

Differential Retention of α -Vitamin E Is Correlated With Its Transporter Gene Expression and Growth Inhibition Efficacy in Prostate Cancer Cells

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BACKGROUND. Epidemiological studies showed Vit E has protective effects against prostate cancer (PCa). Interestingly, different prostate cancer cells have different sensitivity to α -Vit E or VES treatment. The goal of this study is to determine whether cellular Vit E bioavailability and its transport proteins are important contributing factors.

METHODS. α -Vit E and its ester form, VES, were used to treat prostate cancer LNCaP, PC3, and DU145 cells, and their growth rates were determined by MTT assay. Cellular levels of Vit E were quantified using HPLC as the index of bioavailability. The expression levels of Vit E transport proteins were determined by real-time PCR.

RESULTS. Among these PCa cells, only LNCaP cells were sensitive to 20 μ M α -Vit E treatment, while both LNCaP and PC3 cells were sensitive to 20 μ M VES treatment. Coordinately, cellular levels of α -Vit E and VES positively correlated to their inhibitory effects. Further study found expression levels of Vit E transport proteins, including tocopherol associated protein (TAP), scavenger receptor class B type I (SR-BI), α -tocopherol transfer protein (TTP), and ATP binding cassette transporter A1 (ABCA1), were different in various PCa cells, which may contribute to cellular Vit E bioavailability. This notion is further supported by the findings that over-expression or knockdown of TTP could coordinately alter cellular α -Vit E levels in PCa cells.

CONCLUSION. Antiproliferative efficacy of α -Vit E is correlated with its cellular bioavailability in PCa cells. Modulating the expression of the efflux or influx transporters could sensitize the growth inhibition efficacy of Vit E in prostate cancer cells. *Prostate* © 2007 Wiley-Liss, Inc.

KEY WORDS: vitamin E succinate; prostate cancer; TAP; TTP; ABCA1; SR-BI

INTRODUCTION

Prostate cancer is the second leading fatal cancer in American men [1]. While initially androgen dependent and responsive to androgen ablation therapy, prostate cancer will eventually relapse and become androgen-independent. There is no cure for patients with androgen-independent prostate cancer [2].

Epidemiological studies show that daily supplementation of Vit E may reduce the incidence and mortality of prostate cancer [3–5]. The Vit E family contains eight members, α -, β -, γ -, δ -tocopherol and α -, β -, γ -, δ -tocotrienol. Among them, α -tocopherol (α -Vit E) is the most bioactive form in the body, making up more than 90% of Vit E in mammalian tissues [6]. Because Vit E is structurally unstable and easily oxidized, some Vit E derivatives have been synthesized

to increase stability, including VES. VES is one of the most effective Vit E analogs to suppress prostate cancer cells in vitro. Moreover, VES selectively inhibits the growth of cancer cells, but not non-malignant cells [7]. We previously showed that VES suppresses prostate

Abbreviations: PCa, prostate cancer; α -Vit E, α -tocopherol; VES, α -vitamin E succinate, α -tocopherol succinate; TAP, α -tocopherol associated protein; ABCA1, ATP binding cassette transporter A1; TTP, α -tocopherol transfer protein; SR-BI, scavenger receptor class B type I; HPLC, high performance liquid chromatography.

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cancer LNCaP cells, but not primary-cultured prostate fibroblasts [8]. VES suppresses prostate cancer cell growth via multiple mechanisms, including down-regulation of the AR/PSA signaling pathway [8], blocking cell cycle progression [9,10], suppressing invasion [11], and inducing apoptosis [12,13].

Interestingly, α -Vit E analog suppresses different prostate cancer cells with different efficacy. We hypothesize this may be, at least partly, due to differential accumulation of Vit E in prostate cancer cells.

Although Vit E is fat-soluble, its uptake into the cells is not just through passive transport. Instead, several Vit E-binding proteins/transport proteins mediate Vit E uptake and efflux in vivo and in vitro [14]. For example, α -tocopherol transfer protein (TTP) can selectively bind and release α -Vit E from the liver into the serum. Deficiency of TTP will increase Vit E accumulation in the liver and deplete α -Vit E from peripheral tissue in mice [15]. Functional loss of TTP can result in ataxia with Vit E deficiency (AVED) in humans [16]. α -Tocopherol associated protein (TAP), another α -Vit E-binding protein, mediates α -, γ -, δ -Vit E, and VES uptake into the prostate cancer cells to facilitate Vit E anti-proliferation function [17].

In this study, we mainly focused on comparing the retention of α -Vit E and VES in prostate cancer cells. Our results suggest that VES had much better anti-proliferation effects on prostate cancer cells than α -Vit E. Moreover, various prostate cancer cells have varying sensitivity to α -Vit E and VES treatment. We found that a greater amount of cellular Vit E is correlated with Vit E anti-proliferative activity, and differential expression of Vit E transported genes in prostate cancer cells may contribute to their different cellular Vit E amounts. Therefore, targeting on the expression of Vit E-binding protein is another strategy for facilitating the chemopreventive and chemotherapeutic effects of α -Vit E analog on prostate cancer.

MATERIALS AND METHODS

Chemicals and Reagents

α -, δ - tocopherol, VES, and ascorbic acid (vitamin C) were purchased from Sigma.

Cell Culture and Culture Condition

The LNCaP, PC3, and DU145 cells were obtained from the American Type Culture Collection (Manassas, VA). The LNCaP and PC3 cells were maintained in RPMI 1640 medium supplemented with 8% fetal bovine serum (FBS) and 100 μ g/ml penicillin-streptomycin. The DU145 cells were maintained in DMEM medium supplemented with 8% fetal bovine serum

(FBS) and 100 μ g/ml penicillin-streptomycin. All cells were incubated in a humidified atmosphere of 5% CO₂ in air at 37°C.

MTT Assay

The cells were seeded into 12- or 24-well plates. Thirty-six hours later, the cells were treated with α -Vit E or VES. At the indicated time, 0.5 ml of MTT (0.5 mg/ml) was added. After 3 hr incubation at 37°C, 1 ml 0.04 M HCl in isopropyl alcohol was added to each well. Absorbency was read at a wavelength of 595 nm. The experiment was performed in quadruplicate.

Extraction and HPLC Analysis

After α -Vit E or VES treatment, the cells were harvested and counted. Cell pellets were lysed with 0.3 ml 1% ascorbate in 0.1 M SDS plus internal standard 1 nmol δ -tocopherol, followed by 0.4 ml ethanol, then frozen, and thawed four times. Vit E isoforms were extracted with 0.8 ml hexane. The hexane extract was taken to dryness under N₂ in a TurboVap[®]LV concentration workstation (Zymark, MA). The residue was dissolved in 2.5% ascorbate in methanol (1 ml) and analyzed (50 μ l) by HPLC.

Cellular VES concentrations were determined by measuring α -Vit E concentrations before and after base hydrolysis. The cell extractions were divided into two equal portions. The second portion was hydrolyzed using 0.2 ml of 4 M KOH overnight, then neutralized with 0.2 ml 4 M HCl, and extracted with 0.8 ml hexane. The hexane extract was taken to dryness under N₂, the residue dissolved in 2.5% ascorbate in methanol and analyzed (50 μ l) by high-performance liquid chromatography (HPLC) [18].

Measurements of α -Vit E were made on a C¹⁸ column eluted with MeOH: H₂O 96:4 (V/V) at a flow rate of 1.8 ml/min. Signal was detected by Waters 2475 multi λ fluorescence detector (Waters). The data were analyzed by Empower software (Waters).

Real Time Quantitative RT-PCR

According to the manufacturer's instructions, total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA), 2 μ g RNA was then subjected to reverse transcription using Superscript III (Invitrogen). The real-time PCR was performed with SYBR Green PCR Master Mix (Biorad, Hercules, CA). Specific primers (Table I) were designed according to Beacon Designer software.

PCR was performed at 94°C for 3 min, and 40 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30–40 sec on an iCycler iQ Multi-color real-time PCR detection system (Biorad). Each sample was run in triplicate. Data were analyzed using iCycler iQ software (Biorad).

TABLE I. The Primers of Vit E Transport Proteins for Real-Time PCR

Gene symbol	GenBank number	Forward primer (5'-3')	Reverse primer (5'-3')
ABCA1	AB055982	tctgaagccaatcctgagaac	cctcctgtcgcattgctactc
SR-BI1	NM_005505	ggtgcggcggtgatgatg	cccagagtcggagttgtgag
TAP	NM_012429	agtctcgggagaatgtccaggatg	cactcaggaagggtttgatgaggt
TTP	D49488	tcagcggaaatggaatcaagg	atccgtaagtacagcagcaatc
36B4	NM_007475	tacacctcccacttactg	gattctccgactcttcc

Construction of TTP cDNA and siRNA Expression Plasmids

Full length TTP was isolated from cDNA of prostate cancer DU145 cells and cloned into pGEM-T vector and then subcloned into the *Kpn I* and *Xba I* of the pCDNA4-HisMax (Invitrogen). TTP siRNA was constructed using a DNA-based vector pSuperior-retro-puro (OligoEngine, WA) that contained the puromycin resistance marker. The oligonucleotides containing the siRNA sequences targeting hTTP, GTGGCATCCA-TTTGATAAAA, or GGAACGGATTCACATGCAT, was subcloned into the *BglIII*–*HindIII* site of pSuperior-retro-puro vector to generate TTPsi593 and TTPsi675, respectively. All constructions were verified by sequencing.

Transfections

Full length hTTP plasmid was transfected into LNCaP cells using an efficient electroporation system that yielded more than 80% infection [17,19]. TTPsiRNA were transduced into DU145 cells by retroviral system.

RESULTS

α -Vit E and VES Inhibit PCa Cell Growth

As an initial attempt to compare the effect of α -Vit E on prostate cancer cells with VES, we treated prostate cancer LNCaP, PC3, and DU145 cells with α -Vit E or VES at 20 μ M, the physiological concentration of α -Vit E in human serum [20]. Treatment with 20 μ M α -Vit E yielded growth inhibition of AR-positive LNCaP cells by 14% within 6 days, but no inhibition of AR-negative PC3 and DU145 cells. In contrast, 20 μ M VES showed significant anti-proliferative activity on LNCaP cells and PC3 cells up to 55%, but no inhibition on DU145 cells. These data suggested that VES has better anti-proliferation effects than α -Vit E. DU145 cells are insensitive to 20 μ M α -Vit E and VES treatment.

The Differential Retention of α -Vit E and VES in Cultured PCa Cells

To test if the higher efficacy of α -Vit E in LNCaP cells is due to its greater bioavailability, we assessed the

kinetics of Vit E uptake by prostate cancer LNCaP, PC3, and DU145 cells. As shown in Figure 2A, within 48 hr, α -Vit E can be accumulated in LNCaP cells at least 50% more than that in PC3 and DU145 cells. These data indicate that the higher cellular retention ability of α -Vit E is associated with increased α -Vit E-mediated growth inhibition in LNCaP cells.

Next, we examined cellular VES levels after the cells were exposed to 20 μ M VES for 12, 24, and 48 hr. As shown in Figure 2B, within 48 hr, cellular VES concentrations were approximately 10 nmol/10⁶ cells in LNCaP and PC3 cells, as opposed to ~4 nmol/10⁶ cells in DU145 cells. These data correlated well with the observation that 20 μ M VES significantly suppressed the growth of LNCaP and PC3 cells, but not DU145 cells (Fig. 1), suggesting higher cellular retention ability of VES might be associated with the increased VES sensitivity in LNCaP and PC3 cells.

VES can be hydrolyzed to α -Vit E by esterase, which could reduce VES's growth inhibitory activity (Fig. 1). However, the efficiency of VES hydrolysis in PCa cells remains unknown. Here, we also measured the total Vit E (VES + α -Vit E) amount. As shown in Figure 2C, after VES treatment for 48 hr, total Vit E amount was ~10 nmol/10⁶ cells in both LNCaP and PC3 cells, whereas it was only ~5 nmol/10⁶ cells in DU145 cells, suggesting that DU145 cells retain much less total Vit E. We then calculated the ratio between α -Vit E and total Vit E to reflect the VES hydrolysis rate. In LNCaP and PC3 cells, 7–10% of VES can be converted to α -Vit E, while in DU145 cells, the rate was 15–23% (Fig. 3D). Uptake, efflux, and metabolism of VES will determine the final cellular VES levels. The high VES hydrolysis rate in DU145 cells can contribute to the low cellular VES levels. Furthermore, amount of total Vit E (VES + α -Vit E) in DU145 cells is much lower than that in LNCaP and PC3 cells, suggesting that low uptake or/and high efflux process may be the main factors for the low cellular VES amount in DU145 cells. Taken together, low accumulation of cellular VES in DU145 cells may be one of the reasons that DU145 cells are insensitive or less sensitive to 20 μ M VES.

To determine whether DU145 cells are sensitive to higher dose of VES treatment, we evaluated the IC₅₀ of VES in prostate cancer cells. As shown in Figure 3, VES

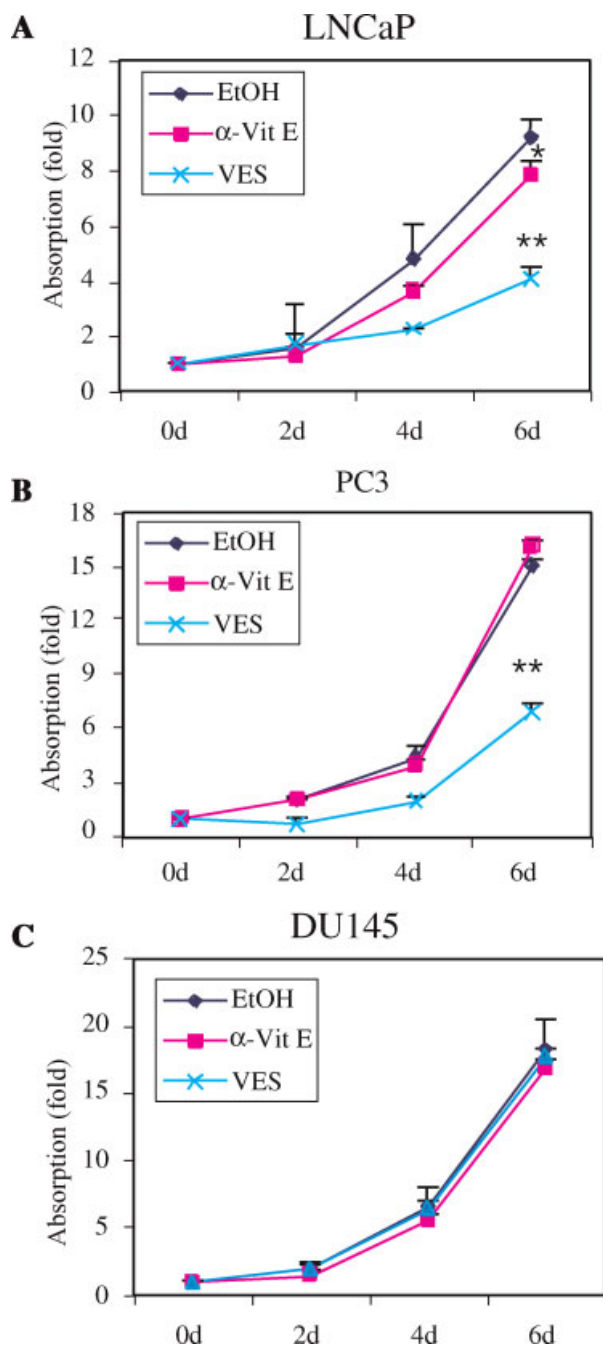


Fig. 1. The growth effect of 20 μ M α -Vit E and 20 μ M VES on PCa cells. The PCa LNCaP (A), PC3 (B), and DU145 (C) cells were treated with α -Vit E (20 μ M) or VES (20 μ M) for the indicated time periods. Cell growth was determined by MT Tassay. The data of Day 0 was set as 1. The data were represented as mean value \pm SD of triplicate samples. *, $P < 0.05$, and **, $P < 0.01$.

inhibited prostate cancer cell growth with IC₅₀ 20 μ M for both LNCaP cells and PC3 cells, and 50 μ M for DU145 cells, indicating a 2.5-fold difference in the dose of IC₅₀. Further study found cellular VES levels reached 17 nmol/10⁶ cells inside the DU145 cells after

50 μ M VES treatment (Fig. 3D), but 4 μ mol/10⁶ cells after 20 μ M VES treatment. There is 4-fold increase of the cellular VES levels when using 2.5-fold higher dose to treat cells. It appears that the intracellular VES levels is a better indicator for VES anti-proliferative effects.

Taken together, the efficacy of α -Vit E or VES among different prostate cancer cells may be, at least partly, dependent on cellular α -Vit E or VES bioavailability.

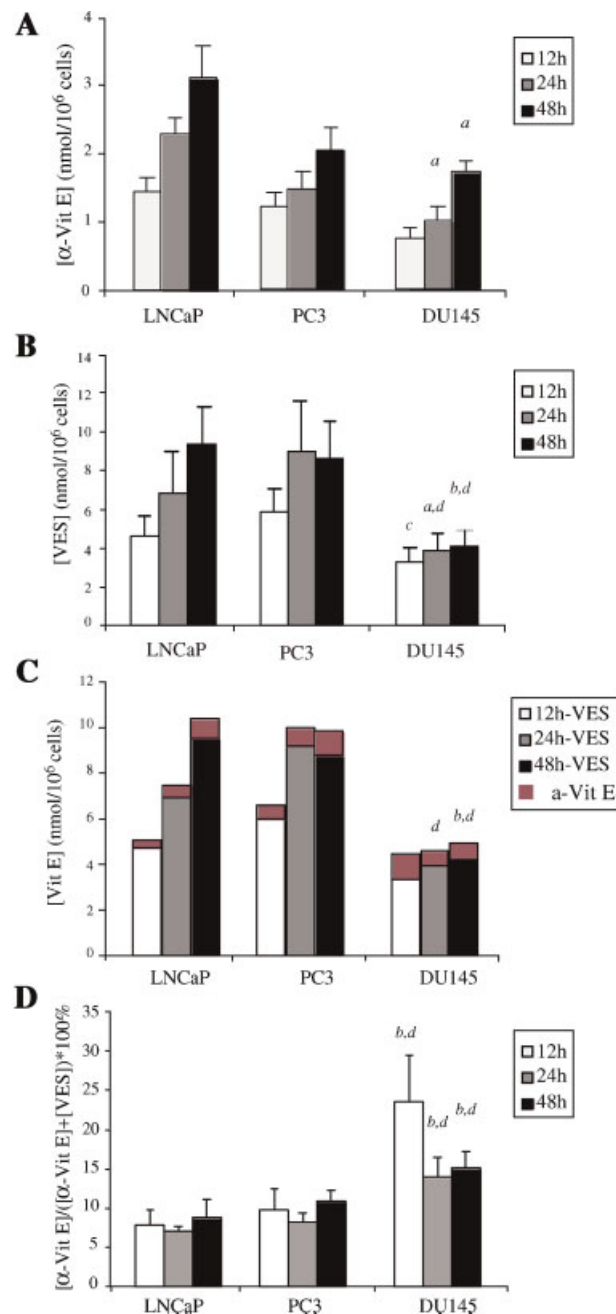


Fig. 2.

Differential Expression Levels of Vit E Transporter Genes in PCa Cells

Vit E is a fat-soluble nutrient, however, its transport into the cells is not solely via a passive process. One of the potential mechanisms for the differential retention of Vit E in various prostate cancer cells may be due to different expression levels of Vit E transporters with varying capacity to transport Vit E. It has been reported that TTP and lipid efflux gene ATP binding cassette transporter A1 (*ABCA1*) can transfer the α -Vit E out of the cells [15,21,22], while TAP and high density lipoprotein receptor class B scavenger receptor (SR-BI), which mainly mediates selective cholesterol uptake, may facilitate α -Vit E uptake inside the cells [17,23,24]. Furthermore, we also found VES uptake in the PCa cells can be mediated by TAP [17]. Thus, we first compared these genes' mRNA expression in different PCa cell lines. As shown in Figure 4A, TAP had the highest expression in PC3 cells. The expression of SR-BI was similar in LNCaP, PC3, and DU145 cells (Fig. 4B). On the other hand, Vit E efflux protein TTP had the highest expression in DU145 cells, modest expression in PC3 cells, and least expression in LNCaP cells (Fig. 4C). *ABCA1* expression levels in DU145 and PC3 cells were similar, and much higher than that in LNCaP cells (Fig. 4D). These data suggested that high ability to efflux with low ability to uptake Vit E may be the major reason why DU145 cells have such low retention of Vit E. Similarly, it is likely that high efflux with high uptake of Vit E in PC3 cells and low efflux with low uptake of Vit E in LNCaP cells may balance the retention of vitamin E which contributes to their relatively high accumulations of Vit E.

Fig. 2. The cellular concentrations of Vit E/VES in PCa cells. **A:** The content of α -Vit E in PCa cells after α -Vit E treatment. LNCaP, PC3, and DU145 cells were treated with 20 μ M α -Vit E. Cells were harvested and counted at the indicated time point. Cellular α -Vit E was extracted with hexane and detected by HPLC. The data were represented as the mean values \pm SEM of at least five independent experiments. Statistics *P* value is compared to the data of LNCaP cells at the same time point. *, *P* < 0.05. The concentrations of VES (**B**), total Vit E (**C**), and cellular VES hydrolysis rate (**D**) in PCa cells. LNCaP, PC3, and DU145 were treated with 20 μ M VES. Cells were harvested at the indicated time point. Cellular VES concentrations were determined by measuring the amount of α -Vit E in cellular extracts before and after base hydrolysis. Cellular VES hydrolysis rate is calculated by the ratio between cellular α -Vit E to total Vit E (VES + α -Vit E). The data were represented as the mean values \pm SD of at least four independent experiments. *a*, *b* are the statistical *P* value by which the data of DU145 cells is compared with the data of LNCaP cells at the same time point. *a* < 0.05; *b* < 0.01. *c*, *d* is the statistical *P* value by which the data of DU145 cells is compared with the data of PC3 cells at the same time point: *c* < 0.05; *d* < 0.01.

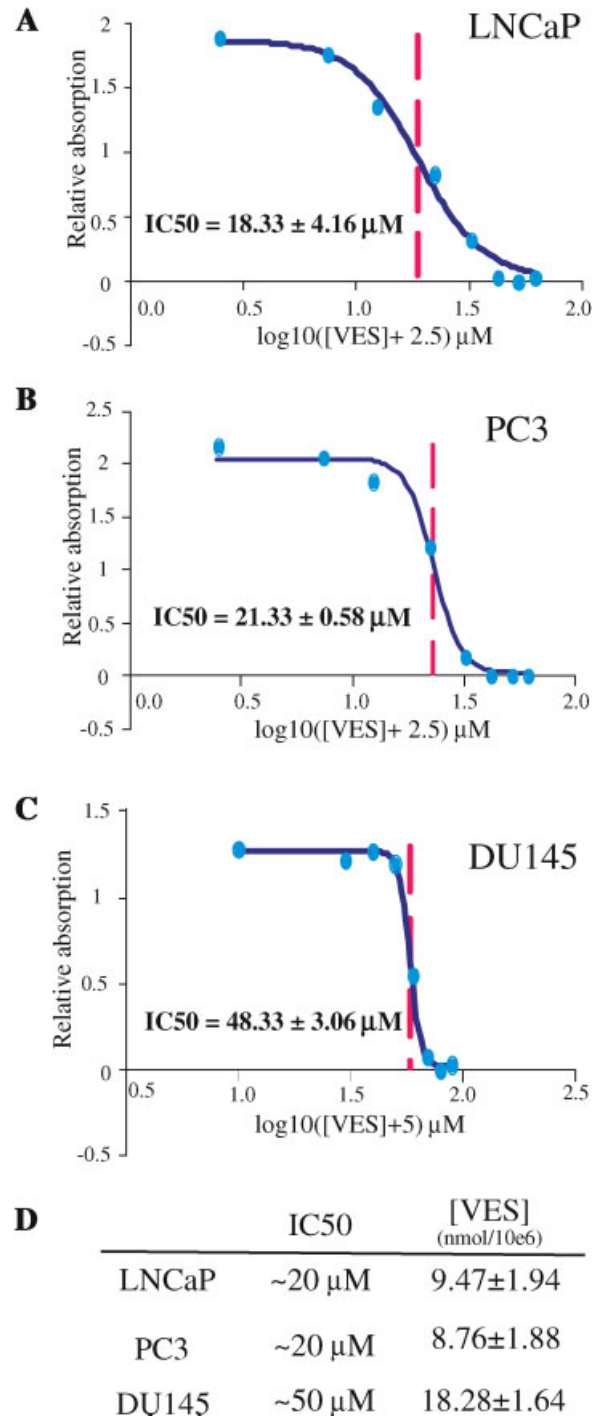


Fig. 3. IC₅₀ and cellular VES amount in PCa cells. VES IC₅₀ in PCa cells. The PCa LNCaP (**A**), PC3 (**B**), and DU145 (**C**) cells were treated with 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μ M VES for 6 days. Cell growth was determined by MTT assay. The diagram represented one set of experiments. The IC₅₀ were represented as mean value \pm SD of at least three independent experiments. **D:** The cellular VES amount after VES IC₅₀ treatment for 2 days in PCa cells. The data is mean values \pm SD.

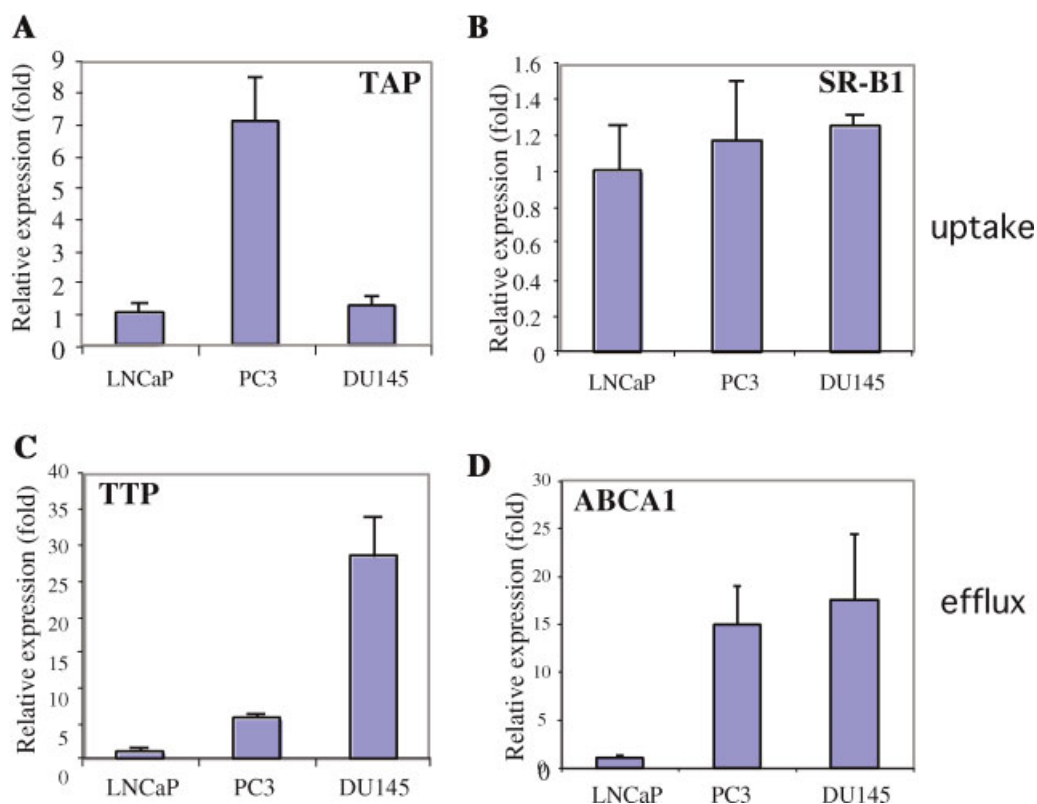


Fig. 4. The differential expression levels of Vit E transport genes in PCa cells. The mRNA expression levels of Vit E absorption genes *TAP* (A), *SR-BI* (B), and efflux genes *TTP* (C), *ABCA1* (D) were evaluated in LNCaP, PC3, and DU145 cells by real time PCR methods. The expression of those genes in LNCaP was set as 1. The data were represented as mean value \pm SD of triplicate samples.

TTP Mediates Cellular Vit E Levels in Prostate Cancer Cells

We further asked if TTP affects Vit E accumulation in PCa cells by knockdown of TTP in DU145 cells. Two DNA-based siRNAs constructs targeting TTP (TTPsi573 and TTPsi675) had been generated. As shown in Figure 5A, the pooled TTPsiRNA-transfected DU145 cells, both DU-TTPsi573 and DU-TTPsi675 cells, reduced TTP expression by around 40%, while the empty vector control (DU-V) did not significantly change TTP mRNA levels, compared to parental DU145 cells. We then treated these cells with 20 μ M α -Vit E or VES for 24 h, and examined the total cellular Vit E concentration. As shown in Figure 5B,C, DU-TTPsi573 and DU-TTPsi675 cells can increase cellular α -Vit E concentration about 1.8-fold compared to the parental DU145 cells. Interestingly, there is no significant difference on VES concentration among DU-TTPsi573, DU-TTPsi675, DU-V, and DU145 cells (Fig. 5C). To further characterize TTP function in PCa cells, we ectopically restored TTP expression back in LNCaP cells that have low endogenous TTP expression (Fig. 5D). The cellular Vit E and VES concentrations were then examined after 24 h of treatment. As shown

in Figure 5E and F, the concentration of α -Vit E, but not VES, was significantly reduced in TTP-transfected LNCaP cells (LN-TTP) compared to vector control cells (LN-V). Therefore, our data indicated that TTP mediates the efflux of α -Vit E but not VES in PCa cells.

Taken together, our data suggested that the expression levels of TTP and probably other Vit E transport proteins might influence cellular Vit E concentrations in different PCa cells.

DISCUSSION

The androgen receptor (AR) is required for normal prostate development and prostate cancer cell proliferation and survival [25–27]. We have identified that downregulation of AR signaling contributes to the VES anti-proliferation effect [8]. However, both AR-positive LNCaP cells and AR-negative PC3 cells are equally sensitive to VES treatment, indicating that AR is not the only genetic difference responsible for VES-mediated inhibition of prostate cancer cells growth. In this study, we found that cellular Vit E bioavailability may represent another factor for Vit E inhibition effects on prostate cancer cells. This notion was supported by the observation that the cellular Vit E bioavailability is

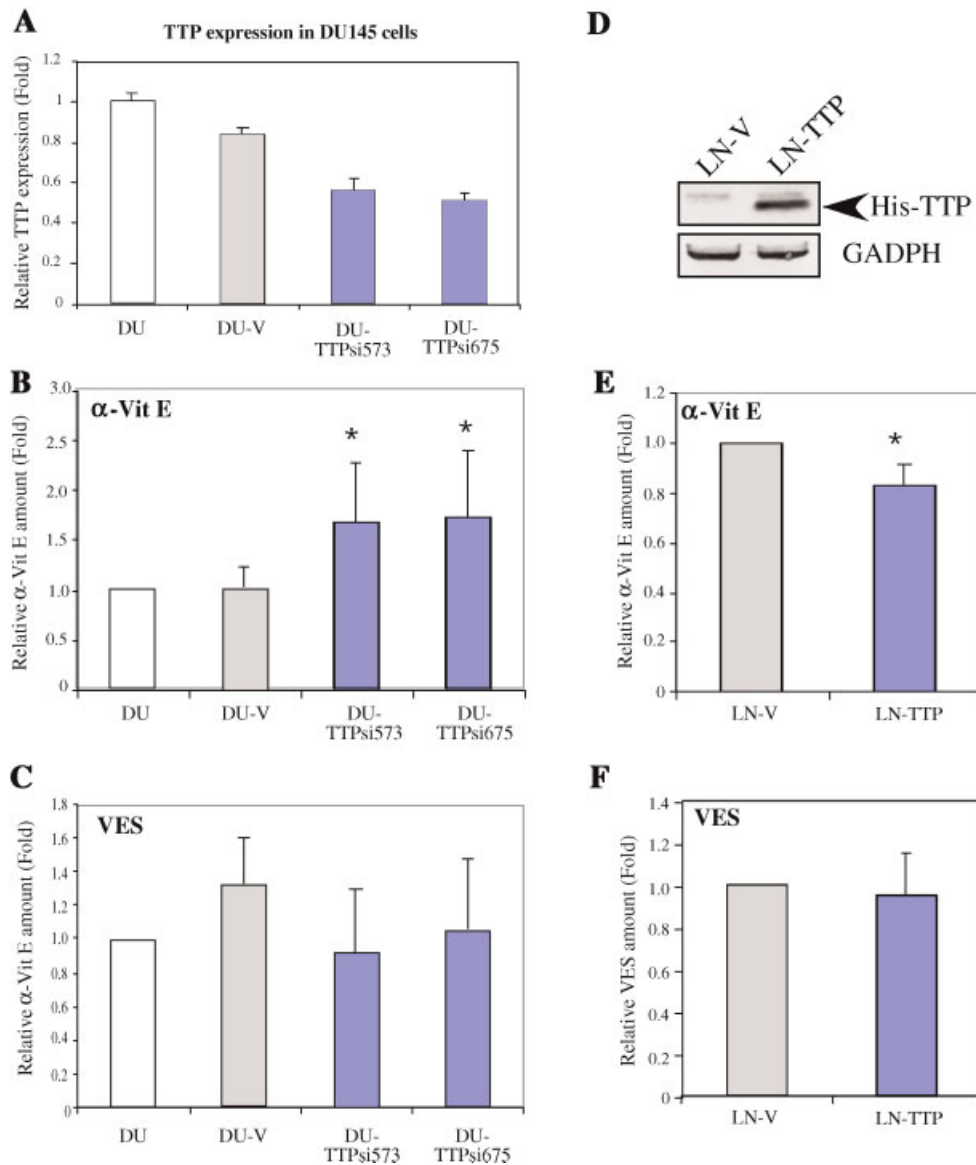


Fig. 5. TTP modulates the cellular Vit E content in prostate cancer cells. **A:** TTP expressions were knocked down in DU145 cells by siRNA strategies. DU145 cells were infected by TTPsiRNA573, TTPsiRNA675, or empty vector. After 1 μ g/ml puromycin selection, the expressions of TTP in the pooled DU-TTPsiRNA573, DU-TTPsiRNA675, empty vector cells DU-V, and parental DU145 cells were examined by real time PCR. The expression of TTP in DU145 cells was set as 1. The data were represented as mean value \pm SD of triplicate samples. The Vit E (**B**) and VES (**C**) content in DU-TTPsiRNA cells after α -Vit E or VES treatment. DU-TTPsiRNA573, DU-TTPsiRNA675, empty vector cells DU-V, and parental DU145 cells were treated with 20 μ M α -Vit E or VES for 1 day. Cells were harvested and counted at the indicated time point. Cellular α -Vit E was extracted with hexane and detected by HPLC. The data of DU145 cells were set as 1. The data were represented as the mean values \pm SD of at least three independent experiments. Statistics *P* value is compared to the data of DU145 cells. **D:** TTP was ectopically expressed in LNCaP cells. LNCaP cells were transfected with pCDNA4-His-TTP, or empty vector by electroporation. After 2–3 days, the cells LN-TTP and LN-V were harvested to examine TTP expression by Western blot using His antibody. α -Vit E (**E**) and VES (**F**) content in LN-TTP cells after α -Vit E or VES treatment. LN-TTP or empty vector cells LN-V were treated with 20 μ M α -Vit E or VES for 1 day. Cells were harvested and counted at the indicated time point. Cellular α -Vit E and VES were extracted with hexane and detected by HPLC. The data of LN-V was set as 1. The data were represented as the mean values \pm SD of at least three independent experiments. Statistics *P* value is compared to the data of LN-V cells. *, *P* < 0.5.

positively correlated with Vit E anti-proliferative activity in prostate cancer cells.

TTP has high expression in liver and low expression in brain, intestine and other organs [28]. However, little information is available to show the expression status

of TTP in prostate. Using RT-PCR, we isolated TTP full length cDNA and did not identify any mutation in benign prostate and prostate cancer cells. TTP function in prostate is not well known. With the help of TTP, α -Vit E may be supplied from the prostate epithelial

cells to the seminal fluid to nourish sperm. Interestingly, the mRNA expression level of TTP is much higher in prostate cancer cell line DU145 compared to other prostate cancer cells. We used knockdown strategy to study the function of TTP in prostate cancer cells, however, our siRNA only knocked down 40% of TTP mRNA levels, still higher than that in prostate cancer LNCaP and PC3 cells. Moreover, since the cultured medium contains excess amount of Vit E, the function of TTP to efflux α -Vit E may be inhibited. Nevertheless, our data suggested that TTP mediated the transport of α -Vit E, but not VES in prostate cancer cells.

We observed high VES retention compared to α -Vit E in PCa cells after the same dose treatment. TTP selectively transports α -Vit E from the liver into the serum after Vit E absorption in the intestine [29,30]. Our current studies suggested that TTP can mediate the transport of α -Vit E, but not the α -Vit E ester analog, VES, in cultured PCa cells. In comparison, our previous studies show that TAP not only mediates α -Vit E, but also mediates VES uptake [17]. The specificity of TTP may contribute to higher VES amount than α -Vit E levels after same dose treatment. However, we do not exclude that other genes may regulate Vit E/Vit E analog transport.

Rate of uptake, efflux, and metabolism all contribute to the final cellular Vit E amount. In this study, we also found DU145 cells have a higher rate of hydrolysis of VES to α -Vit E (Fig. 2D), therefore increased resistance to VES treatment. It is likely esterase expression levels are higher in DU145 cells. Supportively, Kline et al. reported that higher content of esterase in some ovarian cancer cells might cause them to be insensitive to VES treatment [31]. On the other hand, it is also possible that the rate of α -Vit E metabolism (converting α -Vit E into other metabolites) is faster in DU145 cells than that in LNCaP and PC3 cells. Thus, the total cellular concentration of α -Vit E and VES is lower in the DU145 cells. Further studies are needed to verify this possibilities in the future.

CONCLUSION

Different prostate cancers have different sensitivities to α -Vit E treatment. This difference was, at least partly, associated with the cellular accumulation or stability of α -Vit E in different PCa cells. Our study also suggested that Vit E-binding proteins and other transport proteins might contribute to the retention of α -Vit E in PCa cells.

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