PADI1 contributes to epithelial-mesenchymal transition in pancreatic ductal adenocarcinoma through activating ERK1/2-p38 signaling pathway

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Research Article

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Abstract

Background

Peptidylarginine deiminase 1 (PADI1) may be relative with the progression of epithelial-mesenchymal transition (EMT) in pancreatic ductal adenocarcinoma (PAAD). We aim to explore the role of PADI1 in PAAD.

Methods

The expression pattern of PADI1 in PAAD tissues and normal tissues was analyzed using The Cancer Genome Atlas (TCGA) dataset. PADI1 was knocked down in CFPAN-1 and HPAC cells, while overexpressed in PANC-1 and Bxpc-3 cells by RNA interference. Wound healing assay was performed to analyze relative cell migration distance. Cell migration and invasion were assessed by Transwell assay. Related protein expression levels were measured by western blot and immunofluorescence.

Results

Bioinformatics analysis showed that PADI1 was overexpressed in PAAD tissues and associated with worse survival prognosis. Knockdown of PADI1 suppressed the cell migration, invasion and activated ERK1/2-p38 signaling pathway in CFPAN-1 and HPAC cells. Overexpression of PADI1 obtained the opposite results in PANC-1 and Bxpc-3 cells. Moreover, treatment with MEK1/2 inhibitor significantly recovered the effects of PADI1 knockdown on cell migration, invasion, EMT process and p-ERK1/2 and p38 expression in CFPAN-1 and HPAC cells.

Conclusions

Our data suggested that PADI1 may function as an oncogene in regulating metastasis *in vitro* in PAAD.

Introduction

Pancreatic ductal adenocarcinoma (PAAD), accounting for nearly 85% of pancreatic cancer, represents one of the most leading cause of digestive tract malignancy-related death with less than 10% five-year survival rate [1, 2]. Despite great progress has been made in the traditional therapeutic treatments, including surgery resection, chemotherapy or radiotherapy against PAAD, the prognosis of patients with PAAD still remain poor, which mainly attributed distant metastasis and local invasion [3, 4]. Therefore, it is of great importance to explore the underlying molecular mechanisms for improving the prognosis of this deadly disease.

Metastasis has been widely considered as the major challenge in the clinical therapy of PAAD, which begins with the epithelial-mesenchymal transition (EMT), including loss of cell-cell adhesion, unrestrained cell migration and invasion [5, 6]. Activation of EMT is associated with changed expression of many genes, including downregulation of epithelial markers (e.g. occluden-1 and E-cadherin) and upregulation of mesenchymal markers (e.g. N-cadherin and vimentin) [7]. In addition, MAPK pathway has significant roles in the initiation of EMT, which is prevalent in many cancers, including pancreatic cancer [8] and ovarian cancer [9]. MAPK family includes three subfamilies (ERK1/2, p38 and JNK), all of which can be triggered by growth or stress factors, thus playing a vital part in signal transduction inside the cells [9, 10]. Therefore, ERK1/2 and p38 appear to be closely correlated with the EMT process in PAAD cells.

The peptidylarginine deiminase (PADI) family consists of five members (PAD1–4, 6) located within a highly organized gene cluster at 1p36.13 in humans and on the orthologous region of mouse chromosome 4, which could post-translationally convert arginine residues into neutrally charged citrulline [11]. Interestingly, recent studies have reported the important role of PADI-mediated protein citrullination in various cancers [12]. For instance, Stadler et al [13] reported that PADI4 overexpression inhibited breast cancer cell migration via its role in modulating EMT, while Xin et al [14] reported that silencing PADI4 suppressed cell proliferation, cell cycle progression and invasion in gastric cancer cells. PADI2 has been identified as a potential breast cancer biomarker and therapeutic target [15]. Inhibition of PADI2 could enhance the anti-tumor effect of docetaxel in tamoxifen-resistant breast cancer cells [16]. To our best knowledge, a recent report by Qin et al [17] not only found PADI1 depletion suppressed tumorigenesis in triple negative breast cancer, but also demonstrated that these changes were correlated with activated ERK1/2 and p38 MAPK signaling pathways. However, whether PADI1 regulating metastasis and EMT in PAAD by modulating ERK1/2-p38 signaling pathway still remain unclear.

In the present study, we first investigated the expression pattern and survival prognosis of PADI1 in PAAD patients. Then, we performed loss-of-function and gain-of-function assays to analyze the effects of PADI1 on PAAD cell migration, invasion, EMT and ERK1/2-p38 signaling. Moreover, rescue experiments were performed to confirm whether PADI1 regulated PAAD cell migration and invasion via regulating ERK1/2-p38 signaling pathway.

Materials And Methods

Database analysis

The mRNA expression pattern of PADI1 in primary PAAD tissues and normal tissues during the study is available in the public repository from the Cancer Genome Atlas (TCGA: https://portal.gdc.cancer.gov/) database. Based on the survival data of PAAD patients obtained from TCGA database, we investigated the effect of PADI1 expression level on PAAD patient survival rate using Kaplan–Meier method with log-rank test.

Cell lines and treatments

Total five PAAD cell lines, including CFPAC-1, PANC-1, PL45, Bxpc-3 and HPAC, as well as human pancreatic nestin-expressing cells (hTERT-HPNE) were obtained from the Cell Resource Center of Shanghai Institute of Life Sciences, Chinese Academy of Sciences. All cell lines were cultured in the recommended conditions supplemented with 10% fetal bovine serum (FBS, Hyclone Co., Logan, UT, USA) in a humidified 5% CO_2 air incubator at 37 °C.

The small interfering RNA targeting PADI1 (siPADI1), PADI1 mimics overexpression plasmid and corresponding controls were synthesized by Shanghai Genepharma, Co., Ltd (Shanghai, China). For cell transfection, CFPAC-1, PANC-1, Bxpc-3 and HPAC cells were seeded into six-well plates at a density of 1×10^7 cells per well. CFPAC-1 and HPAC cells were transfected with siPADI1 or siNC as control, while PANC-1 and Bxpc-3 cells were transfected with PADI1 mimics or empty vector under the instructions provided Lipofectamine 3000 (Life Technology, MD, USA) for 48 h. For the validated experiments, CFPAC-1 and HPAC cells were treated with ERK1/2 inhibitor (Compound 27, Sigma-Aldrich, St.Louis, MO, USA) for 2 h, followed by siPADI1 transfection.

Quantitative real time PCR

Total RNA from cell lines was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized from 2 μg of RNA with PrimeScriptTM RT Reagent Kit (Takara Biotechnology, Dalian, China) following the manufacturer's protocols. Quantitative real time PCR was performed on the ABI StepOnePlus Real-time PCR system with SYBR Premix Ex Taq™ (Takara, Dalian, China) according to the following cycling conditions: 95 °C for 30 sec, followed by 40 cycles of 95 °C for 5 sec and 60 °C for 34 sec. The primer sequences used in this study were as follows: PADI1 forward: 5′-TTTCGGTGCTTTGAGTGGGTC-3′ and reverse: 5′-TGTTGGCATATTTGTGGCAGG-3′; GAPDH forward: 5′-CACCCACTCCTCCACCTTTG-3′ and reverse: 5′-CCACCACCCTGTTGCTGTAG-3′. Reactions were performed in triplicate and relative fold changes in PADI1 mRNA expression were calculated with the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Total protein sample was extracted from cell lines using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) and protein concentration was determined with a BCA protein assay kit (Beyotime). Equal amount of protein sample was separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore). The membranes were blocked with 5% non-fat milk for 2 h and incubated with primary antibodies against PADI1, E-cadherin, N-cadherin, Vimentin, p-ERK1/2, p38, p-p38 and GAPDH (all dilution: 1:1000, Abcam, UK), followed by incubated with horseradish peroxidase-conjugated secondary antibody. Afterwards, immunoreactive bands were analyzed with an electrochemiluminescence system (GE Healthcare).

Immunofluorescence

Transfected cells from different groups were seeded into chamber slides and cultured overnight. Next day, cells were fixed with 4% formaldehyde for 30 min and permeabilized with 0.5% Triton X-100. After blocked with 1% BSA for 30 min, cells were incubated with PADI1 and p-ERK1/2 antibodies overnight at 4 °C. Then, cells were washed with PBS and incubated with appropriated secondary antibody. The nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole, Sigma). The stained images were observed and analyzed via immunofluorescence microscopy.

Wound healing assay

In brief, transfected cells were plated into 24-well plates and grown to 100 confluence. Afterwards, cells from different groups were scratched with a 10 μl pipette tip (time 0). Then cells were washed with PBS to remove isolated cells, which were further incubated with complete growth medium. After 24 h, the images of cells migrated to the injured area were obtained with an inverted microscope.

Transwell assay

Cell migration and invasion ability were assessed by 24-well transwell chamber precoated without and with 1 mg/ml Matrigel (Millipore, MA, USA), respectively. Briefly, approximately 2.0×10^4 transfected cells from different groups suspended in 100 μl of serum-free medium were inoculated in the upper chamber and 500 μl of complete medium was added to the lower chamber as the chemoattractant. After 48 h, the cells that migrated into the lower chamber were fixed with 75% ethanol, stained with 0.5% crystal violet and counted under a light microscope (BD Biosciences, Heidelberg, Germany).

Statistical analysis

Statistical data were analyzed with GraphPad prism 6 and expressed as means ± SD from three independent experiments. Paired Student's t-test was applied to determine the statistical significance of differences for two groups. One-way ANOVA, followed by Dunnett's test was used to assess the statistical significance of differences for multiple groups. Differences are considered statistically significant at $p < 0.05$.

Results

PADI1 was overexpressed in PAAD tissues and associated with worse survival prognosis

To elucidate the role of PADI1 in PAAD pathogenesis, we first searched the expression pattern of PADI1 in primary tumor and normal tissues derived from PAAD patients on TCGA database. As presented in Fig. 1A, PADI1 was identified to be significantly overexpressed in PAAD tumor samples (n = 178) compared with in the normal tissues (n = 4). Analysis of TCGA datasets indicated that high PADI1 expression levels in PAAD samples were associated with poor survival prognosis (Fig. 1B). In order to further validate the expression of PADI1, we performed quantitative real time PCR and western blot

analysis to determine the expression of PADI1 in PAAD cell lines. The results showed that the expression of PADI1 mRNA (Fig. 1C) and protein (Fig. 1D) was obviously upregulated in PAAD cells lines, including CFPAC-1, PANC-1, PL45, Bxpc-3 and HPAC, compared with normal pancreatic cell line hTERT-HPNE. Consistently, immunofluorescence staining of PADI1 confirmed the overexpression of PADI1 in PAAD cell lines, in comparison with hTERT-HPNE cells (Fig. 1E).

Knockdown of PADI1 suppressed the PAAD cell migration, invasion and activated ERK1/2-p38 signaling pathway

To determine the functional role of PADI1 in PAAD cells, we first performed loss-of-function assays in CFPAN-1 and HPAC cells with relatively higher PADI1 expression by transfection with siPADI1. As demonstrated by PCR (Fig. 2A) and western blot analysis (Fig. 2B), the expression of PADI1 was significantly decreased in CFPAC-1 and HPAC cells after siPADI1 transfection compared with control group. The results from wound healing assay indicated that knockdown of PADI1 obviously suppressed the healing rate of CFPAN-1 and HPAC cells (Fig. 2C). Transwell assay further demonstrated that knockdown of PADI1 significantly suppressed the migration (Fig. 2D-E) and invasion (Fig. 2F-G) ability in both CFPAN-1 and HPAC cells. In molecular level, knockdown of PADI1 suppressed the EMT progress, as reflected by elevated E-cadherin expression and reduced N-cadherin and Vimentin expression, as well as upregulated p-ERK1/2 and p-38 expression in CFPAN-1 and HPAC cells (Fig. 2H). Immunofluorescence assay further confirmed the protein expression of p-ERK1/2 was increased after PADI1 knockdown in CFPAN-1 and HPAC cells (Fig. 2I).

Overexpression of PADI1 promoted the PAAD cell migration, invasion and ERK1/2-p38 signaling pathway

To further confirm the oncogenic role of PADI1 in PAAD cells, we selected PANC-1 and Bxpc-3 cells with relatively lower PADI1 expression to transfect with PADI1 overexpression plasmid. As shown in Fig. 3A-B, the expression of PADI1 was remarkedly upregulated after PADI1 mimics plasmid transfection compared with empty vector in PANC-1 and Bxpc-3 cells. In contrast with PADI1 knockdown, we observed that overexpression of PADI1 notably promoted healing rate (Fig. 3C), cell migration (Fig. 3D-E) and invasion (Fig. 3F-G) ability in PANC-1 and Bxpc-3 cells. Similarly, PADI1 overexpression obtained the opposite effects on the protein levels of E-cadherin, N-cadherin, Vimentin, p-ERK1/2, p38 and p-p38, as depicted by western blot analysis (Fig. 3H) and immunofluorescence assay (Fig. 3I).

PADI1 affected the EMT process via regulating ERK1/2-p38 signaling pathway in PAAD cells

Next, we explored whether PADI1 regulated PAAD cell migration and invasion via regulating ERK1/2-p38 signaling pathway. CFPAC-1 and HPAC cells were first transfected with siPADI1 and then treated with ERK1/2 inhibitor (Compound 27). As depicted by wound healing assay, inhibition of p-ERK1/2 obviously promoted the cell healing rate in PADI1 silenced CFPAC-1 and HPAC cells (Fig. 4A). Similarly, the cell migration (Fig. 4B-C) and invasion (Fig. 4D-E) ability were enhanced in PADI1 silenced CFPAC-1 and HPAC cells after p-ERK1/2 inhibition. Furthermore, we found that activated EMT process and suppressed

ERK1/2-p38 signaling pathway following inhibition of p-ERK1/2 in PADI1 silenced CFPAC-1 and HPAC cells (Fig. 4F-G).

Discussion

Here, we first observed that the expression of PADI1 was upregulated in PAAD tissues compared with normal tissues and correlated with worse survival prognosis in the TCGA database. In fact, the PADI enzyme family has been reported to be linked with carcinogenesis and tumor progression [15, 18, 19]. Similar to our findings, Qin et al [17] searched Oncomine and TCGA database and found the expression of PADI1 was upregulated in breast cancer patients and positively correlated with triple negative breast cancer. Wang et al [20] showed that PADI2 as an androgen-repressed gene is upregulated in castration-resistance prostate cancer. Liu et al [21] revealed that PADI4 was overexpressed in lung cancer tissues and cell lines. According to these evidences, we thus speculated that PADI1 may be involved in regulating PAAD progression.

We next investigated the functional role of PADI1 in PAAD in vitro. As expected, PADI1 knockdown supressed the migration and invasion aibiilty in PAAD cells, while PADI1 overexpression obtained the opposite results. Our in vitro data suggsted that PADI1 exerted oncogenic effects on metastasis in PAAD cells. In molecular level, we observed that knockdown of PADI1 elevated the expression of E-cadherin, p-ERK1/2 and p-38, and reduced N-cadherin and Vimentin expression in CFPAN-1 and HPAC cells. On contrast, PADI1 overexpression generated the opposite results on the above protein expression levels in PANC-1 and Bxpc-3 cells. ERK and p38 are the extensively studied two major MAPK protein kinases in mammals [22]. Here, we showed that both p-ERK1/2 and p-38 are activated in PADI1 depleted cells, indicating an inhibitory effect of PADI1 on the activation of ERK1/2-p38 signaling. Consistent with our data, PADI1 silencing reduced cell proliferation, suppressed epithelial-mesenchymal transition, prevented metastasis, and activated ERK1/2 and P38 MAPK signaling pathways in MDA-MB-231 cells [17]. PADI4 promoted EMT while PADI4 inhibitor suppressed EMT in osteosarcoma cells [23]. Downregulating PADI2 suppressed colony formation, proliferation, migration and invasion of A2780 and SKOV3 cells [24]. On the contrary, overexpression of PADI4 constrains the activity of EMT via suppressing ETS-domain containing protein (Elk1) expression, and inhibits resistance of NSCLC to gefitinib [25]. From these reports, the different effects of PADIs in different tumor cells might be mainly ascribed to different tissue sources.

Furthermore, we provided evidence showing that ERK1/2 and P38 play distinct roles in migration, invasion and EMT process in PADI1 depleted CFPAC-1 and HPAC cells. Our data described the activated migration, invasion and EMT process following inhibition of p-ERK1/2 in PADI1 silenced CFPAC-1 and HPAC cells, which indicated that ERK1/2 inactivation appears to primarily be responsible for upregulation of N-cadherin and Vimentin, and downregulation of E-cadherin expression in PAD1 knockdown cells. Actually, it still remains controversial on the precise role of MAPK signaling in regulating migration, invasion and EMT. Epidermal growth factor (EGF) down-regulates E-cadherin expression through production of H_2O_2, activation of p38 MAPK, and up-regulation of Snail in human ovarian cancer cells

[26]. Pirfenidone is able to attenuate EMT and fibrosis in vivo and in vitro through antagonizing the MAPK pathway [27]. Different cell types are not doubt the major reason explaining these controversies.

Conclusion

Taken together, PADI1 exerted oncogenic effects on the PAAD cells in vitro by promoting cell migration, invasion and EMT process, which all might be associated with the inactivation of ERK1/2-p38 signaling pathway. Our research indicates that targeting PADI1 in PAAD may serve as a promising treatment.

Abbreviations

PADI1: Peptidylarginine deiminase 1; EMT: epithelial-mesenchymal transition; PAAD: Pancreatic ductal adenocarcinoma

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed in this research are available in the published article.

Competing interests

The authors do not have competing interest.

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Authors' contributions

T.J designed, performed the experiments and drafted the manuscript. K.M and L.C performed and analyzed the data. T.C designed the experiments and had a critical scientific revision on the manuscript.

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Not applicable.

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Figures

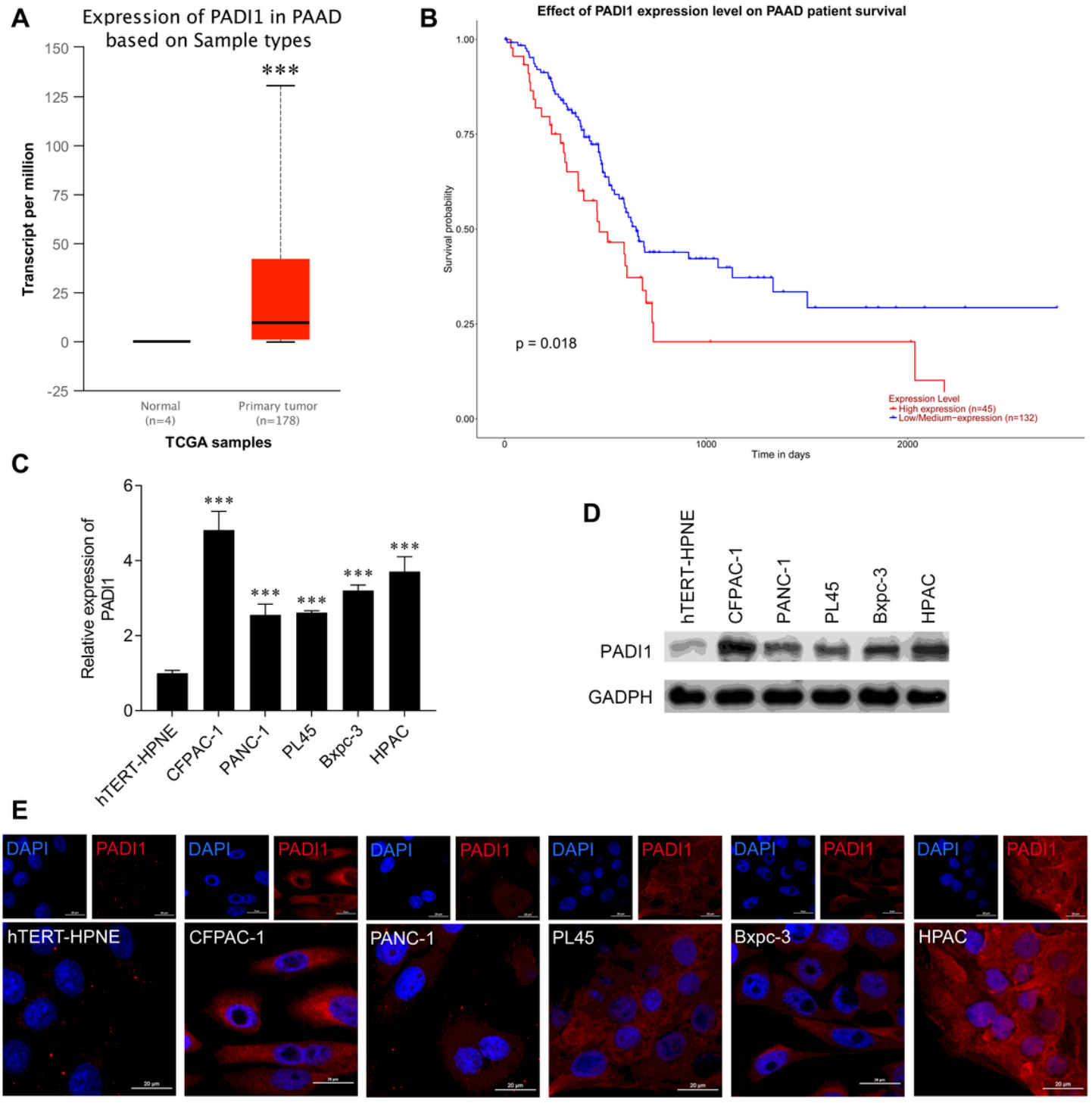

Figure 1

The expression of PADI1 and prognosis in PAAD patients. TCGA database was searched to investigate (A) the expression of PADI1 in tumor and normal tissues, as well as (B) the effects of PADI1 on survival prognosis in PAAD patients. (C) Quantitative real time PCR and (D) western blot analysis was applied to determine the expression of PADI1 mRNA and protein level, respectively in five PAAD cell lines (CFPAC-1, PANC-1, PL45, Bxpc-3 and HPAC) and human pancreatic cell line hTERT-HPNE. (E) PADI1 was observed using immunofluorescence (400×) in PAAD cell lines and hTERT-HPNE.

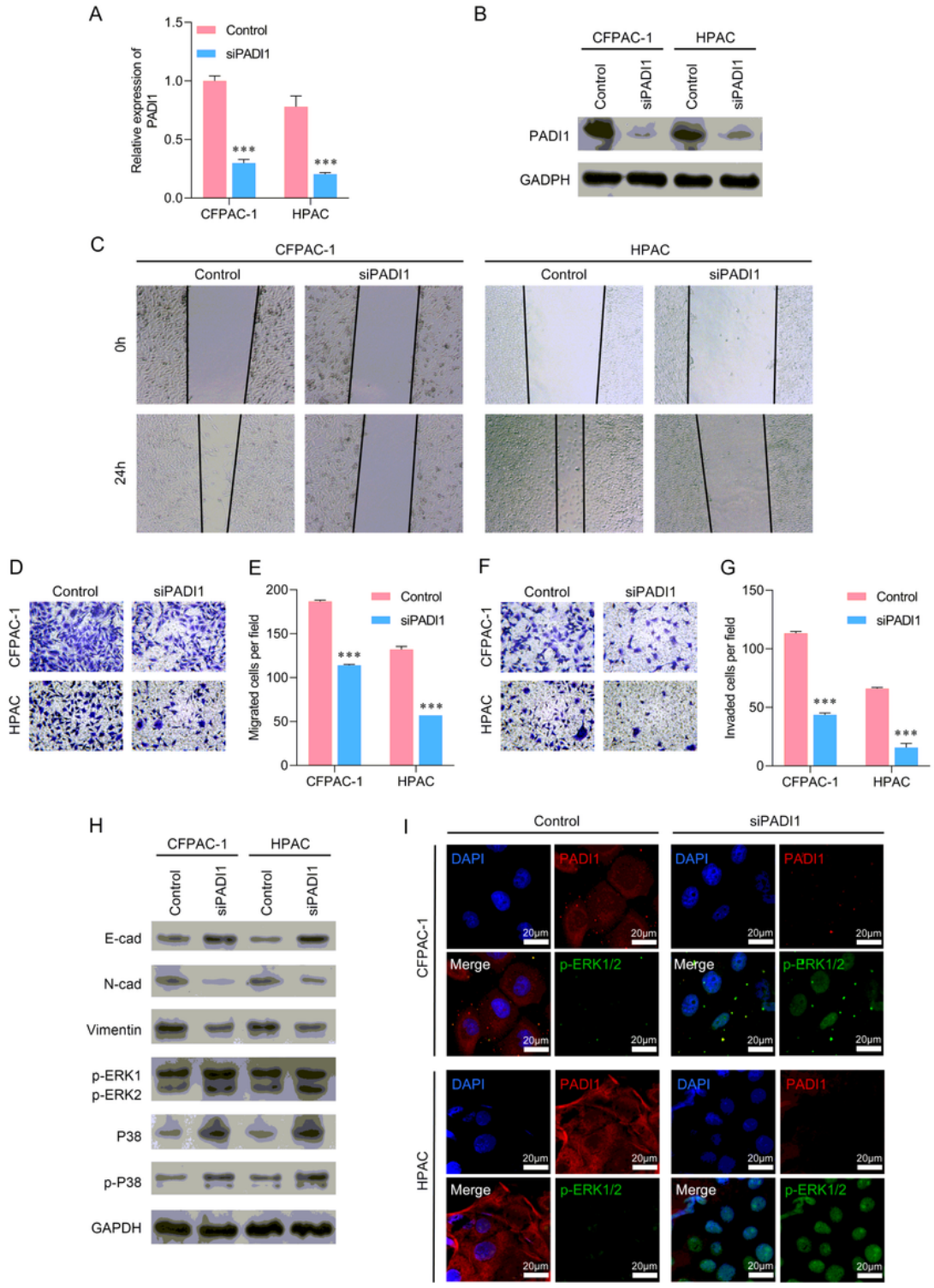

Figure 2

Knockdown of PADI1 suppressed the PAAD cell migration, invasion and activated ERK1/2-p38 signaling pathway. CFPAC-1 and HPAC cells were transfected with siPADI1 or control. (A) Quantitative real time PCR and (B) western blot analysis was applied to determine the expression of PADI1 mRNA and protein levels in the above two cell lines. (C) Wound healing assay was performed to assess cell migration in the above two cell lines. Transwell assay was used to evaluate cell migration (D-E) and invasion (F-G) in the

above two cell lines. Data were expressed as means ± SD from three independent experiments. ***p < 0.001, compared with control; (H) The protein expression of E-cadherin, N-cadherin, Vimentin, p-ERK1/2, p38 and p-p38 was measured in the above two cell lines by western blot analysis. (I) PADI1 and p-ERK1/2 expression levels were observed using immunofluorescence (400×) in the above two cell lines.

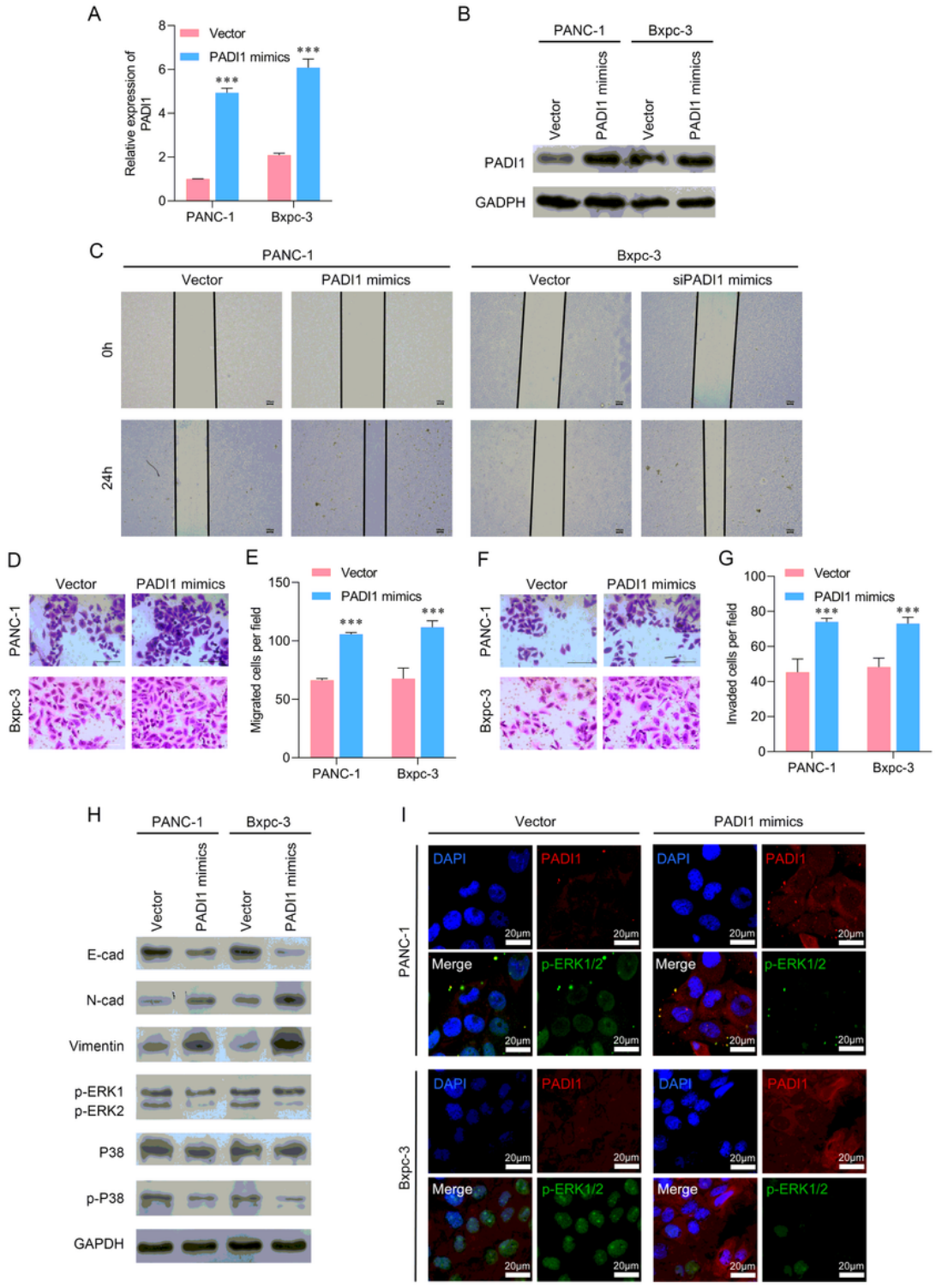

Figure 3

Overexpression of PADI1 promoted the PAAD cell migration, invasion and ERK1/2-p38 signaling pathway. PANC-1 and Bxpc-3 cells were transfected with PADI1 mimics overexpression plasmid or vector. (A) Quantitative real time PCR and (B) western blot analysis was applied to determine the expression of PADI1 mRNA and protein levels in the above two cell lines. (C) Wound healing assay was performed to assess cell migration in the above two cell lines. Transwell assay was used to evaluate cell migration (D-E) and invasion (F-G) in the above two cell lines. Data were expressed as means ± SD from three independent experiments. ***$p < 0.001$, compared with vector; (H) The protein expression of E-cadherin, N-cadherin, Vimentin, p-ERK1/2, p38 and p-p38 was measured in the above two cell lines by western blot analysis. (I) PADI1 and p-ERK1/2 expression levels were observed using immunofluorescence (400×) in the above two cell lines.

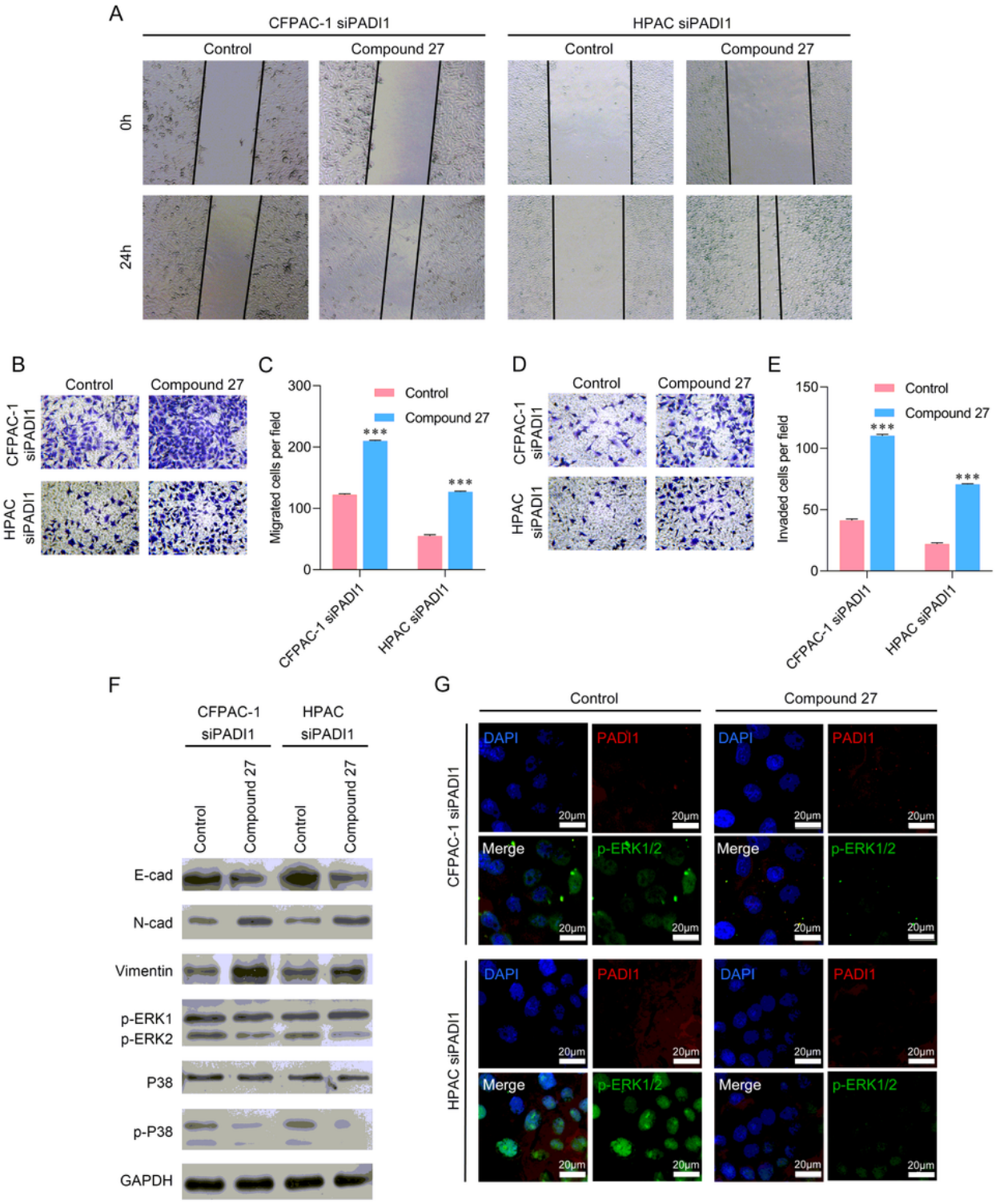

Figure 4

PADI1 affected the EMT process via regulating ERK1/2-p38 signaling pathway in PAAD cells. CFPAC-1 and HPAC cells were first treated with ERK1/2 inhibitor (Compound 27) and then transfected with siPADI1. (A) Wound healing assay was performed to assess cell migration in the above two cell lines. Transwell assay was used to evaluate cell migration (B-C) and invasion (D-E) in the above two cell lines. Data were expressed as means ± SD from three independent experiments. ***p < 0.001, compared with

control; (F) The protein expression of E-cadherin, N-cadherin, Vimentin, p-ERK1/2, p38 and p-p38 was measured in the above two cell lines by western blot analysis. (G) PADI1 and p-ERK1/2 expression levels were observed using immunofluorescence (400×) in the above two cell lines.