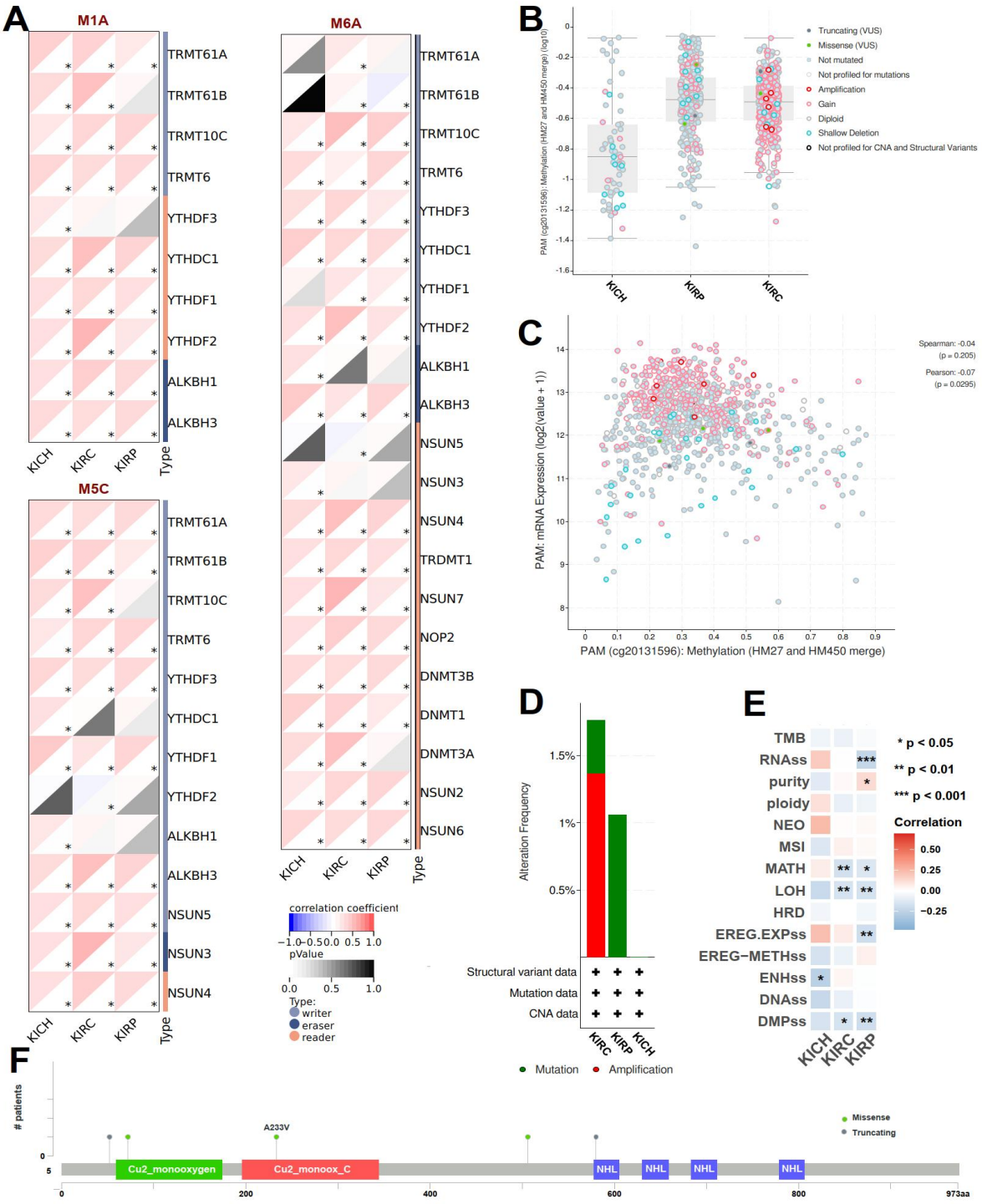

Figure 2. Methylation Modification and Genomic Pattern of PAM in ccRCC. **(A)** Correlation analysis of PAM with mRNA expression of RNA methylation-related genes in RCC cohorts. **(B)** Differences in DNA methylation levels of PAM in different types of RCC. **(C)** Correlation analysis between PAM mRNA expression and DNA methylation in ccRCC. **(D)** Genomic alterations of PAM in different types of RCC. **(E)** Correlation analysis between PAM and stemness scores, tumor heterogeneity in different types of RCC. **(F)** Bar plot of PAM protein domains. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; NS, not significant.

3.4 PAM Expression is Associated with Immune Regulatory Genes and Immune Cell Infiltration Levels in ccRCC

Figure 3

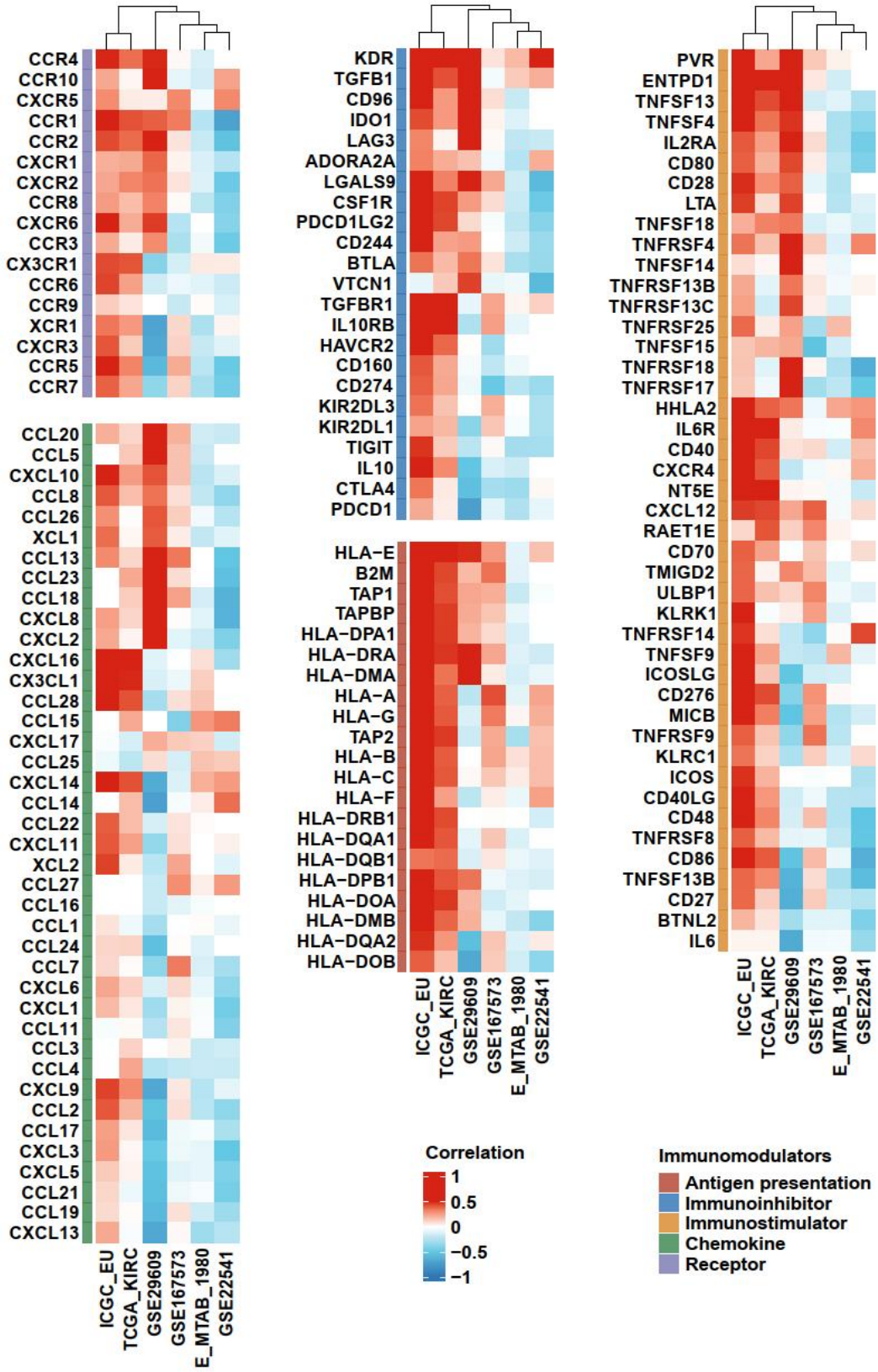

Figure 3. Correlation analysis of PAM with mRNA expression of immune regulatory factors in multiple ccRCC cohorts.

Figure 4

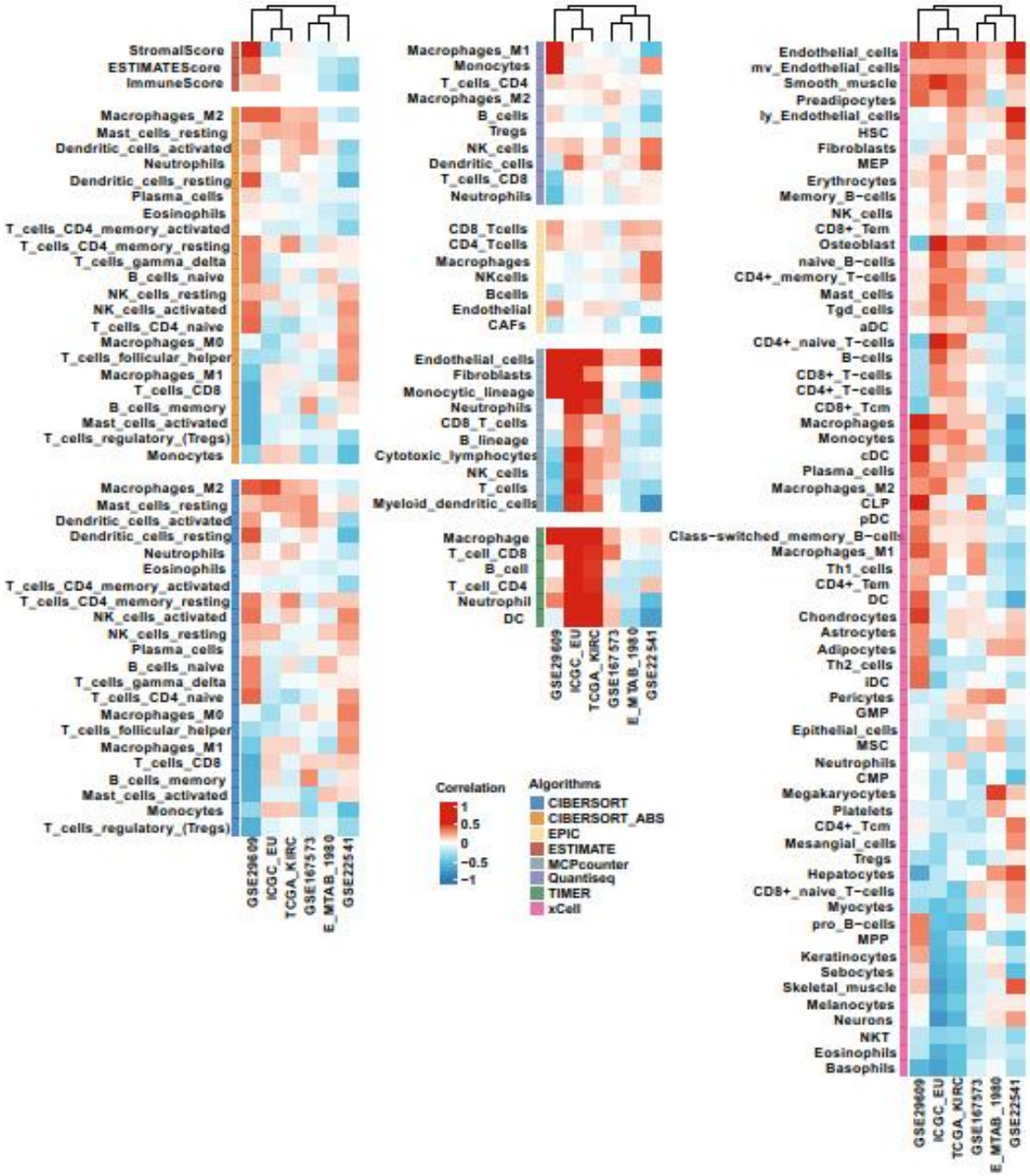

Figure 4. Correlation between PAM mRNA expression and the level of immune cell infiltration in multiple ccRCC cohorts.

It has previously been shown that immune-related genes are important for maintaining self-tolerance and preventing excessive immune responses (which could lead to damage to healthy tissue). However, some cancer cells can exploit these checkpoints to escape immune system attack [45]. Therefore, we investigated the correlation between the expression levels of immune-related genes and PAM in ccRCC, to characterize the potential role of PAM in immunotherapy. The results showed that in most ccRCC cohorts, particularly ICGC-EU and TCGA-KIRC, the expression of PAM was widely positively correlated with immunoinhibitor, immunostimulator, chemokines, and receptors (Figure 3).

We also analyzed the correlation between PAM expression and immune cell infiltration in ccRCC using various methods, including CIBERSORT, CIBERSORT_ABS, EPIC, ESTIMATE, MCPcounter, Quantiseq, TIMER, and xCell. The results showed that PAM expression was positively correlated with various immune cell infiltrates, including macrophages, fibroblasts, endothelial cells, and CD8+ T cells, and negatively correlated with NKT cells, eosinophils, basophils, and Treg cells (Figure 4). This suggests that PAM is involved in immune infiltration and plays an important role in the immune-tumor interaction.

3.5 PAM Affects Immune Cell Function and Tumor Immunotherapy Response

Single-cell data analysis of cell subpopulations showed that PAM is primarily expressed in malignant cells and CD8+ T exhausted cells in ccRCC, which can lead to tumor progression (Figure 5A). In addition, PAM is positively correlated with the T cell exhaustion score and with immunosuppressive cells such as CAF FAP and MDSC, and is considered a negative regulator of NK cells in multiple CRISPR Screen cohorts, consistent with our previous analysis (Figure 5B). In multiple mouse immunotherapy cohorts, we analyzed the differences in PAM mRNA expression before and after PD1 and PDL1 treatment. The results showed that after PD1 treatment, PAM expression decreased, and the PAM expression in responders was lower than that in non-responders (Figure 5C). However, after PDL1 treatment, PAM expression increased (Figure 5D). This result is surprising, and there may be some unknown regulatory mechanisms among PD1, PDL1, and PAM. We also analyzed the KM curves of the PD1/PDL1 treatment cohorts and found that patients with higher PAM expression had a poorer prognosis after treatment (Figure 5E). These results suggest that PAM may affect immune cell function, regulate the response to tumor immunotherapy, and is a potential target for immunotherapy.

Figure 5

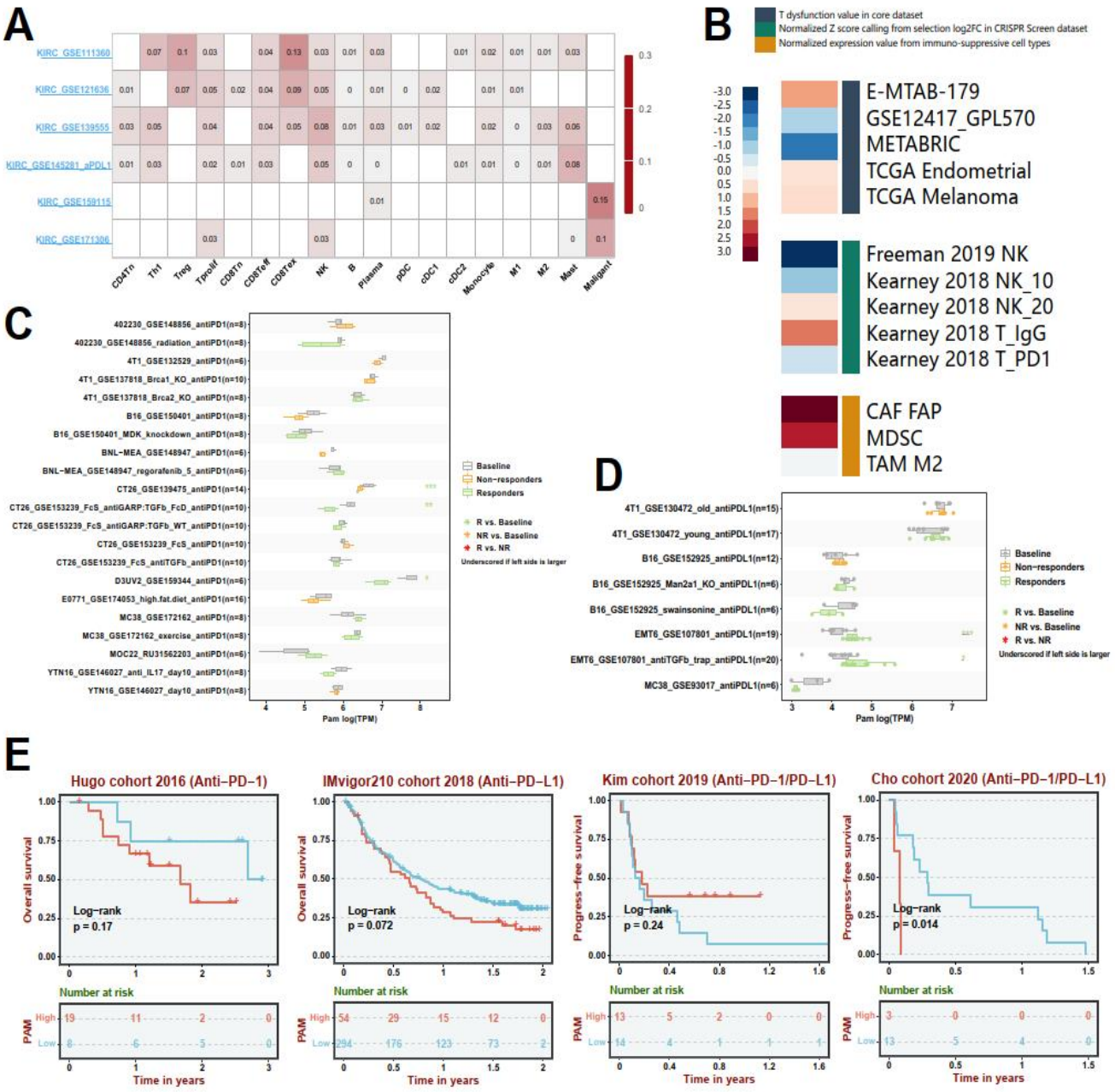

Figure 5. The relationship between PAM and immune cell function and immunotherapy response. **(A)** Expression of PAM in different cell types within the single-cell sequencing cohorts of ccRCC. **(B)** Correlation analysis between PAM and immune cell function in immunotherapy cohorts and CRISPR screening cohorts. **(C-D)** mRNA expression of PAM before and after immunotherapy in mouse PD1 and PDL1 immunotherapy cohorts. **(E)** Kaplan-Meier curves for patients with high and low PAM expression in human PD1 and PDL1 immunotherapy cohorts. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; NS, not significant.

3.6 Functional Pathways of PAM in ccRCC

Figure 6

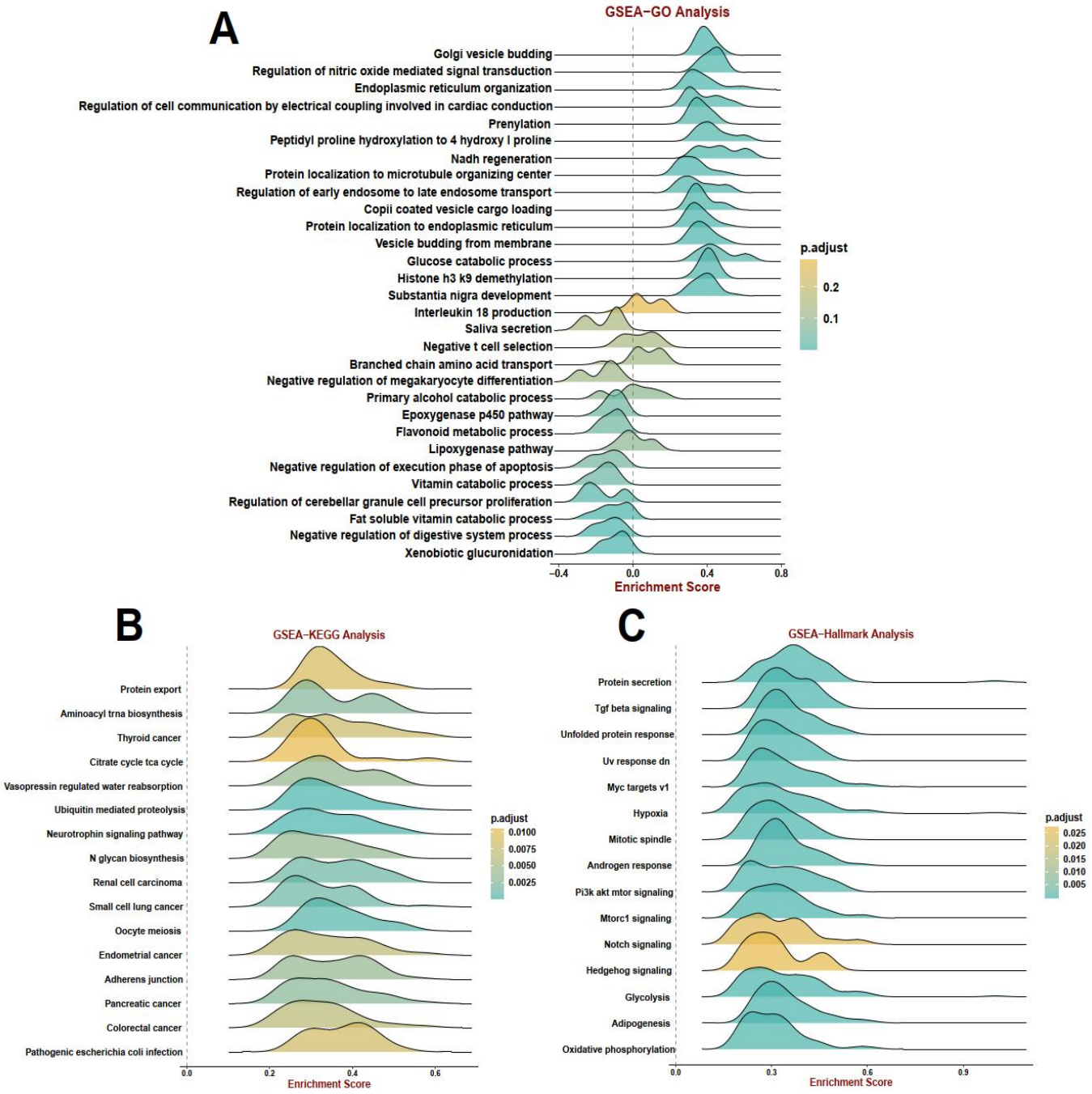

Figure 6. Functional analysis of PAM in ccRCC. GSEA enrichment analysis of GO **(A)**, KEGG **(B)**, and Hallmark **(C)** pathways.

To explore the pathways through which PAM mediates its oncogenic effects in ccRCC, we performed extensive enrichment analyses. In the GO enrichment analysis, PAM was associated with the negative regulation of cell apoptosis execution, negative regulation of megakaryocyte differentiation, and T cell negative selection pathways, although some pathways did not meet the significance hypothesis after multiple p-value correction (Figure 6A). In the KEGG enrichment analysis, PAM was significantly enriched in various cancer pathways, including colorectal cancer, pancreatic cancer, endometrial cancer, small cell lung cancer, ccRCC, and thyroid cancer, and it was related to ubiquitin-mediated proteolysis, regulation of water reabsorption by antidiuretic hormone, and citrate cycle (TCA cycle), which demonstrated the important link between PAM and tumorigenesis and development (Figure 6B). Hallmarks pathways are considered to be universally present in cancer cells during their development, survival, and metastasis [46, 47]. In the GSEA enrichment analysis of Hallmarks pathways, PAM was significantly enriched in a large number of pathways, including Notch, Mtorc1, mtor, etc., indicating that the association between PAM and cancer is robust (Figure 6C).

3.7 High Expression of PAM is Associated with the Occurrence of ccRCC and Promotes the Proliferation and Migration of ccRCC

To validate the role of PAM in promoting the development of ccRCC, we conducted in vitro tumor phenotype experiments and collected a certain number of ccRCC patient tissue samples. In paired ccRCC tissues and adjacent non-cancerous tissues, the protein expression of PAM in the tumor tissues was significantly higher than that in the adjacent non-cancerous tissues (Figure 7A). Subsequently, we performed PAM knockdown in two ccRCC cell lines, ACHN and OS, and verified it using qPCR (Figure 7B). The cell scratch assay indicated that the migratory ability of the tumor cell lines with PAM knockout was significantly reduced (Figure 7C). The CCK-8 assay revealed a decrease in the proliferation ability of the tumor cell lines with PAM knockout (Figure 7D). The Transwell assay showed similar results (Figure 7E).

Figure 7

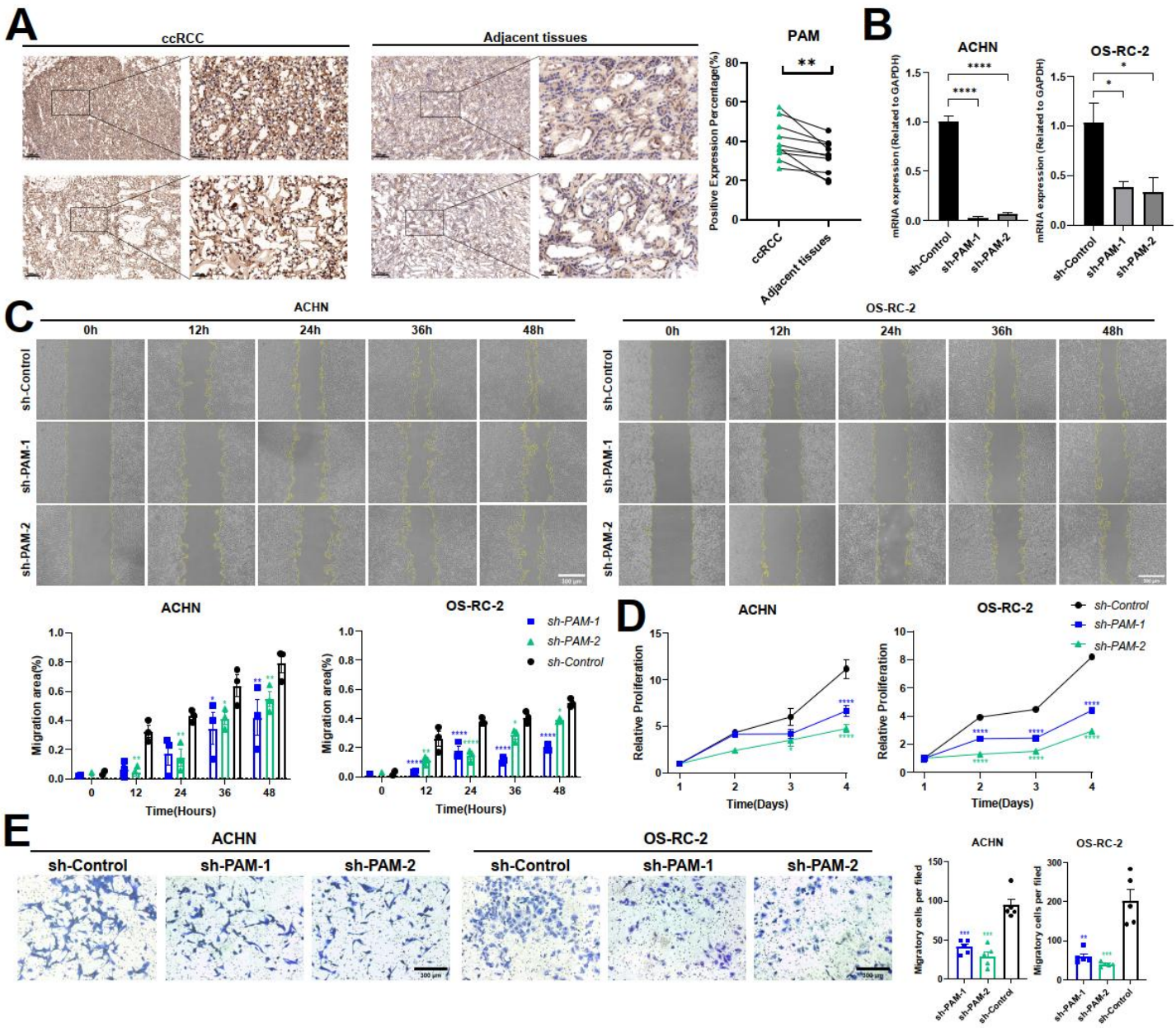

Figure 7. The impact of PAM on ccRCC proliferation and migration. **(A)** Immunohistochemistry of PAM in paired ccRCC tissues and adjacent non-cancerous tissues. **(B)** Quantitative PCR was used to verify the PAM knockout in ACHN and OS-RC-2 cells. **(C)** Cell scratch assay of ACHN and OS-RC-2 cell lines after PAM knockout. **(D)** CCK-8 assay of ACHN and OS-RC-2 cell lines after PAM knockout. **(E)** Transwell assay of ACHN and OS-RC-2 cell lines after PAM knockout. All data are expressed as SEM ± mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; NS, not significant.

4 Discussion

RCC is the second most common malignancy in the urinary system [3]. Radical nephrectomy continues to be the primary treatment for RCC, yet postoperative metastasis and recurrence significantly impact therapeutic outcomes, leading to dramatically reduced overall survival rates [9]. This underscores the clinical urgency for novel biomarkers to predict RCC progression and prognosis. ccRCC is initiated and progresses through various mechanisms, including oncogene activation, tumor suppressor gene inactivation, and dysregulated growth factor expression [10, 11]. The enzymatic amidation process, mediated by the PAM gene-encoded enzyme, may interact with these pathways, though conclusive studies are insufficient.

PAM is a monooxygenase that catalyzes the conversion of peptide hormone precursors into their active α-amidated forms, requiring oxygen, ascorbic acid, and copper ions for activity [17, 18]. Monooxygenases, also referred to as mixed-function oxidases, integrate an oxygen atom into substrate molecules and play a pivotal role in multiple biochemical reactions in the body [48]. As a byproduct of monooxygenase activity, reactive oxygen species (ROS) can activate the PI3K-AKT signaling pathway, a frequently activated pathway in human cancers [49]. This pathway reprograms cellular metabolism to support the anabolic demands of proliferating cells by increasing the activity of nutrient transporters and metabolic enzymes [50]. It is integral in regulating tumor cell proliferation, invasion, and metastasis [51]. ROS, produced by monooxygenases, converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn recruits and activates AKT, phosphorylating various downstream target proteins involved in cell survival, proliferation, and migration. ROS can modulate PI3K-AKT signaling by regulating the activity of PI3K or AKT proteins or by affecting upstream or downstream regulatory molecules [51], thus promoting tumor growth and metastasis, including in renal cancer.

PAM is critical for life, as it is the only known enzyme that catalyzes C-terminal α-amidation [16]. It is expressed in most mammalian cells, with peak activity in the pituitary gland and hypothalamus [19], and plays a key role in regulating physiological and pathological processes in humans. PAM modifies the stability, activity, and receptor-binding capacity of peptide hormones by converting their precursors into active α-amidated forms [17, 18]. Beyond its role in C-terminal amidation, PAM is necessary for the formation of atrial secretory granules, as shown by Bäck et al. [52].

In the context of disease, mutations leading to reduced PAM activity have been linked to an increased risk of type 2 diabetes, potentially by disrupting insulin granule packaging and secretion in β-cells [53-55]. Decreased PAM activity is also evident in the cerebrospinal fluid of Alzheimer's disease patients compared to controls [56] and is implicated in conditions such as multiple sclerosis and post-polio syndrome [21-24]. As such, PAM is considered a potential therapeutic target and biomarker for a variety of clinical conditions. Timothy M. et al. conducted a retrospective study on PAM immunoreactivity in primary neuroendocrine tumors (NENs), finding that lower PAM immunoreactivity correlates with reduced survival. Specifically, negative PAM staining is linked to higher mortality risk and shorter survival times, suggesting that PAM loss may signal dedifferentiation in neuroendocrine tumors [25].

Our research utilized bioinformatics to explore the complex relationships between gene expression and ccRCC, identifying eight ccRCC-associated genes, including PAM. Comprehensive analysis of mRNA and protein expression, as well as prognostic significance, revealed that PAM is differentially expressed in tumors versus normal tissue and that high PAM expression is associated with poor ccRCC prognosis. Integrative analysis of gene expression data, clinical information (e.g., survival, disease staging), genomic variation (e.g., CNV, SNPs), and methylation data indicated that PAM is involved in immune infiltration and significantly contributes to tumor-immune interactions. PAM negatively regulates

apoptosis and is associated with multiple cancer pathways, highlighting its pivotal role in tumorigenesis and progression. In vitro experiments with tumor cells and clinical sample analyses validated our findings, showing that PAM expression is elevated in ccRCC tissues compared to adjacent normal tissue and that high expression levels are linked to increased tumor cell proliferation and migration, as well as poor patient prognosis.

The strength of our study lies in the integration of large-scale GWAS data from the UK Biobank (encompassing 463,009 participants) and whole-blood eQTL data (338 samples), which facilitated genome-wide screening for potential pathogenic genes. This approach successfully pinpointed several ccRCC-related genes, including PAM. The application of Mendelian randomization-based SMR analysis and HEIDI testing bolstered the reliability of our results. By synthesizing genomic, clinical, and multi-omic data, including gene expression, survival, genomic variation, and methylation information, we thoroughly characterized PAM's role and mechanisms in ccRCC. Additionally, in vitro tumor cell assays (cell culture, viability assays, Transwell migration assays) and clinical sample analyses bridged the gap between basic molecular research and clinical relevance, providing robust evidence for our conclusions and enhancing the translational value of our findings.

Nevertheless, our study has limitations. Variability in data quality across different databases and inherent limitations of certain data sources may impact the accuracy of our results. Future analyses should explore PAM's role in interconnected pathways and identify upstream and downstream targets in signal transduction. The limited sample size of clinical data necessitates further studies with larger cohorts, incorporating both retrospective and prospective analyses, to validate the clinical significance of PAM expression in ccRCC and its prognostic implications.

In conclusion, our findings demonstrate that PAM is upregulated in ccRCC tissues and promotes tumor cell proliferation and migration. High PAM expression levels are associated with poor patient prognosis, identifying PAM as a potential prognostic biomarker and therapeutic target for ccRCC. This study provides valuable insights into the prognosis and treatment of ccRCC, offering a new direction for future research.

5 Conclusions

This study demonstrates that PAM, a monooxygenase enzyme, is overexpressed in ccRCC and is associated with tumor progression and poor patient prognosis. High PAM expression promotes ccRCC cell proliferation and migration, and is involved in immune infiltration and tumor-immune interactions. These findings identify PAM as a potential prognostic biomarker and therapeutic target for ccRCC.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

X. W., H. W., and Y. G. designed this research. X. W. and H. W. organized the processing flow. Y. L., H. L., Y. Z., C. D., X. M., X. Y., K. L., B. L., Z. X., Y. G., and H. X. completed the whole analytic process of this study. X. W. and Y. G. organized and presented the results. X. W., H. W., and Y. G. contributed to the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Tables

Table1. Basic information of the GWAS and eQTL data.

Data source	Total number of participants	Number of eligible genetic variants
eQTL data		
Whole blood	338	4490
GWAS data		
Kidney cancer	463010	9851867

Table 2. The probes identified in the SMR analysis of whole blood data.

Gene	*CHR*	*Top SNP*	*SMRFDR*	*PHEIDI*	*Nsnp*
RERE	1	rs2292242	0.00220617	NA	NA
CASP9	1	rs12691551	0.00220617	NA	NA
PLEKHM2	1	rs10492987	0.002581221	0.2019583	3
PPIG	2	rs2592791	0.002812971	0.5002909	8
HTRA3	4	rs7678398	0.002225746	0.4425886	4
RP11-448G15.3	4	rs6826888	0.002833143	NA	NA
PAM	5	rs2431530	0.001346517	0.1311256	12
PPIP5K2	5	rs468024	0.002581221	0.02643236	7
HIST1H4H	6	rs3999544	0.002833143	0.04996774	4
CDCA7L	7	rs7790135	0.00220617	NA	NA
CTD-3064M3.1	8	rs55846720	0.003037622	NA	NA
IQSEC3	12	rs10849575	0.002833143	NA	NA
RP4-673D20.1	20	rs507582	0.002833143	NA	NA

Figure legends

Figure 1. mRNA, Protein Expression, and Prognostic Analysis in ccRCC. **(A)** Differential mRNA expression analysis between tumor and normal tissues in the TCGA-KIRC and GSE167573 cohorts. **(B)** Differential protein expression analysis between tumor and normal tissues in the CPTAC-CCRCC cohort. **(C)** Cox regression analysis in ccRCC cohorts. **(D)** Kaplan-Meier curves for PAM high and low expression groups in the TCGA-KIRC cohort. **(E)** Kaplan-Meier curves for PAM high and low expression groups in the CPTAC-CCRCC cohort. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; NS, not significant.

Figure 2. Methylation Modification and Genomic Pattern of PAM in ccRCC. **(A)** Correlation analysis of PAM with mRNA expression of RNA methylation-related genes in RCC cohorts. **(B)** Differences in DNA methylation levels of PAM in different types of RCC. **(C)** Correlation analysis between PAM mRNA expression and DNA methylation in ccRCC. **(D)** Genomic alterations of PAM in different types of RCC. **(E)** Correlation analysis between PAM and stemness scores, tumor heterogeneity in different types of RCC. **(F)** Bar plot of PAM protein domains. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; NS, not significant.

Figure 3. Correlation analysis of PAM with mRNA expression of immune regulatory factors in multiple ccRCC cohorts.

Figure 4. Correlation between PAM mRNA expression and the level of immune cell infiltration in multiple ccRCC cohorts.

Figure 5. The relationship between PAM and immune cell function and immunotherapy response. **(A)** Expression of PAM in different cell types within the single-cell sequencing cohorts of ccRCC. **(B)** Correlation analysis between PAM and immune cell function in immunotherapy cohorts and CRISPR screening cohorts. **(C-D)** mRNA expression of PAM before and after immunotherapy in mouse PD1 and PDL1 immunotherapy cohorts. **(E)** Kaplan-Meier curves for patients with high and low PAM expression in human PD1 and PDL1 immunotherapy cohorts. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; NS, not significant.

Figure 6. Functional analysis of PAM in ccRCC. GSEA enrichment analysis of GO **(A)**, KEGG **(B)**, and Hallmark **(C)** pathways.

Figure 7. The impact of PAM on ccRCC proliferation and migration. **(A)** Immunohistochemistry of PAM in paired ccRCC tissues and adjacent non-cancerous tissues. **(B)** Quantitative PCR was used to verify the PAM knockout in ACHN and OS-RC-2 cells. **(C)** Cell scratch assay of ACHN and OS-RC-2 cell lines after PAM knockout. **(D)** CCK-8 assay of ACHN and OS-RC-2 cell lines after PAM knockout. **(E)** Transwell assay of ACHN and OS-RC-2 cell lines after PAM knockout. All data are expressed as SEM ± mean. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; NS, not significant.