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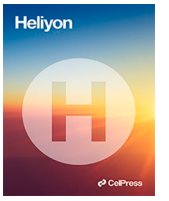

Research article

PTOV1 facilitates colorectal cancer cell proliferation through activating AKT1 signaling pathway

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ABSTRACT

Background: Colorectal cancer is a predominant contributor to global cancer-related morbidity and mortality. The oncogene PTOV1 has been linked to various human malignancies, yet its specific role in CRC pathogenesis requires further elucidation.

Methods: Our study used a comprehensive array of authoritative bioinformatics tools, such as TIMER, UCSC Xena, GEO, Human Protein Atlas, UALCAN, CIBERSORTx and others which used to investigate the complex effects of PTOV1 on gene expression profiles, diagnostic and prognostic biomarkers, tumor immunology, signaling pathways, epigenetic alterations, and genetic mutations. Gene expression validation was conducted using Western blot and qRT-PCR. The in vitro proliferative and migratory potentials of CRC cells were evaluated using CCK-8 assays, colony formation, and transwell migration assays, respectively. MSP was applied to assess the methylation status of the PTOV1 promoter region.

Results: Our results reveal a significant association between increased PTOV1 expression, driven by promoter hypomethylation, and poor patient prognosis in CRC. Elevated PTOV1 levels were positively correlated with the enrichment of diverse immune cell subsets and immune-related molecules within the tumor microenvironment. In vitro assays demonstrated that PTOV1 knockdown markedly reduced CRC cell proliferation, colony formation, and migration, while ectopic PTOV1 expression had the opposite effect. Importantly, PTOV1 was shown to regulate the PI3K-AKT signaling pathway, significantly influencing the phosphorylation of AKT1 and the expression of cell cycle regulators P21 and P27. The pharmacological inhibition of AKT1 phosphorylation using MK2206 effectively counteracted the proliferative effects induced by PTOV1 overexpression.

Conclusion: The ability of PTOV1 to enhance CRC cell proliferation via modulation of the AKT1 signaling pathway establishes it as a potential therapeutic target and a promising biomarker for prognostic stratification in CRC.

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1. Introduction

Colorectal cancer (CRC), a malignant neoplasm arising in the colonic and rectal tissues, is the third most prevalent malignancy globally and the second leading contributor to cancer-related mortality worldwide, with an estimated 930,000 fatalities in 2020 [1]. Despite considerable progress in diagnostic modalities and therapeutic strategies for CRC, the mortality rate remains elevated due to the propensity for metastasis and recurrence, which significantly impairs patient prognosis. Thus, elucidating the molecular underpinnings of CRC pathogenesis and pinpointing pivotal genes that drive this process is of paramount importance.

Prostate tumor overexpressed 1 (PTOV1), a 46-kDa protein encoded by a gene situated on chromosome 19q13.3 [2], was initially characterized in the context of prostate cancer as a gene that exhibits elevated expression in neoplastic tissues compared to normal prostate tissue [3,4]. The expression levels of PTOV1 have been found to correlate positively with tumor cell proliferation and histological grade, suggesting its potential as a biomarker for aggressive tumor behavior [5–9]. Extant literature has documented the association of PTOV1 with a spectrum of malignancies, including hepatocellular carcinoma, breast cancer, esophageal cancer, and cervical cancer, underscoring its multifaceted role in oncogenesis and tumor progression [10–20]. Furthermore, PTOV1 has been implicated in the development of resistance to chemotherapeutic agents such as cisplatin, retinoic acid (RA), and docetaxel [21–26]. However, the specific contribution of PTOV1 to CRC pathophysiology remains to be fully delineated. The present study is designed to explore the potential roles and underlying mechanisms of PTOV1 in the progression of CRC.

2. Methods

2.1. TIMER database

The TIMER database, a preeminent bioinformatics resource, facilitated a systematic exploration of gene expression profiles across a spectrum of tumor types [27,28]. Employing the "DiffExp" module, we discerned variations in gene expression, adeptly illustrated through box plots. The statistical significance of these variations was rigorously assessed using the non-parametric Wilcoxon test. Furthermore, the "Gene" module delineated the interplay between gene expression and immune cell infiltration, presenting scatterplots that incorporated purity-corrected Spearman's rho correlation coefficients alongside their attendant statistical significance.

2.2. Data acquisition

Our study leveraged the Colon Adenocarcinoma (COAD) and Rectum Adenocarcinoma (READ) cohorts from The Cancer Genome Atlas (TCGA), accessed via the UCSC Xena repository. The RNA sequencing data were subjected to a rigorous normalization process to transcripts per million (TPM) to ensure the integrity of our analytical approach. For gene expression profiles, we sourced raw data from the Gene Expression Omnibus (GEO) under the accession numbers GSE41258 [29,30], GSE23878 [31], GSE20842 [32], and GSE110223 [33], which were retrieved from the NCBI GEO databases. Furthermore, immunohistochemical staining data for PTOV1 in both normal human colorectal tissues and CRC tissues were procured from the Human Protein Atlas (HPA) [34]. This data was retrieved by querying the term "PTOV1″ within the "Tissue Atlas" and "Pathology Atlas" sections of the HPA website. The staining intensity scores were extracted from the "Antibody staining" section, providing a quantitative assessment of PTOV1 protein expression levels.

2.3. MEXPRESS

The MEXPRESS tool was engaged to scrutinize the methylation status of the PTOV1 promoter, a potentially pivotal epigenetic modifier in CRC patients [35].

2.4. Kaplan-Meier plotter database analysis

The Kaplan-Meier plotter served as an invaluable online analytical instrument for assessing the prognostic implications of PTOV1 expression in the context of CRC [36].

2.5. CIBERSORTx

CIBERSORTx, an algorithm designed for estimating the relative abundance of various cell types within heterogeneous cell populations based on gene expression data [37], was utilized to evaluate the immune response in CRC. This analysis hinged on the TCGA dataset and further dissected the abundance of tumor-infiltrating immune cells (TIICs) across groups stratified by PTOV1 expression levels. Statistical significance was stringently determined at a p-value threshold of <0.05.

TISIDB database

The TISIDB database, a user-friendly platform for dissecting tumor-immune system interactions [38], was instrumental in our analysis of gene expression patterns in relation to tumor immune subtypes, immunotherapy potential, and immunomodulatory factors. This resource proved invaluable for our cancer immunology research and therapeutic explorations.

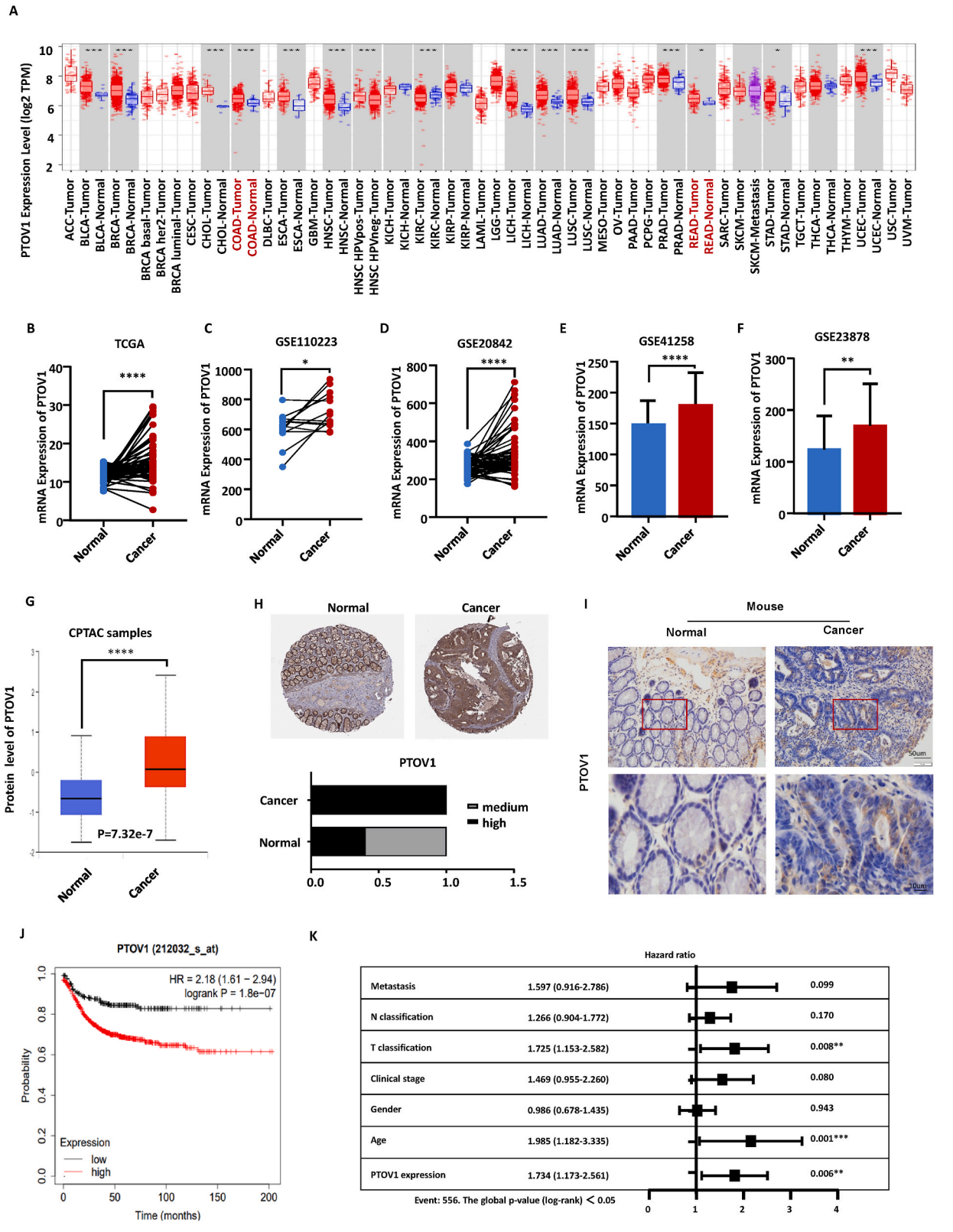

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Fig. 1. The High PTOV1 Expression is Associated with Poor Survival in CRC Patients. (A) High or low expression of PTOV1 in different human cancer tissues compared with normal tissues using the TIMER database. (B) PTOV1 mRNA expression levels in CRC and normal tissue samples analyzed in the TCGA databases. (C–F) The expression levels of PTOV1 in colorectal cancer and normal tissue samples in GSE110223, GSE20842, GSE41258 and GSE23878 datasets. (G) PTOV1 protein was significantly upregulated from CPTAC samples. (H) The protein expression of PTOV1 in CRC tissues and normal tissues from the Human Protein Atlas. (I) IHC analysis PTOV1 expression in mouse tissues (each group: n = 6). (J) Survival curves of the CRC patients (n(high) = 1524, n(low) = 565). (K) Forest plot illustrated the results of multivariate Cox regression analyses. Note: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Scare bar = 100 μm.

Table 1
Correlation between PTOV1 expression and clinicopathologic characteristics of CRC patients.

Characteristics	Total	PTOV1		$\chi 2$	p
		Low expression	High expression		
Age					
< 60	194 (32.5)	90	104	0.118	0.731
≥60	403 (67.5)	193	210		
Gender					
Male	323 (54.1)	164	159	3.206	0.073
Female	274 (45.9)	119	155		
Clinical stage					
I	104 (17.4)	48	56	3.922	0.270
II	214 (36.3)	112	102		
III	172 (28.8)	76	96		
IV	86 (14.4)	36	50		
T classification					
T1	20 (3.4)	11	9	1.057	0.788
T2	104 (17.4)	46	58		
T3	406 (68.0)	195	211		
T4	64 (10.7)	29	35		
N classification					
N0	337 (56.4)	169	168	2.889	0.236
N1	146 (24.5)	64	82		
N2	112 (18.8)	48	64		
Metastasis					
M0	442 (74.0)	214	228	0.564	0.453
M1	145 (24.3)	65	80		
Vital states					
Alive	470 (78.7)	238	232	**9.271**	**0.002***
Dead	127 (21.3)	45	82		

Note: T, Tumor; N, Lymph Node. P < 0.05, statistically significant.

Table 2
Univariate and multivariate Cox regression analysis of prognostic factors in CRC patients for overall survival.

	Univariate analysis		Multivariate analysis	
	HR (95%CI)	P	HR (95%CI)	P
Overall survival				
Age	1.636 (1.085–2.467)	**0.019***	1.985 (1.182–3.335)	**0.001***
Gender	1.042 (0.734–1.479)	0.818	0.986 (0.678–1.435)	0.943
Clinical stage	2.180 (1.765–2.693)	**0.000***	1.469 (0.955–2.260)	0.080
T classification	2.561 (1.806–3.633)	**0.000***	1.725 (1.153–2.582)	**0.008***
N classification	1.986 (1.615–2.443)	**0.000***	1.266 (0.904–1.772)	0.170
Metastasis	3.142 (2.185–4.519)	**0.000***	1.597 (0.916–2.786)	0.099
PTOV1 expression	1.636 (1.137–2.356)	**0.008***	1.734 (1.173–2.561)	**0.006***

Note: T, Tumor; N, Lymph Node. P < 0.05, statistically significant.

2.6. Gene set enrichment analysis (GSEA)

We conducted gene set enrichment analysis (GSEA) on the TCGA-COADREAD cohorts, which included a cohort of 598 CRC patients. The dataset was stratified according to PTOV1expression. This stratification allowed us to apply GSEA to discern differentially enriched signaling pathways associated with varying levels of PTOV1 expression.

2.7. Immunohistochemistry (IHC) staining assay

Paraffin-embedded mouse CRC tissue sections were deparaffinized in xylene and rehydrated through graded alcohols. Antigen

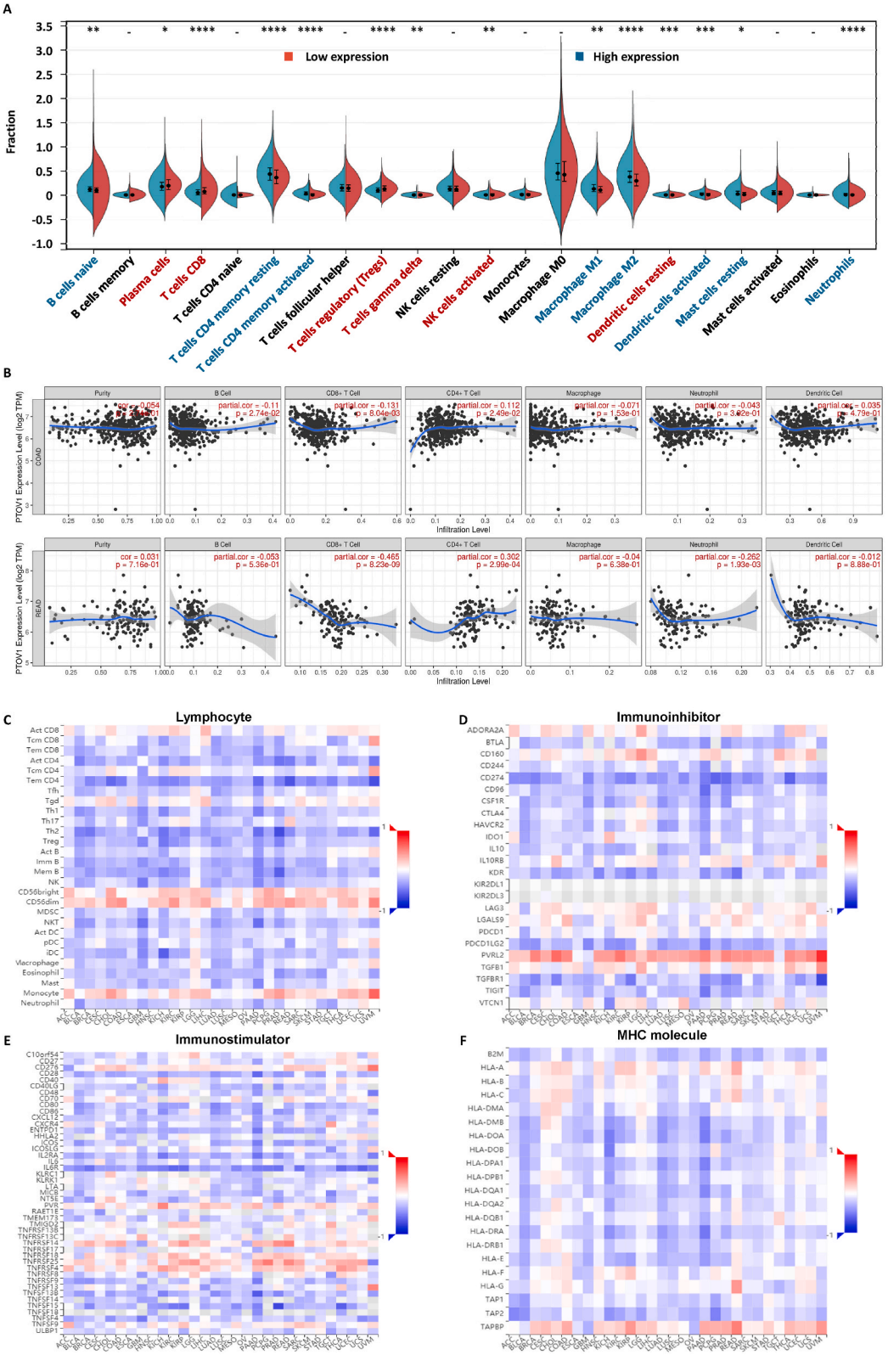

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Fig. 2. The relationship between PTOV1 and immune in colorectal cancer. (A) Different proportions of sorts of immune cells in high- and low-PTOV1 groups in CRC specimens by CIBERSORTx methods. (B) The relationship between PTOV1 expression and the abundance of immune cells in CRC. (C–F) The relationship between PTOV1 expression and the abundance of TILs, immunoinhibitor, immunostimulators and MHC molecules in CRC. Note: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Table 3
The correlation between PTOV1 expression and lymphocyte subtypes.

Lymphocyte subtypes	COAD		READ	
	rho	P value	rho	P value
Activated $CD8^+$ T cell (Act_CD8)	−0.035	0.45	−0.118	0.129
Central memory $CD8^+$ T cell (Tcm_CD8)	−0.037	0.428	−0.098	0.206
Effector memory $CD8^+$ T cell (Tem_CD8)	−0.1	**3.18e-2**	−0.144	0.064
Activated CD4 T cell (Act_CD4)	−0.32	**<2.71e-12**	−0.506	**<2.2e-16**
Central memory $CD4^+$ T cell (Tcm_CD4)	−0.036	0.437	−0.186	**1.61e-2**
Effector memory $CD4^+$ T cell (Tem_CD4)	−0.497	**<2.2e-16**	−0.664	**<2.2e-16**
T follicular helper cell (Tfh)	−0.193	3.4e-5	−0.111	0.152
Gamma delta T cell (Tgd)	−0.113	1.52e-2	−0.079	0.309
Type 1 helper cell (Th1)	−0.207	8.19e-6	−0.188	**1.49e-2**
Type 17 helper cell (Th17)	0.024	0.61	0.214	**5.46e-3**
Type 2 helper cell (Th2)	−0.219	2.25e-6	−0.442	**3.39e-9**
Regulatory T cell (Treg)	−0.258	2.43e-8	−0.233	**2.47e-3**
Activated B cell (Act_B)	−0.146	1.68e-3	−0.126	0.105
Immature B cell (Imm_B)	−0.218	2.71e-6	−0.197	**1.07e-2**
Memory B cell (Mem_B)	−0.358	3.26e-15	−0.311	**4.61e-5**
Natural killer cell (NK)	−0.139	2.86e-3	−0.227	**3.27e-3**
CD56bright natural killer cell (CD56bright)	−0.004	0.939	−0.007	0.928
CD56dim natural killer cell (CD56dim)	0.435	<2.2e-16	0.41	**4.98e-8**
Myeloid derived suppressor cell (MDSC)	−0.084	0.0709	−0.051	0.513
Natural killer T cell (NKT)	−0.184	7.69e-5	−0.248	**1.25e-3**
Activated dendritic cell (Act_DC)	−0.102	2.93e-2	−0.082	0.294
Plasmacytoid dendritic cell (pDC)	−0.214	4.12e-6	−0.378	**5.81e-7**
Immature dendritic cell (iDC)	−0.333	3.11e-13	−0.274	**3.6e-4**
Macrophage	−0.189	4.7e-5	−0.063	0.422
Eosinophil	−0.191	3.84e-5	−0.324	**2.15e-5**
Mast cell (Mast)	−0.238	2.57e-7	−0.149	0.0543
Monocyte	0.224	1.26e-6	0.234	**2.4e-3**
Neutrophil	−0.208	7.65e-6	−0.025	0.747

Note: COAD, colon adenocarcinoma; READ, rectal adenocarcinoma. P < 0.05, statistically significant.

retrieval was performed under high pressure, followed by quenching of endogenous peroxidase with 3 % hydrogen peroxide. Sections were incubated with a primary antibody against PTOV1 (4 °C, overnight) and subsequently with a secondary antibody (room temperature, 1.5 h). Visualization was achieved using a diaminobenzidine (DAB) substrate kit, with hematoxylin counterstaining for nuclear clarity.

2.8. Cell culture

Human cell lines were sourced from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cultured in DMEM with 10 % FBS and antibiotics (penicillin/streptomycin, 1000 U/ml), cells were maintained in a 5 % CO2 humidified atmosphere at 37 °C. Experiments utilized cells within three passages post-thawing.

2.9. Antibodies and reagents

Rabbit polyclonal antibodies against PTOV1 were sourced from Abcam (ab81173). Rabbit polyclonal antibodies to total AKT (2938S), phospho-AKT (S473, 4060S), P21 (2947S), P27 (3686S), and PI3K (4249S) were from Cell Signaling Technology (CST). A rabbit monoclonal Flag tag antibody was from Sigma (F2555). GAPDH antibody from Easybio (BE0024). The AKT1 inhibitor MK2206 (10 μM, 72 h) and DNMT inhibitor 5-Aza-dC (20 μM, 72 h) were from MedChemExpress (MCE) and Selleckchem, respectively.

2.10. Transfection

For PTOV1 knockdown, cells were transfected with specific siRNAs (siPTOV1 #1 and #2) or scrambled siRNA using Lipofectamine 2000 (Invitrogen). The siRNA sequences used were as follows: siPTOV1 #1 (5′-GAGACUGCGACUCGCUCAAGG-3′) and siPTOV1 #2 (5′-GGAGCAGGAGCAACAGCAACG-3′). Overexpression was induced using the pcDNA3/Flag-PTOV1 plasmid (UNIBIO) which were performed using Lipofectamine 2000.

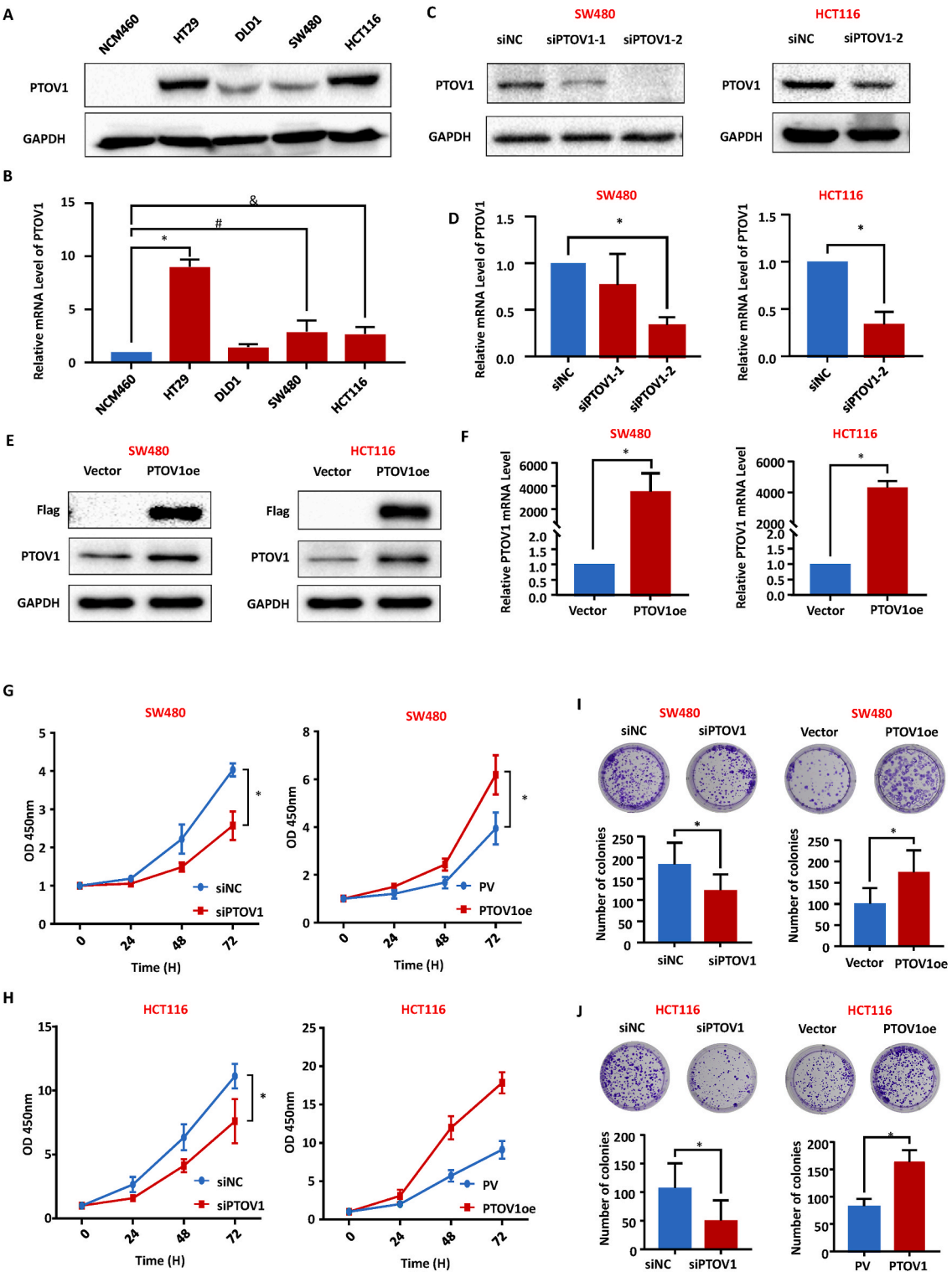

Fig. 3. PTOV1 Promotes CRC Cell Proliferation Ability. (A–B) The baseline protein and mRNA level of PTOV1 in NCM460 and CRC cells (n = 3 and n = 5 each group respectively). (C–D) The protein and mRNA expression level of PTOV1 in SW480 (n = 4 and n = 5 each group respectively) and HCT116 (n = 4 each group) cells transfected PTOV1 specific siRNA (n = 4 and n = 5 each group respectively). (E–F) The protein and mRNA expression level of PTOV1 in SW480 (n = 4 and n = 3 each group respectively) and HCT116 (n = 4 each group) cells transfected PTOV1 over-expressed plasmid. (G) Cell proliferation after PTOV1 over-expression (n = 6 each group) or suppressed by siRNA (n = 6 each group) in SW480 cells was determined by CCK-8 assay. (n = 6). (H) Cell proliferation after PTOV1 over-expression (n = 5 each group) or suppressed by siRNA (n = 5 each

group) in HCT116 cells was determined by CCK-8 assay. (n = 6). (I) Colony formation assays for SW480 cells showed PTOV1 knockdown (n = 6 each group) decreased cell viability and overexpression of PTOV1 (n = 6 each group) increased cell viability. (J) Colony formation assays for HCT116 cells showed PTOV1 knockdown (n = 3 each group) decreased cell viability and overexpression of PTOV1 (n = 3 each group) increased cell viability. Note: *P < 0.05, #P < 0.05, &P < 0.05; Data are presented as means ± SD.

2.11. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol Reagent and reverse transcribed using the TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech). qRT-PCR employed SYBR Green mix (Invitrogen), with GAPDH as the endogenous control. Relative mRNA expression was determined using the 2ˆ−ΔΔCtˆ method. Primer sequences for the genes of interest are listed in Supplementary Table 1.

2.12. Western blot

Protein lysates were prepared in RIPA buffer, quantified using the BCA Protein Assay Kit (Thermo Fisher), and equalized for SDS-PAGE and transfer to nitrocellulose membranes. Primary and HRP-conjugated secondary antibodies were used, with detection via an enhanced chemiluminescence system (BIO-RAD).

2.13. CCK-8 assay

The effect of PTOV1 on CRC cell proliferation was assessed using the CCK-8 assay kit (Beyotime Biotechnology). Cells were plated in 96-well plates (2000 cells/well), and the proliferation rate was determined in relation to a 0-h time point control.

2.14. Colony formation and cell cycle assays

The cells after transfection were plated into 6-well plates (1000 cells/well) and incubated for two weeks to allow colony formation. Crystal violet staining was used for visualization, followed by counting under an optical microscope. For cell cycle analysis, cells were stained with propidium iodide (PI) and analyzed using a BD FACSVerse system (Olympus). Cell cycle assay was further analyzed with FLOWJO software.

2.15. Transwell migration assay

To assess the migration capacity of CRC cells, transwell chambers equipped with polycarbonate membranes of 8 μm pore size (Corning Costar) were utilized. A suspension containing 10,000 cells in 500 μl of serum-free medium was seeded into the upper compartment of the chamber. The lower compartment was filled with 500 μl of complete growth medium. Following incubation, cells that had invaded through the membrane to the lower surface were fixed and stained with crystal violet. The number of invasive cells was quantified using ImageJ software.

2.16. Methylation specific PCR

DNA methylation status was evaluated following treatment with 5-aza-dC or DMSO. Bisulfite conversion was performed with EpiMark sulfite kit (NEB), and methylation-specific PCR (MSP) was conducted using designed primers (Fig. 5E and Supplementary Table 1). PCR products were resolved by agarose gel electrophoresis. UV irradiation showed bands.

2.17. Statistical analyses

Data from a minimum of three independent experiments were analyzed using GraphPad Prism version 9. Results are expressed as mean ± SD, with the central tendency indicated by the mean. Error bars in graphical representations denote standard deviation. Differential expression of PTOV1 mRNA between CRC tissues and adjacent normal tissues was assessed using independent samples t-tests for unpaired comparisons and paired sample t-tests for matched samples. Associations between PTOV1 expression and clinical characteristics were evaluated using the Pearson chi-squared test for categorical data. Prognostic significance was determined through Cox proportional hazards regression models for both univariate and multivariate analyses. Gene expression analysis from the TIMER database included calculation of ranks, fold changes, and P-values. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Elevated PTOV1 expression in CRC tissue

The oncogene PTOV1, recognized for its role in prostate adenocarcinoma, exhibits significant upregulation across a spectrum of human cancers, with particular relevance to CRC ($P < 0.05$, Fig. 1A). Comprehensive bioinformatics analysis leveraging TCGA and GEO datasets consistently revealed heightened PTOV1 mRNA expression in CRC tissues, achieving statistical significance ($P < 0.05$,

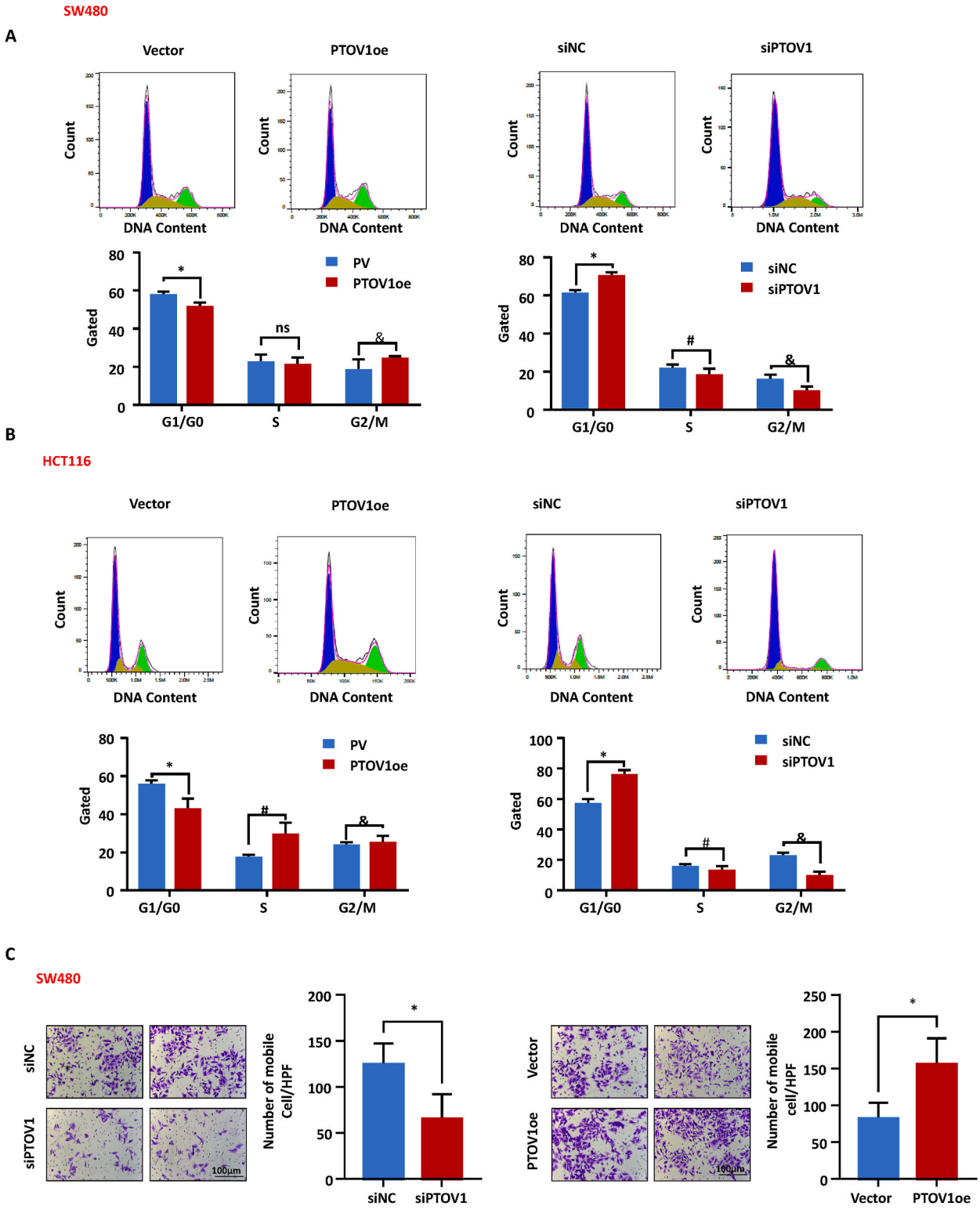

Fig. 4. Knockdown of PTOV1 induces cell cycle arrest and inhibit cell migration ability. (A–B) Cell cycle analysis with flow cytometry at 48 h after transfection. The bar chart represents the percentage of cells in G0/G1, S, or G2/M phase, as indicated. (C) The migration ability of CRC cells was measured by transwell assay (n = 3). Note: *P < 0.05, #P < 0.05, &P < 0.05; Data are presented as means ± SD.

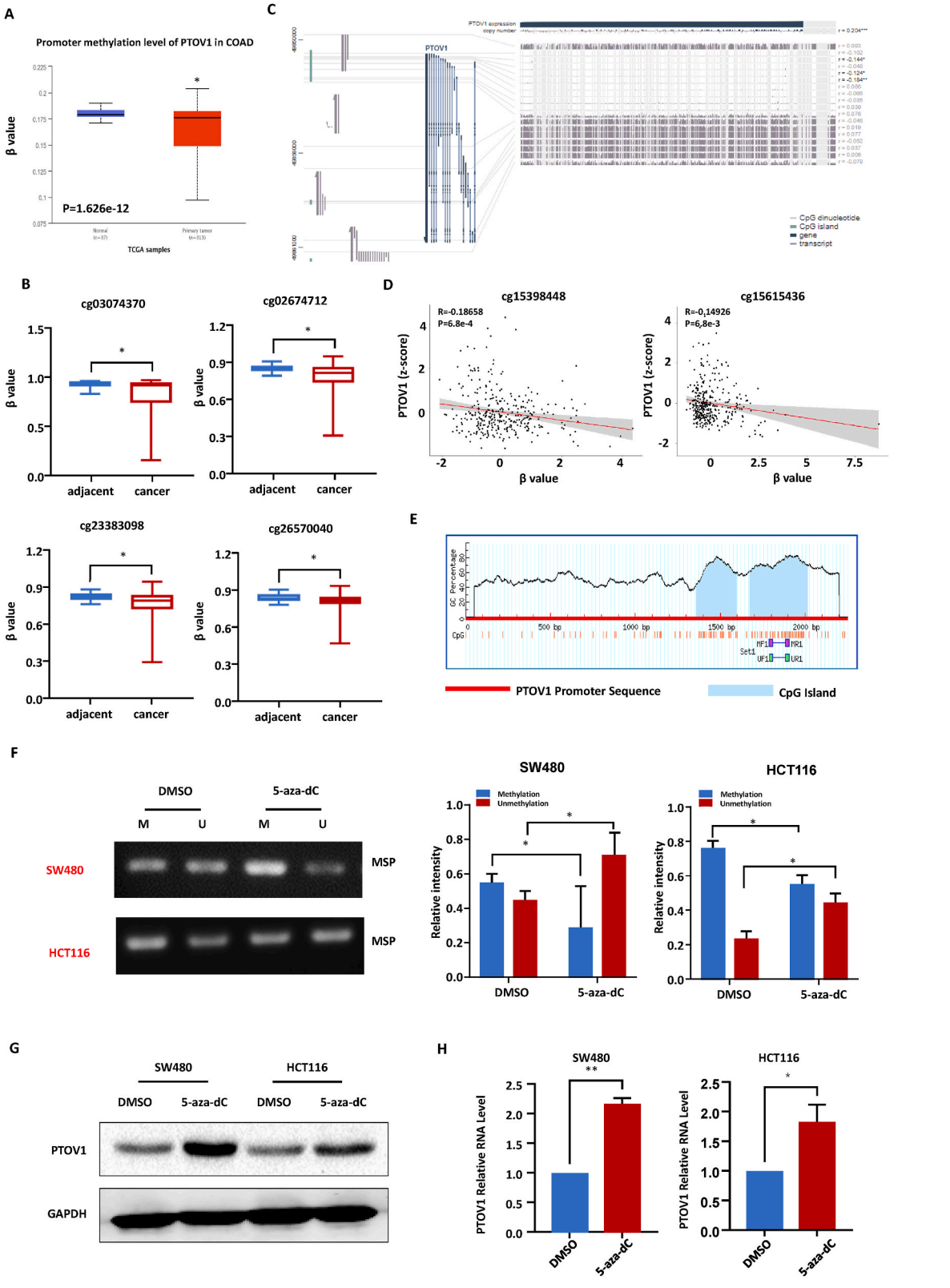

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Fig. 5. PTOV1 expression is negatively regulated by the DNA methylation of PTOV1 promoter in CRC. (A) Methylation of the PTOV1 promoter is lower in CRC compared with normal tissue in TCGA-COAD datasets collected by UALCAN. (B) MEXPRESS tool analysis for the relationship between the degree of PTOV1 promoter methylation and its expression level in COAD. (C) The CpG methylation level of PTOV1 in normal and primary tumor tissue among cancer types in COAD (n = 38 and n = 309 respectively). (D) The correlation between methylation of the two CpG sites and PTOV1 expression, respectively (n = 347). (E) The genomic coordinates of PTOV1 promoter region CpG island and MSP primer locations. (F) PTOV1 promoter methylation levels in SW480 (n = 5 each group) and HCT116 (n = 6 each group) with or without 5-aza-dC treatment by conducted MSP. (G–H) The protein (n = 3) and mRNA (n = 5) level of PTOV1 in SW480 and HCT116 with or without 5-aza-dC treatment. Note: *P < 0.05, **P < 0.01. U: unmethylated; M: methylated.

Fig. 1B–F). This transcriptional upregulation is paralleled by an increase in PTOV1 protein levels, as evidenced by CPTAC sample analysis and corroborated by the Human Protein Atlas database, with a marked distinction between CRC and adjacent normal tissues ($P < 0.05$, Fig. 1G and H). In a murine model of primary CRC, a pronounced elevation of PTOV1 was observed relative to normal tissue counterparts (Fig. 1I). A clinicopathological correlation analysis utilizing TCGA data set forth a significant association between elevated PTOV1 mRNA expression and vital status in CRC patients ($P = 0.002$), with no correlation to other factors such as age or gender (Table 1).

3.2. PTOV1 as an adverse prognostic indicator

To investigated the correlation between the expression of PTOV1 and prognosis in CRC patients. The prognostic significance of PTOV1 was delineated through Kaplan-Meier survival curve analysis, which illustrated a negative prognosis for patients with high PTOV1 expression levels (Fig. 1J). Additionally, Univariate and multivariate Cox regression analyses further substantiated the impact of age, clinical staging, T and N classification, metastasis, and PTOV1 expression on CRC patient survival, with PTOV1 emerging as an independent prognostic factor ($P < 0.05$, Table 2 and Fig. 1K) (see Table 3).

3.3. Immune infiltration correlated with PTOV1 expression

Many evidences confirm that the infiltration of tumor-related immune cells is considered to be an independent factor affecting the survival and prognosis in various tumors. Immune cell infiltration, acknowledged as an independent prognostic factor in various tumors [39,40], was scrutinized for its relationship with PTOV1 expression. Utilizing CIBERSORTx and TIMER database analyses, an inverse relationship was observed between PTOV1 expression and the abundance of immune cells such as naive B cells and $CD4^+$ T cell subsets, while an increase was noted in plasma cells and $CD8^+$ T cells ($P < 0.05$, Fig. 2A and B). TISIDB database interrogation extended these findings, revealing associations between PTOV1 and a spectrum of 16 immune cell subtypes, along with immunomodulators, within the CRC microenvironment ($P < 0.05$, Fig. 2C–F, Table 3 and Supplementary Tables 2–4).

3.4. PTOV1's impact on CRC cell proliferation and migration

In vitro experimentation within CRC cell lines (HT29, DLD1, SW480, HCT116) demonstrated that PTOV1 upregulation than normal colonic epithelial cell line (NCM460) in mRNA and protein level (Fig. 3A and B). Then, PTOV1 was manipulated by transfecting specific siRNA or specific plasmid in SW480 and HCT116 (Fig. 3C–F). The knockdown efficiency of siRNA-2 was higher than siRNA1 and was selected for the subsequent experiments (termed siPTOV1). Abnormal upregulation of PTOV1 was positively linked to cellular viability and proliferative capacity, as ascertained by CCK-8 and colony formation assays (Fig. 3G–J). Flow-cytometric analysis demonstrated that PTOV1 knockdown could induce cell cycle arrest (Fig. 4A and B). The knockdown of PTOV1 attenuated cell migration, as indicated transwell assays (Fig. 4C).

3.5. Regulation of PTOV1 by DNA methylation

Given the importance of DNA methylation in CRC progression and the dys-regulation of DNA methylation in CRC [41], the interplay between DNA methylation and PTOV1 regulation was explored. Uncovering reduced methylation at the PTOV1 promoter in CRC, which correlates with increased gene expression in TCGA database and MEXPRESS database (Fig. 5A–D). Importantly, in colon reduced PTOV1 promoter DNA methylation was significantly associated with increased PTOV1 mRNA expression (Fig. 5D). As shown in Fig. 5E, there are two CpG islands located in the promoter region of PTOV1, the relationship between methylation status of PTOV1 promoter region and PTOV1 expression was further analyzed. The demethylating agent 5-aza-dC, when applied, resulted in diminished promoter methylation of PTOV1 and a concomitant rise in PTOV1 expression (Fig. 5E–H).

3.6. PTOV1's modulation of CRC-relevant signaling pathways

To discover the signaling pathways with which PTOV1 was involved in CRC. we screened out PTOV1 related genes based on PTOV1 expression using TCGA dataset. Fig. 6A shows a volcanic map of gene changes associated with PTOV1 expression in CRC. Heat maps of the top 30 genes of PTOV1 positively and negatively associated in CRC were also presented (Fig. 6B and C). Bioinformatics-driven pathway analysis highlighted PTOV1's involvement with key signaling cascades, particularly the PI3K-Akt pathway, which is pivotal for CRC cell proliferation (Fig. 6D). Gene Set Enrichment Analysis (GSEA) underscored the enrichment of the PI3K-AKT-mTOR

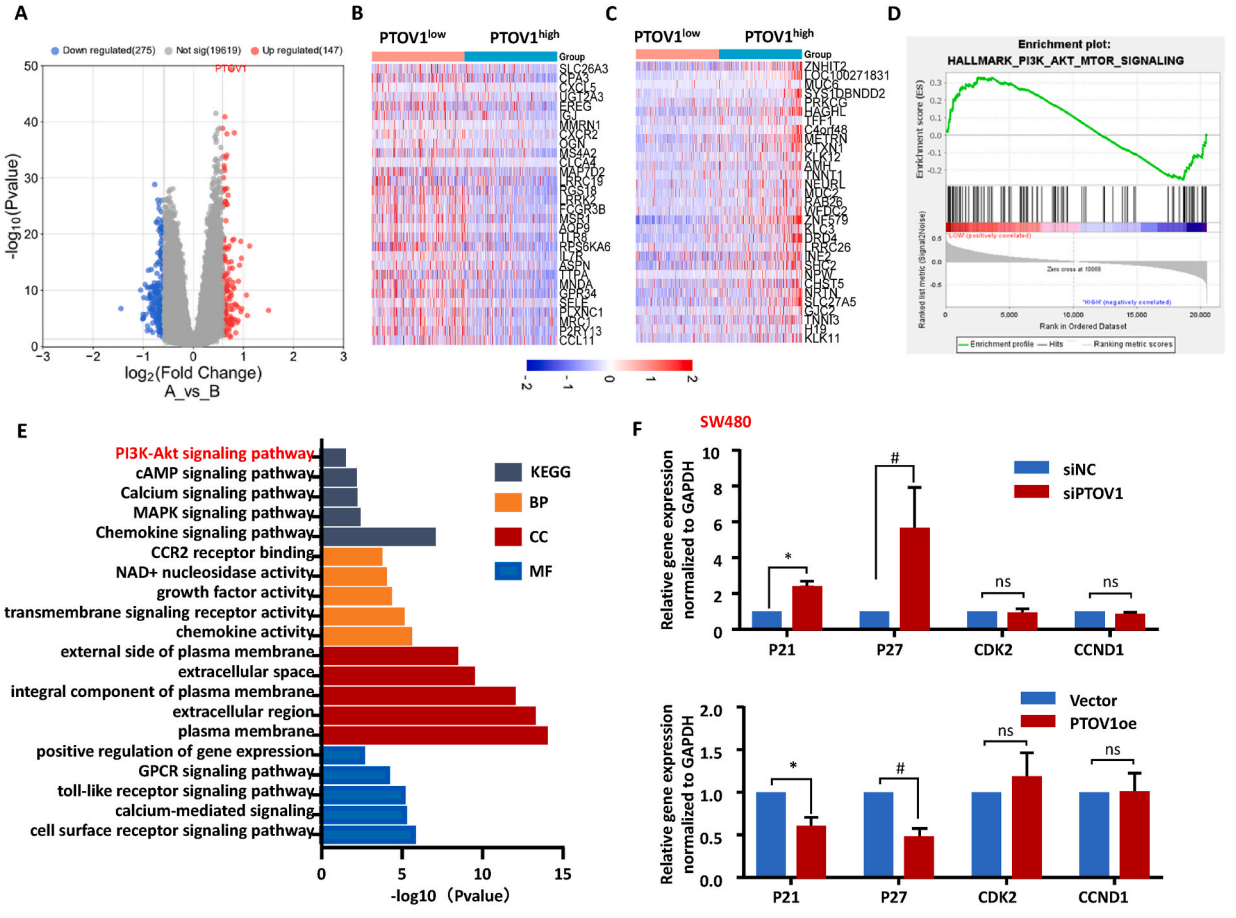

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Fig. 6. Analysis of the biological functions and signaling pathways of PTOV1 in CRC A volcanic map of gene changes associated with PTOV1 expression in CRC. (B–C) Heat maps of the top 30 genes of PTOV1 positively and negatively associated in CRC. (D) GO terms in the biological process (BP), cellular compartment (CC), molecular function (MF) and KEGG pathways. (E) GSEA was performed using the high- and low-PTOV1 expression groups stratified by the median PTOV1 expression in TCGA-COADREAD. GSEA reveal significant differences (NOM $P < 0.05$, FDR <0.25) in enrichment of MSigDB Collection. (F) qRT-PCR analysis for expression of P21, P27, cyclin D1, and CDK2 in SW480 cells (n = 5). (G–H) P27, P21 and AKT1 protein were measured by Western blot in SW480 (n = 3) and HCT116 (n = 3) cells. Note: *$P < 0.05$, #$P < 0.05$; Data are presented as means ± SD.

pathway in PTOV1-high CRC contexts (p-value <0.05; FDR< 0.25; Fig. 6E).

3.7. PTOV1-mediated proliferation via AKT1 signaling

Bioinformatics analysis points to a potential correlation between the AKT1 signaling pathway and the regulatory effects of PTOV1 on CRC progression. Given the established significance of the AKT pathway in cell proliferation during tumorigenesis, we investigated PTOV1 modulates AKT pathway to influence CRC cell expansion. The results revealed that PTOV1 knockdown significant diminishes AKT1 phosphorylation and upregulated P21 and P27, established downstream targets of AKT1, while the expression levels of CDK2 and CCND1 remained unaltered by PTOV1 levels (Fig. 6F–H).

To substantiate the role of AKT1 in PTOV1-induced cell proliferation, we utilized MK2206, a specific inhibitor of AKT1 activation. This compound was deployed to counteract the effects of PTOV1 overexpression on AKT1 activity (Fig. 7A). Our results indicate that MK2206 effectively reversed the downregulation of P21 and P27, indicative of a restoration of cell cycle control in the presence of excessive PTOV1 (Fig. 7A and B). Furthermore, MK2206 treatment rescued the proliferative effects induced by PTOV1 overexpression, as evidenced by both colony formation and CCK-8 assays (Fig. 7C and D). Collectively, these findings underscore the mechanism by which PTOV1 drives CRC cell proliferation through the AKT1 signaling cascade.

4. Discussion

CRC, a formidable malignancy globally, is characterized by an insidious onset and a dearth of distinctive clinical indicators during its early stages. This leads to a generally poor prognosis [42]. Despite advancements in endoscopic screening and the implementation of multimodal therapies that have improved patient survival, the overall treatment outcomes and prognoses remain suboptimal. Identifying biomarkers with high specificity and sensitivity for early detection and adjunct therapy is thus a critical and effective strategy in the battle against CRC.

PTOV1, an oncogene with documented upregulation across various human cancers, is significantly linked to tumor progression and an unfavorable prognosis [16–18]. Its involvement in the regulation of tumor progression has been noted [19–23]. However, its specific role in CRC has remained unexplored. Our study reveals a significant increase in PTOV1 expression in CRC tissues, which correlates with a poor prognosis. By manipulating PTOV1 levels in CRC cell lines through specific siRNA or plasmid transfection, we demonstrate PTOV1's promotion of cell proliferation and migration. PTOV1, known to shuttle between the nucleus and cytoplasm in a cell cycle-dependent manner [2–4]. This study confirmed that PTOV1 facilitate AKT1 phosphorylation, thereby driving CRC cell proliferation through the downregulation of P21 and P27. However, how PTOV1 regulates AKT1 phosphorylation still needs to be explored.

The tumor immune microenvironment (TIME), encompassing non-neoplastic cells within and surrounding tumors, is increasingly recognized for its prognostic implications in cancer, including immune cell infiltration[43–45]. Our analysis of PTOV1's role in TIME in CRC indicates a negative correlation with $CD4^+$ T cell and B cell infiltration levels. A substantial body of literature supports the notion that immune cell infiltration in CRC is significantly associated with patient prognosis and disease progression [45–47]. Furthermore, PTOV1 shows significant correlations with numerous immunoregulators and immunomodulatory genes in CRC. These findings suggest a potential role for PTOV1 in CRC's tumor immunity, hinting at its capacity to influence immune response activation or suppression. This study marks the first to elucidate the relationship between PTOV1 and immune cell infiltration, highlighting the need for further investigation into the underlying mechanisms. However, the molecular mechanism of PTOV1 in tumor immunity still needs to be explored.

AKT1, a pivotal regulator in CRC progression, is activated by PTOV1. Our study confirms that PTOV1 suppresses the expression of P21 and P27, downstream targets of AKT1, while leaving CCND1 and CDK2 unaffected. This mechanism by which PTOV1 activates AKT1 suggests its influence on cell cycle progression and other cellular processes integral to CRC development. The aberrant proliferation of tumors is a well-established hallmark of cancer progression, reinforcing PTOV1's role as a promoter of CRC advancement. The PTOV1-AKT1 axis presents a novel perspective on the regulation of CRC cell behavior and a potential target for therapeutic intervention [48]. Further research is imperative to fully understand how PTOV1 modulates AKT phosphorylation.

Our findings position PTOV1 as an oncogene linked to poor prognosis and the promotion of proliferation in CRC. Its correlation with immune responses suggests PTOV1's potential as a target for anti-tumor immunotherapy, offering new avenues for CRC immunotherapy research. Our study also affirms that PTOV1 expression is regulated by DNA methylation in CRC. Moreover, PTOV1 is involved in activating AKT1 signaling pathway. Further investigation into the molecular mechanisms of PTOV1 overexpression in CRC is warranted. This study clarifies the molecular underpinnings of PTOV1's involvement in the proliferative characteristics of CRC cells, offering a novel angle for research and therapeutic development.

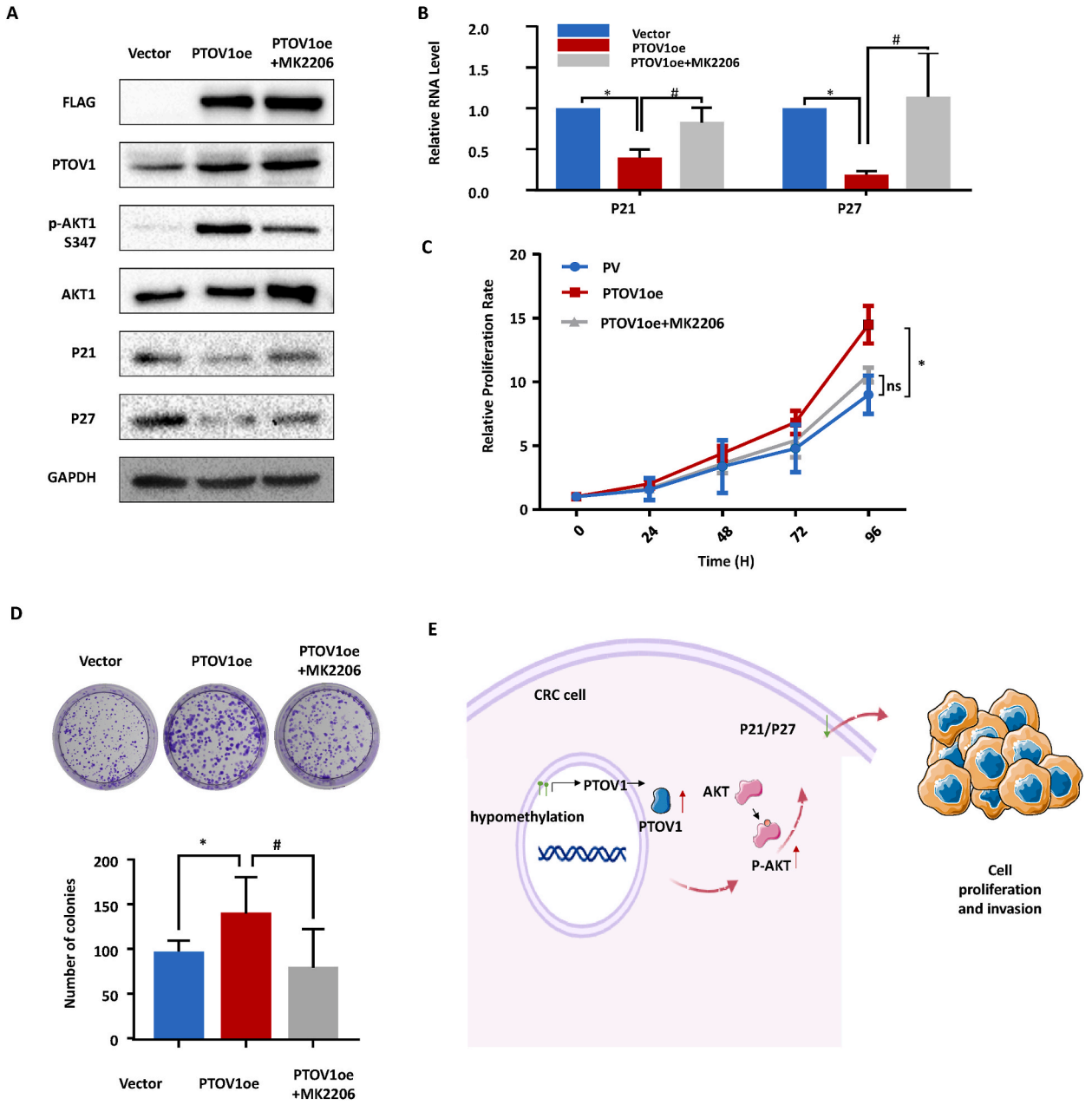

Fig. 7. POTV1 promotes CRC cells proliferation via regulating AKT signaling pathway. (A) AKT1, P21 and P27 expression within PTOV1-overexpressed SW480 cell line and normal control with or without incubation with MK2206 (n = 3). (B) qRT-PCR analysis of P21 and P27 in PTOV1-overexpressed SW480 cells and normal control with or without incubation with MK2206. (C) CCK8 and colony formation assay in PTOV1-overexpressed SW480 cells and normal control with or without exposure to MK2206 (n = 3). (E) Graphical abstract. high level of PTOV1 mediated by hypomethylated in promoter region promotes the proliferation of CRC cells through activating of AKT1. Note: *P < 0.05, #P < 0.05; Data are presented as means ± SD; Supplementary Fig. 1. Resulting heat map visualizing the correlation matrix of diverse tumor-infiltrating immune cells subpopulations.
Each tile indicates coefficients calculated by Pearson's correlation test. Note: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

5. Conclusion

Collectively, our findings underscore the significance of PTOV1 in the neoplastic progression and adverse outcomes of CRC. PTOV1 exhibits pronounced upregulation in CRC tissues. Moreover, our study reveals an inverse relationship between PTOV1 expression levels and the degree of promoter hypomethylation, suggesting that epigenetic regulation significantly influences PTOV1 activity in CRC. Notably, PTOV1 has been shown to augment the phosphorylation of AKT1, a critical signaling molecule, and to modulate the

expression of cell cycle regulators P21 and P27. These effects indicate a pivotal role for PTOV1 in mediating CRC cell proliferation. These convergent lines of evidence collectively propose PTOV1 as a promising candidate for a prognostic biomarker and a potential therapeutic target in CRC. The elucidation of these mechanisms provides novel insights into the regulatory network of oncogenes in CRC, offering avenues for future research and the development of targeted interventions.

Data availability statement

All data generated in this study are available from the corresponding author upon reasonable request. **Ethics declarations** This study was approved by the Ethics Committee of Capital Medical University with the approval number: AEEI-2022-306.

CRediT authorship contribution statement

Si-An Xie: Writing – original draft, Project administration, Data curation, Conceptualization. **Wen Zhang:** Validation. **Feng Du:** Methodology. **Si Liu:** Software, Resources, Conceptualization. **Ting-Ting Ning:** Resources. **Nan Zhang:** Software, Resources. **Shu-Tian Zhang:** Writing – review & editing. **Sheng-Tao Zhu:** Writing – review & editing.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36017.

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