Int J Clin Exp Med 2016;9(11):20466-20473
www.ijcem.com /ISSN:1940-5901/IJCEM0025363

Original Article

Valproic acid (VPA) suppresses the expression of SMAD4 in prostate carcinoma by up-regulating miR-34a

Wenjun Xia[1*], Xiaopeng Lan[2*], Ji Lv[2], Jun Ma[2], Wenbin Chen[3], Ling Gao[3], Qinghua Xia[4]

[1]Surgical Intensive Care Unit, Provincial Hospital Affiliated to Shandong University, Shandong, China; [2]Department of Urology, Qingdao Central Hospital, Shandong, China; [3]Central Laboratory, Provincial Hospital Affiliated to Shandong University, Shandong, China; [4]Minimally Invasive Urology Center, Provincial Hospital Affiliated to Shandong University, Shandong, China. [*]Equal contributors.

Received February 1, 2016; Accepted April 23, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: SMAD4 plays an important role in epithelial-mesenchymal transition (EMT) and cancer metastasis. Previous studies have reported that valproic acid (VPA) suppresses prostate carcinoma (PCa) cell metastasis and down-regulates SMAD4 protein levels. However, the mechanism by which VPA regulates the expression of SMAD4 in PCa cells remains unknown. We identified miRNAs that can complementarily bind to SMAD4 mRNA using www.targetscan.com and searched PUBMED to identify miRNAs related to VPA. The miRNAs identified by both of the searches were selected. The expression of SMAD4 was analyzed after VPA treatment or transfection of pre-miRNAs or miRNA inhibitors. After VPA treatment, the levels of SMAD4 mRNA and protein were down-regulated whereas the expression of miR-20a, 34a, and 449a was up-regulated. Up-regulation of miR-34a mimicked the SMAD4-inhibiting effect of VPA, whereas down-regulation of miR-34a eliminated this effect in LNCaP and PC3 cells. These results indicate that VPA inhibits the expression of SMAD4 by up-regulating the expression of miR-34a.

Keywords: Valproic acid, SMAD4, prostate carcinoma, micro RNA

Introduction

Prostate carcinoma (PCa) is the most common cancer among men [1], and a high incidence of metastasis is the main reason for PCa mortality [2]. Epithelial-mesenchymal transition (EMT), or the morphological transformation from epithelial cells to mesenchymal cells, is believed to be the major cause of cancer metastasis [3]. The development of EMT is controlled by a complicated network that consists of diverse pathways including TGF-β, Notch, and Wnt [4]. TGF-β is considered the dominant regulator of EMT [5]. After the activation of TGF-β receptors on the cellular membrane, receptor-regulated SMADs (R-SMADs) bind to SMAD4 to comprise SMAD complexes [6]. SMAD4 is essential to the translocation of R-SMADs across the nuclear membrane and is the activation of EMT-related transcription factors [7]. This fact was confirmed previously by suppressing SMAD4 activity, which led to the inhibition of EMT [8].

Non-coding RNAs known as micro-RNAs (miRNAs) regulate the translation and induce the degradation of diverse mRNAs by binding to their 3' UTR. Thousands of miRNAs have been discovered in recent years, and the assorted regulatory effects of miRNAs on cancer have been described [9]. Multiple miRNAs were confirmed to regulate EMT in PCa, including miR-1, 29, 34, and 203 [10].

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) reversibly change the status of histones and ultimately adjust the expression of genes relevant to cancer development [11]. Inhibition of HDACs was confirmed to be a therapeutic approach to cancer [12]. Valproic acid (VPA), a clinically applied anticonvulsant drug, is considered an HDAC inhibitor [13] and been proven to inhibit PCa metastasis by suppressing the invasion and migration of PCa cells [14, 15]. We previously found that VPA down-regulates protein levels of SMAD4 [16]. In view of the interaction between VPA and miRNAs reported previously [17, 18], we believe that there may be miRNAs that mediate the SMAD4-inhibiting effect of VPA. In this study, we searched for miRNAs that could potentially

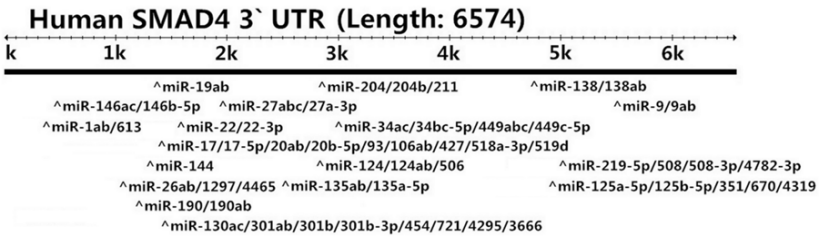

Figure 1. Search results from www.targetscan.org.

be involved in the interplay between SMAD4 and VPA and then tested the correlation between them.

Materials and methods

Cell culture and reagents

The LNCaP and PC3 cell lines (Chinese Academy of Science) were maintained in RPMI-1640 medium (Thermo Fisher Scientific Inc., Waltham, MA) mixed with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Cells were treated with VPA (Sigma Chemical Co., St. Louis, MO) at a concentration of 2.4 mmol/l. SMAD4 antibody (sc-7966, 1:1000 dilution) and β-actin antibody (sc-130301, 1:1000 dilution) were obtained from Santa Cruz Biotechnology (Dallas, TX).

The search for relevant miRNAs

First, we found miRNAs that could complementarily bind to the 3' UTR of SMAD4 mRNA using www.targetscan.com. Second, miRNAs that have been proven to be related to VPA were identified using a text-mining method. We searched PUBMED using the keywords "VPA and (miR or miRNA or microRNA)" and selected the valid literature that described the direct or indirect relationship between VPA and any one of the miRNAs. The overlaps between miRNAs that could bind to SMAD4 mRNA and miRNAs related to VPA were considered relevant miRNAs in our study.

Transfection of pre-miRNAs and inhibitors

Pre-miRNAs and inhibitors of miR-20a, 34a, and 449a were purchased from Genetimes Technology, Inc. (Shanghai, China) and were separately transfected into PC3 cells using Lipofectamine-2000 Transfection Reagent (Life Technologies, Carlsbad, CA).

Western blot analysis

Cells were lysed in RIPA buffer. Extracts were subjected to electrophoresis and membrane transfer. Next, the membranes were consecutively incubated with SMAD4 antibody overnight and secondary antibodies for one hour. Afterwards, Chemiluminescent HRP Substrate (Millipore, Billerica, MA) was used to visualize the proteins using a LAS-4000 Luminescent Image Analyzer (Fujifilm, Tokyo, Japan).

Quantitative RT-PCR

The mRNA levels of SMAD4 and miR-20a, 34a, 124a, 144, and 449a were measured by qRT-PCR using a Real Time (qPCR) Kit (TaKaRa Biotechnology Co., Tokyo, Japan). Primers of SMAD4, GAPDH, U6, and miR-20a, 34a, 124a, 144, and 449a were purchased from Tiangen Biotech Co. (Beijing, China). The levels of SMAD4 mRNA and miRNAs were shown relative to the levels of GADPH and U6.

Statistical analysis

The results are presented as the mean ± standard error (SE). The statistical significance was determined by Student's t test (2-tailed) or the Mann-Whitney U test (2-tailed). $P<0.05$ was considered statistically significant.

Results

Search results

Analysis using www.targetscan.com revealed that tens of miRNAs could bind to the 3' UTR of SMAD4 mRNA (**Figure 1**). Simultaneously, seven articles relevant to the relationship between VPA and miRNA were found in PUBMED. After the main text of each article was reviewed, miRNAs and relevant data were extracted and summarized in **Table 1**. Seven articles reported tens of miRNAs regulated by VPA respectively in human, mice, rats, and hippocampi cells [17-23]. MiRNAs were listed and categorized in the table according to the effects of VPA. Accordingly, five miRNAs (miR-20a, 34a, 124a, 144, and 449a), which are shown both in

Table 1. MiRNAs regulated by VPA

Species	Expression	miRNAs	References
Human	Up-regulation	miR-129, miR-134, miR-182, miR-194, miR-214, miR-221, miR-449a, and miR-519e	[17-19]
	Down-regulation	miR-15a, miR-16, miR-30a-5p, miR-92a-1, miR-144, miR-222, and miR-451	[17, 18, 20]
Mice	Up-regulation	miR-10a, miR-143, miR-145, miR-199a, miR-206, and miR-214	[21]
	Down-regulation	miR-124a, miR-128a, miR-137, miR-383, and miR-491	[21]
Rats	Up-regulation	miR-331	[22]
	Down-regulation	miR-34a and miR-885-3p	[22, 23]
Hippocampi	Up-regulation	miR-15a, miR-20a, miR-144, miR-376a, miR-465, and miR-518b	[23]
	Down-regulation	let-7b, let-7c, miR-23b, miR-24, miR-30c, miR-34a, miR-105, miR-127, miR-128a, miR-143, miR-181a, miR-188, miR-198, miR-216, miR-221, miR-302bc, and miR-410	[23]

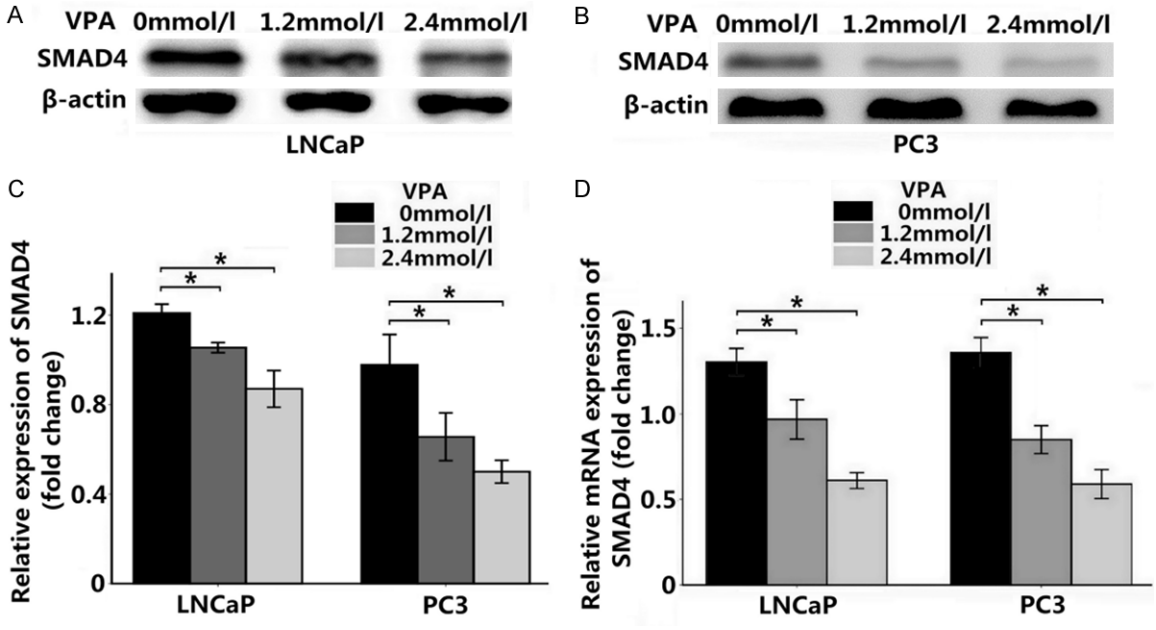

Figure 2. Levels of SMAD4 protein and mRNA after VPA treatment. A and B. Treatment with 1.2 or 2.4 mmol/l VPA for 48 hours decreased SMAD4 expression in LNCaP cells and PC3 cells. C. VPA significantly down-regulated protein levels of SMAD4 in a concentration-dependent manner. D. qRT-PCR revealed that VPA significantly down-regulated mRNA levels of SMAD4 in a concentration-dependent manner. (*P<0.05).

the **Figure 1** and **Table 1**, were included as the target miRNAs in our study.

VPA inhibits the expression of SMAD4

SMAD4 mRNA and protein levels in LNCaP and PC3 cells were analyzed after exposure to VPA. Treatment with 1.2 and 2.4 mmol/l VPA reduced SMAD4 mRNA and protein levels in both cell lines (**Figure 2**), which is consistent with its expression-restraining effect on SMAD4.

VPA up-regulates miR-20a, 34a, and 449a

After VPA treatment, the levels of miR-20a, 34a, 124a, 144, and 449a in both PC3 and LNCaP cells were analyzed. VPA induced the up-regulation of miR-20a, 34a, and 449a in a concentration-dependent manner, whereas the levels of miR-124a and 144 were not significantly altered (**Figure 3**).

MiR-34a inhibits the expression of SMAD4

The transfection of pre-miRNAs of miR-20a or 449a increased the protein levels of SMAD4 in both PC3 and LNCaP cells, whereas the transfection of miR-20a or miR-449a inhibitors led to a decrease in the expression of SMAD4 in PC3 cells. In contrast, up-regulation of miR-34a by the transfection of pre-miRNAs of miR-34a significantly suppressed the expression of SMAD4 in LNCaP and PC3 cells, and the transfection of miR-34a inhibitors induced significantly higher protein levels of SMAD4 in both cell lines (**Figure 4**).

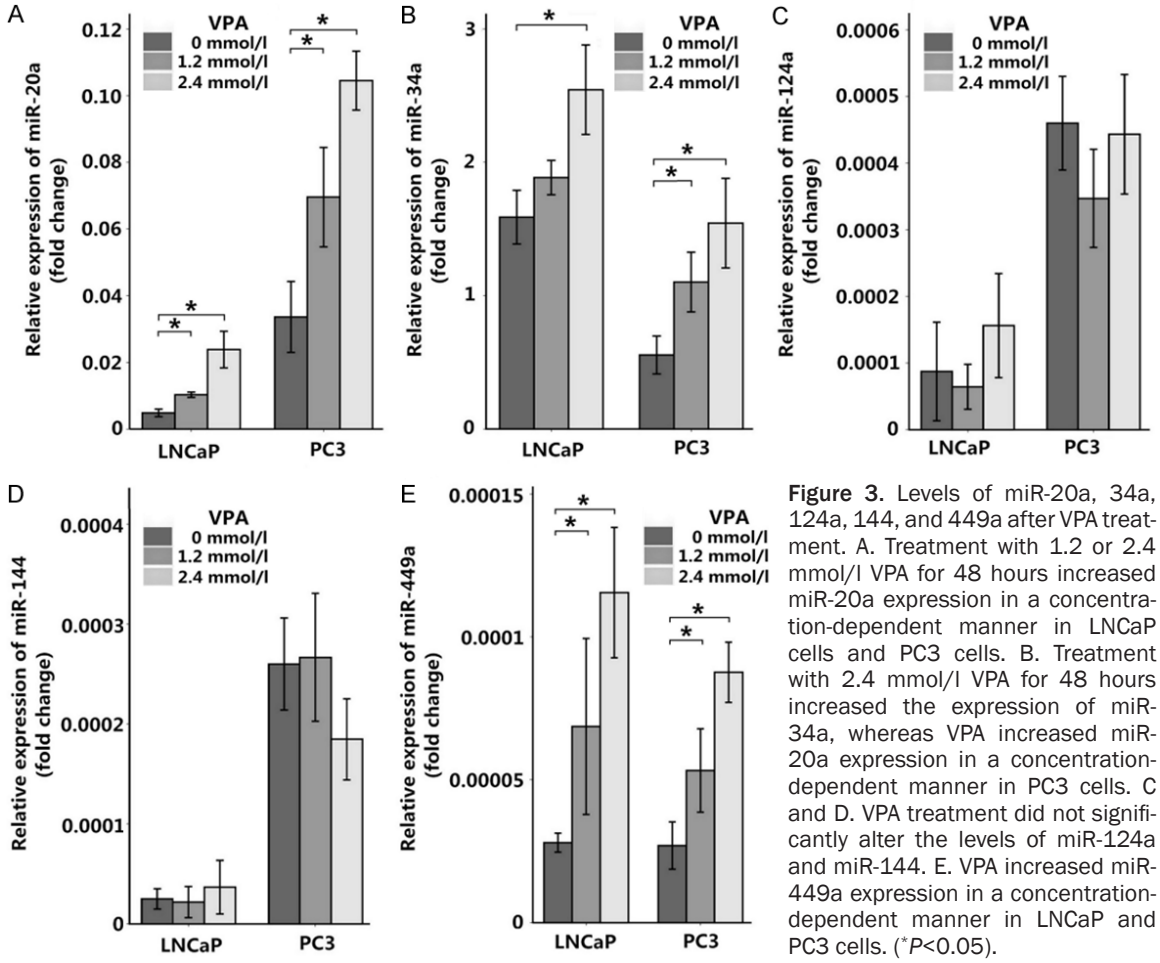

Figure 3. Levels of miR-20a, 34a, 124a, 144, and 449a after VPA treatment. A. Treatment with 1.2 or 2.4 mmol/l VPA for 48 hours increased miR-20a expression in a concentration-dependent manner in LNCaP cells and PC3 cells. B. Treatment with 2.4 mmol/l VPA for 48 hours increased the expression of miR-34a, whereas VPA increased miR-20a expression in a concentration-dependent manner in PC3 cells. C and D. VPA treatment did not significantly alter the levels of miR-124a and miR-144. E. VPA increased miR-449a expression in a concentration-dependent manner in LNCaP and PC3 cells. (*P<0.05).

Down-regulation of miR-34a eliminates the SMAD4-inhibiting effect of VPA

VPA has been shown to inhibit the expression of SMAD4. However, the transfection of miR-34a inhibitors restored SMAD4 expression in VPA-treated cells and eliminated the inhibitory effect of VPA on SMAD4 expression in LNCaP and PC3 cells (**Figure 5**).

Discussion

Thousands of miRNAs have been studied for their regulatory effects on tumorigenesis, cancer cell proliferation and other physiological processes [24]. Metastasis is an important process regulated by many miRNAs, including miR-9 and miR-21 that promote metastasis and the let-7 family that has an inhibitory effect [25]. EMT plays central roles in the regulation of cancer metastasis [26]. Several studies have focused on the link between the miR-200 family and EMT. Park and colleagues proved that the miR-200 family, including miR-200a, 200b, 200c, and 141, are key inhibitors of EMT that target ZEB1/2 [27]. MiR-1, 15b, and 205 were also confirmed to reverse EMT by interacting with p53, BMI1, SIP1, Slug, and ZEB1/2 [28-30].

MiR-34a was reported to inhibit metastasis of PCa [31] and other types of cancers [32, 33]. Du and colleagues investigated the regulation of EMT by miR-34a and reported that miR-34a suppresses EMT in tubular epithelial cells by targeting Notch1 and Jagged1 [34]. ZEB1, a transcription factor that induces the mesenchymal property of cancer cells, is believed to promote EMT by inhibiting miR-34a expression. Consequently, we concluded that miR-34a may be the mechanism underlying several of molecular events relevant to EMT [35]. The search results in our study indicated the possibility that miR-34a binds to the mRNA of EMT-promoting SMAD4, which was confirmed by a previous study showing that miR-34a modu-

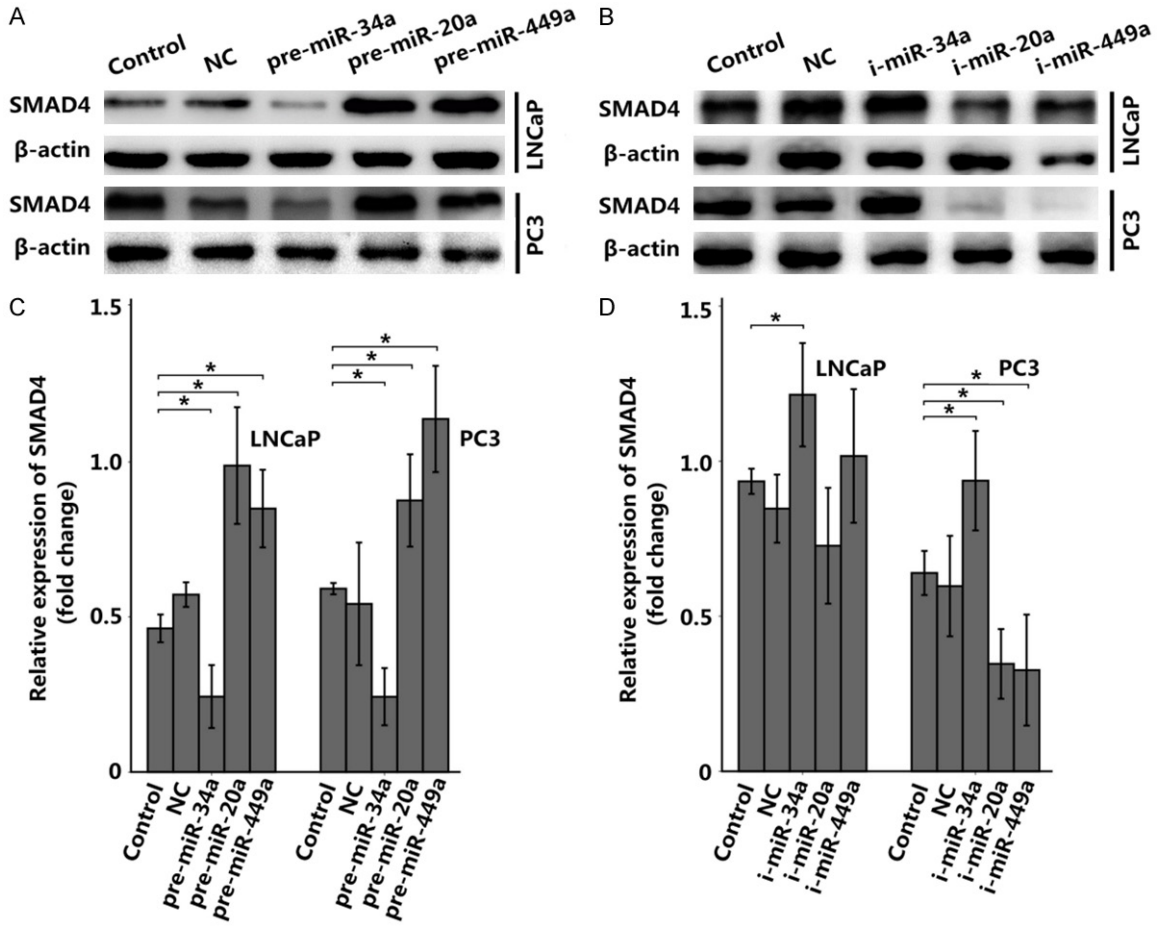

Figure 4. MiR-34a inhibits the expression of SMAD4. A. The transfection of pre-miR-34a decreased the expression of SMAD4, whereas the transfection of miR-20a or miR-449a increased the expression of SMAD4 in LNCaP and PC3 cells. B. The transfection of miR-34a inhibitors (i-miR-34a) increased the expression of SMAD4, whereas the transfection of miR-20a or miR-449a inhibitors decreased the expression of SMAD4 in LNCaP and PC3 cells. C. The change in SMAD4 expression after the transfection of pre-miR-34a, pre-miR-20a, and pre-miR-449a was significant in LNCaP and PC3 cells. D. The change in SMAD4 expression after the transfection of miR-34a inhibitors (i-miR-34a) in LNCaP cells and after the transfection of miR-34a inhibitors (i-miR-34a), miR-20a inhibitors (i-miR-20a), and miR-449a inhibitors (i-miR-449a) in PC3 cells was also significant. (*P<0.05) "Control" represents the group that received no treatment. "NC" (negative control) represents the group that cells were transfected with empty plasmids.

lates cardiac fibrosis by binding to SMAD4 mRNA [36].

As shown in the previous study, VPA inhibits the migration and invasion of PCa cells [15, 16, 37]. The E-cadherin-promoting effect of VPA in endometrial cancer cells was reported by Takai and colleagues [38]. Subsequently, additional evidence has emerged indicating that VPA increases the expression of epithelial markers and decreases the expression of mesenchymal markers, such as Vimentin and N-cadherin, in different cancers [39, 40]. However, the pathways or molecules that contribute to the EMT-inhibiting effect of VPA are still not fully described. Our study found that VPA down-regulates both the mRNA and protein levels of SMAD4 in a concentration-dependent manner in LNCaP and PC3 cells. The expression of miR-34a, which is up-regulated by VPA, is inversely correlated with the expression of SMAD4. Up-regulation of miR-34a mimicked the SMAD4-inhibiting effect of VPA, whereas down-regulation of miR-34a eliminated this effect of VPA in LNCaP and PC3 cells. These results convince us that VPA inhibits SMAD4 expression by up-regulating miR-34a and provide a basis for further studies on the pharmacological mechanism and rationalized utilization of VPA.

MiR-20a, an established oncogenic miRNA, is up-regulated in cancer tissues and promotes

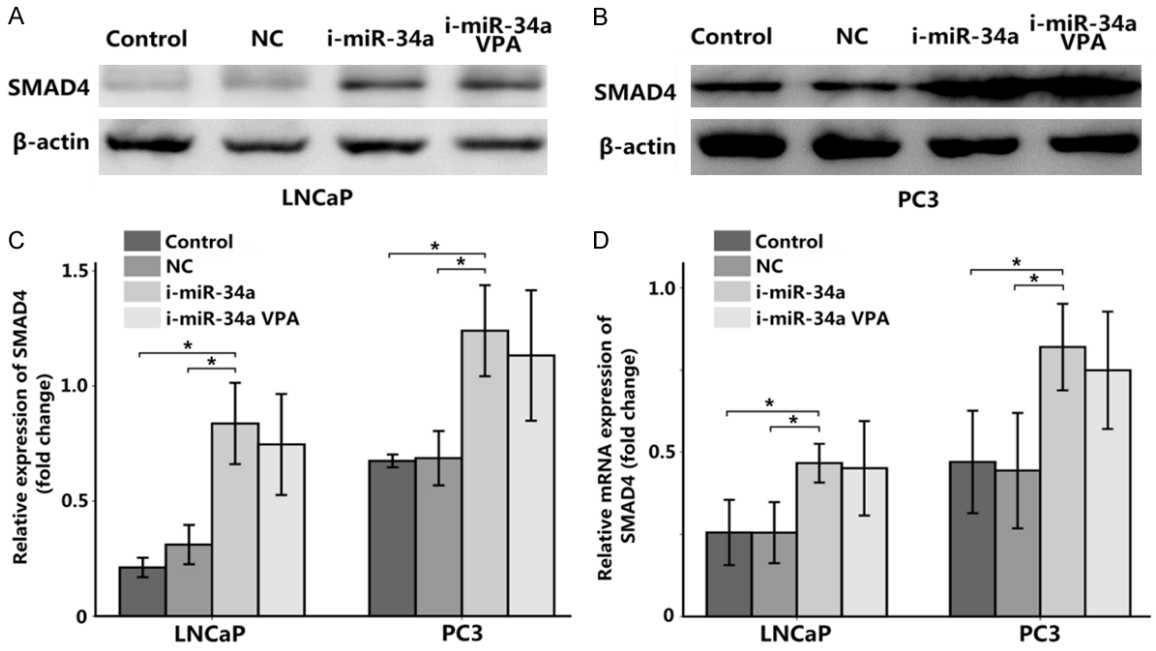

Figure 5. Down-regulation of miR-34a eliminates the SMAD4-inhibiting effect of VPA. A and B. The transfection of miR-34a inhibitors (i-miR-34a) increased the protein level of SMAD4, and additional treatment of VPA induced no significant alterations in either LNCaP or PC3 cells. C and D. The transfection of miR-34a inhibitors (i-miR-34a) combined with VPA treatment did not significantly alter the protein or mRNA levels of SMAD4 in LNCaP and PC3 cells. (*P<0.05). "Control" represents the group that received no treatment. "NC" (negative control) represents the group that cells were transfected with empty plasmids.

the metastasis of gallbladder carcinoma, cervical carcinoma, and PCa [41-43]. Chang and colleagues reported that the up-regulation of miR-20a in gallbladder carcinoma cells using miR-20a mimics or miR-20a-expressing lentivirus significantly increased the levels of N-cadherin and Vimentin while decreasing the levels of E-cadherin, thereby confirming the EMT-promoting effect of miR-20a [42]. Jiang and colleagues reported a contradictory result, showing that over-expression of miR-20a in non-small cell lung cancer cells inhibits migration and EMT [44]. These findings may indicate that there are differential expression levels and regulatory effects between cancers. MiR-449a, which acts as a tumor suppressor, was reported to inhibit the metastasis of non-small cell lung cancer [45] and suppress the proliferation of PCa by targeting HDAC-1 [46]. In our study, the transfection of pre-miR-20a or pre-miR-449a induced the up-regulation of SMAD4 protein levels, which may mainly be due to indirect effects. VPA-induced up-regulation of miR-34a decreased the expression of SMAD4 and concealed the effect of miR-20a and miR-449a on SMAD4. This result is also evidence confirming that miR-34a is a crucial regulator in the process by which VPA inhibits SMAD4 expression. The up-regulated expression of the anti-cancer miRNA miR-449a after VPA treatment implies that miR-449a may participate in other effects of VPA, providing us potential prospects for further research.

In conclusion, we found that VPA up-regulates the expression of miR-20a, 34a, and 449a in LNCaP and PC3 cells. In addition, VPA inhibits the expression of SMAD4 by up-regulating the expression of miR-34a.

Disclosure of conflict of interest

None.

Address correspondence to: Qinghua Xia, Minimally Invasive Urology Center, Provincial Hospital Affiliated to Shandong University, Shandong, China. E-mail: alanhenry14@126.com

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