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Transcriptional regulation of E-cadherin and oncoprotein E7 by valproic acid in HPV positive cell lines

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ABSTRACT

Objective(s): Valproic acid (VPA) has proven to be as one of the most promising useful drug with anticancer properties. In this study, we investigate the VPA effects on E-cadherin expression in HeLa, TC_1, MKN_{45}, and HCT_{116} cell lines. This study assesses the effects of VPA on human papillomavirus E7 expression in HPV positive cell lines.
Materials and Methods: Cell lines were treated by 2 mmol/l VPA and expression of E-cadherin and E7 was analyzed by quantitative real-time PCR. Student's *t* test and ANOVA were used to determine changes in expression levels.
Results: The results revealed that mean of E-cadherin expression is increased by VPA 1.8 times in HCT_{116} and MKN_{45} cell lines, also the mean of E-cadherin mRNA levels is up-regulated 2.9 times in HeLa and TC_1 cell lines. So, E-cadherin augmentation induced by VPA in HeLa and TC-1, HPV positive cell lines, is higher than HPV negative cell lines MKN_{45} and HCT_{116}. The mean of HPV E7 expression is decreased by VPA, 4.6 times in in HeLa and TC-1 cell lines.
Conclusion: This study demonstrates that re-expression of E-cadherin by VPA in HPV positive cell lines is more than HPV negative cell lines. Whereas, HPV E7 reduces the expression of E-cadherin, reduction of HPV E7 expression by VPA is related to more augmentation of E-cadherin in HPV positive cell lines. So, this study demonstrates that VPA has more anticancer properties in HPV positive cell lines, and could potentially be a promising candidate for cervical cancer treatment.

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Introduction

Cervical cancer is one of the most common cancers in women worldwide with more than 200000 deaths, annually (1). Approximately 80% of them live in developing countries where this disease is detected at late stage; so, new treatment drugs for cervical cancer are needed (2, 3).

Development of cervical cancer is caused by high-risk human papillomaviruses (hrHPVs) (4). They are small, nonenveloped, and nonlytic viruses with dsDNA genome that are tropic for squamous epidermis. The cervical carcinogenesis is associated with E6 and E7 oncoproteins from hrHPVs (4). E6 and E7 viral oncogenic proteins inactivate two cellular tumor suppressor proteins p53 and pRB, respectively (5, 6), and are necessary for cells proliferation and DNA damage that eventually lead to cancer (7).

The development of cervical cancer related to changes in epigenetic patterns of cells such as DNA methylation, histone methylation, and histone acetylation (8). Histone acetylation is one of the most important epigenetic mechanisms that neutralizes the positive charge of histone proteins, unfolds the chromatin structure and associated DNA, and induces transcription and gene expression (9).

The acetylation of histone proteins is regulated by histone acetyltransferase (HATs) and histone deacetylases (HDACs). HDACs remove the acetyl groups on the lysine residues of histones and induce positive charge on histone proteins, this leads to aberrant transcription and gene suppression. So, HDACs could reduce the expression of tumor suppressor genes to development of cancer (10).

Histone deacetylase inhibitors (HDACIs) have appeared as the promising drugs for cancer (11, 12). HDACIs have been classified to 5 groups: 1. short chain fatty acids (e.g., butyrates and valproic acid), 2. organic hydroxamic acids (e.g., suberoylanilide bishydroxamine (SAHA) and trichostatin A (TSA), 3. sulfonamide anilides, 4. benzamides (e.g., MS-275), 5. cyclic tetrapeptides (13).

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Among HDACIs, valproic acid (VPA) has been demonstrated as a drug with anticancer properties. VPA is used for treatment of cluster migraine, headaches, and epilepsy (14), but antineoplastic activity of VPA was shown in 1997 (15). VPA increases apoptosis and reduces angiogenic activity in various tumor cells (16). It also inhibits invasion and migration of tumor cells by several mechanisms (16-19).

To start the process of invasion and metastasis, a malignant cell must first break away from the cancerous tumor. In cancer cells, the expression of adhesion molecules seems to be reduced. E-cadherin is one of the most important adhesion molecules encoded by CDH_1 gene, and the dominant epithelial cell-adhesion molecule. This protein is expressed on basal and suprabasal cells and plays important role in epithelial structural integrity (20, 21). The suppression of E-cadherin expression has been widely reported in different epithelial tumors (22-27). The loss of E-cadherin is associated with invasion and metastasis (28).

In this work, we compare the effects of VPA on E-cadherin expression in two groups of cell lines, HPV positive and HPV negative cell lines. Also, we study the effects of VPA on E7 oncoprotein expression in HPV positive cell lines.

Materials and Methods

Cell culture

The HPV positive cell line; HeLa, a cervical carcinoma cell line that contains HPV_{18} DNA, and TC_1 which is transduced to express the E6 and E7 oncogenes of HPV_{16}, were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco). Growth media was supplemented with 10% heat inactivated fetal calf serum (Life Technologies, Camarillo, CA, USA), 100 µg/ml penicillin/streptomycin (Invitrogen, Life Technologies, Camarillo, CA, USA), and 2 mM L-glutamine (Sigma-Aldrich, MO, USA). The HPV negative cell lines; HCT_{116} and MKN_{45}, were also cultured in the same condition. All of the cell lines were maintained in humidified incubator with 5% CO_2.

Valproic acid preparation

Valproate sodium salt was prepared from sigma (Sigma-Aldrich, MO, USA) and was dissolved in phosphate buffered saline (PBS) to 1, 2, and 5 mM concentrations.

Proliferation assay

Cell proliferation was performed using the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye reduction assay. HeLa, TC_1, HCT_{116}, and MKN_{45} cell lines were seeded at a density of 5×10^4 per well in 96 well culture plates and incubated overnight with DMEM containing 10% fetal calf serum. After 24 hr, the cells were treated with medium containing VPA at concentrations of 0, 1, 2, and 5 mmol/L, for three days. After 24, 48, and 72 hr, MTT was added at concentration of 0/5 mg/ml for 4 hr. The absorbance was determined at 570 nm.

RNA preparation

The four cell lines (HCT_{116}, MKN_{45}, HeLa, and TC_1) were cultured in 12 well culture plates to 80 percent confluency and were treated with medium containing 2 mmol/l VPA for 48 hr. According to experimental studies, our experiments were performed three times to achieve valid results; so, we did real-time PCR analysis for each experiment using 3 samples. The cells were harvested and resuspended in 250 µl volume of sterile PBS. Total RNA was extracted from cells using TriPure total RNA isolation Kit according to the manufacturer's procedure (Roche Applied science, Indianapolis, IN, USA). Extracted RNA concentration and purity were analyzed at the wavelength of 260 nm by nanodrop spectrophotometry (Eppendrof, Humburg, Germany). RNA integrity was visually assessed with 1% agarose gel electrophoresis. The RNA with absorption ratio OD 260 nm OD 280 nm between 1/8 and 2 was used for cDNA synthesis.

cDNA synthesis

cDNA was prepared from total RNA by reverse transcription using M-MuLV reverse transcriptase (Fermentas, Vilnius, Lithuania), random hexamers, and RNase inhibitor (Vivantis). One µg of RNA was added by 2 µl of 10 mM dNTPs along with 1 µl of (0.2 µg/µl) random hexamer, 1 µl (20 u/µl) of M-MuLV, and 0.5 µl of RNase inhibitor in a sterile microcentrifuge tube. The final volume was made up to 20 µl by adding sterile distilled water and was mixed well. These tubes were incubated at room temperature for 10 min and then were incubated at 42 °C for one hr. The M-MuLV enzyme inactivation step was done by a step for 10 min at 70 °C. The synthesized cDNA was diluted 10 times in sterile distilled water and was used as a template for quantitative real-time PCR analysis.

Quantitative real-time PCR

Expression of E-cadherin mRNA was analyzed by quantitative real-time PCR using the SYBR Premix Ex Taq II kit (TAKARA Bio INC., Otsu, Japan) on an Applied Biosystems® StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster city, CA, USA). Quantitative real-time PCR was performed using the following primer sequences: E-cadherin, forward: 5′-AGGGGTTAAGCACAACAGCA-3′ and reverse: 5′-GG-TATTGGGGGCATCAGCAT-3′; HPV E7, forward: 5′-ATGCATGGAG ATACACCTAC-3′ and reverse: 5′-TTATGGTTTCTGAGAACAGATG-3′, and glyceral-dehyde 3-phosphate dehydrogenase (GAPDH),

forward: 5′-ATGTTCGTCATGGGTGTGAA-3′ and reverse: 5′- GGTGCTAAGCAGTTGGTGGT-3′. Briefly, 10 µl SYBR Premix Ex Taq II (2x), 0.4 µl ROX Reference Dye (50x), and 0.8 µl of forward and reverse primers with 2 µl of 1/10 diluted cDNA were used in a total volume of 20 µl. The temperature profile for all genes was 95 °C for 1 min; 40 cycles of 95 °C for 15 sec, 55 °C for 30 sec, and 72 °C for 30 sec; melting curve program (60-95 °C). All samples were run in triplicate simultaneously with negative controls. The threshold cycle (ct) of E-cadherin mRNA level of VPA treated cell lines was compared with the threshold cycle generated by the control samples. E-cadherin mRNA levels were normalized to the expression of the endogenous housekeeping gene GAPDH by the $2^{-\Delta\Delta ct}$ method (29).

Statistical analysis

The unpaired, two-tailed student's *t* was done to analyze the statistical differences between groups using Graph-Pad Prism software. mRNA levels comparisons between groups were analyzed using ANOVA test. A *P*-value less than 0.05 ($P<0.05$) was considered to be statistically significant for the differences and determined by asterisk in the corresponding figures.

Results

Optimization of the valproic acid concentration for E-cadherin expression

The cell lines were treated by 1, 2, and 5 mM VPA and harvested 24, 48, and 72 hr after treatment. MMT assay and quantitative real-time PCR results for E-cadherin mRNA levels showed that 2 mM VPA after 48 hr gave the highest amount of E-cadherin expression (data not shown).

Valproic acid up-regulated E-cadherin expression in HPV negative cell lines

Two HPV positive cell lines, HCT_{116} and MKN_{45}, were treated by 2 mM VPA to study the E-cadherin mRNA level changes. Cells were harvested 48 hr after treatment, total RNA was extracted, and cDNA was synthesized by 1 µg RNA. Then E-cadherin expression was analyzed by quantitative real-time PCR. Each experiment was done three times (n=3). The results revealed that E-cadherin expression is increased 1.7 and 2 times in HCT_{116} and MKN_{45} cell lines in comparison to control cells with *P*-value= 0.0423 and *P*-value = 0.0042, respectively (Figure 1A, B).

E-cadherin expression is increased in HPV positive cell lines by valproic acid

The two HPV positive cell lines, HeLa and TC_1 were treated by 2 mM VPA, and E-cadherin mRNA level was analyzed. Each experiment was done three times (n=3). The results showed that E-cadherin

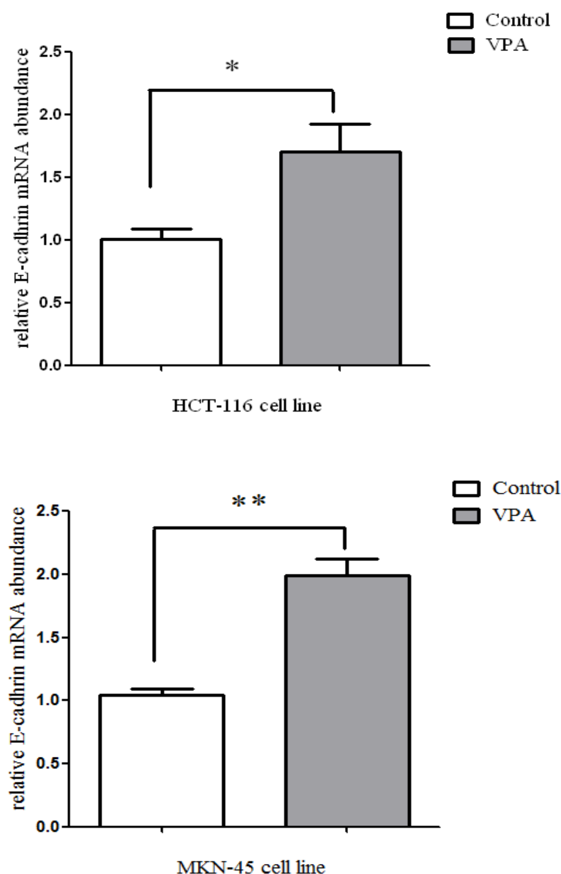

Figure 1. E-cadherin expression is increased in human papillomavirus negative cell lines by valproic acid. HCT_{116} and MKN_{45} cell lines were treated by 2 mM VPA when the cells reached 80% confluency. After 48 hr of treatment, cells were harvested and fold change of E-cadherin expression was analyzed by real time PCR using the $2^{-\Delta\Delta Ct}$ method. **(A)** E-cadherin mRNA level in HCT_{116} cells treated by VPA. **(B)** E-cadherin mRNA level in MKN_{45} cells treated by VPA. Each experiment was done three times (n=3). * $P<0.05$, ** $P<0.01$

mRNA level is significantly up-regulated, 3.2 and 2.6 times in HeLa and TC_1 cell lines with *P*-value = 0.0028 and *P*-value = 0.0089, respectively (Figure 2A, B).

The E-cadherin augmentation is induced by valproic acid in HPV positive cell lines higher than HPV negative cell lines are

The experiments showed that E-cadherin expression is increased by VPA in both in HPV positive and negative cell lines; whereas, E-cadherin augmentation in HeLa and TC_1 (Mean=2.9), HPV positive cell lines, is higher than HPV negative cell lines, MKN_{45} and HCT_{116} (Mean=1.85) (Figure 3).

Valproic acid down-regulated HPV E7 expression in HeLa and TC_1 cell lines

Previous studies revealed the relationship between HPV E7 oncoprotein and E-cadherin expression. One study showed that expression of the HPV_{16} E7 reduces the expression of E-cadherin in

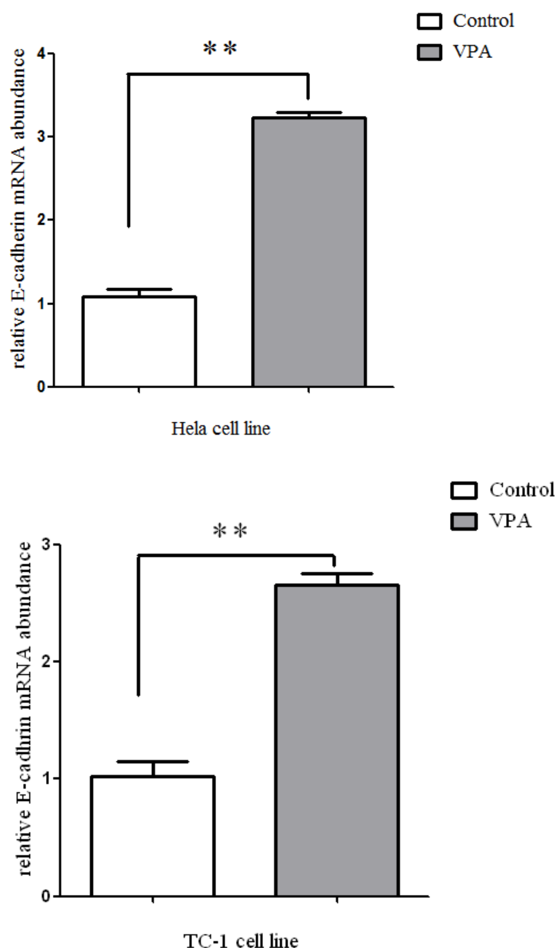

Figure 2. Valproic acid up-regulated E-cadherin expression in human papillomavirus positive cell lines. Hela and TC_1 cell lines were cultured by DMEM containing 2 mM VPA. After 48 hr treatment, cells were harvested and fold change of E-cadherin expression was analyzed by real time PCR using the $2^{-\Delta\Delta Ct}$ method. **(A)** E-cadherin mRNA level in Hela cell line treated by VPA. **(B)** E-cadherin mRNA level in TC_1 cell line treated by VPA. Each experiment was done three times (n=3). * P< 0.05, ** P< 0.01

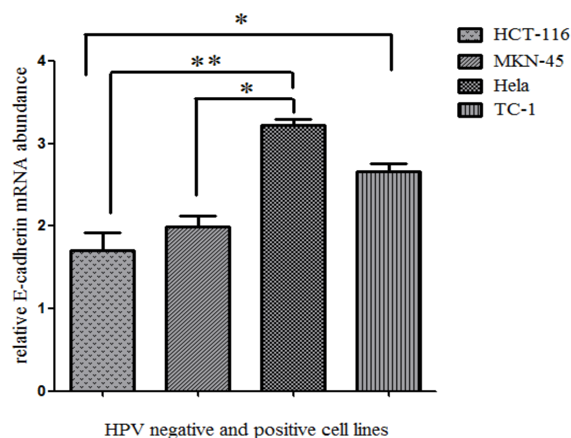

Figure 3. The E-cadherin augmentation induced by valproic acid in HPV positive cell lines is higher than HPV negative cell lines

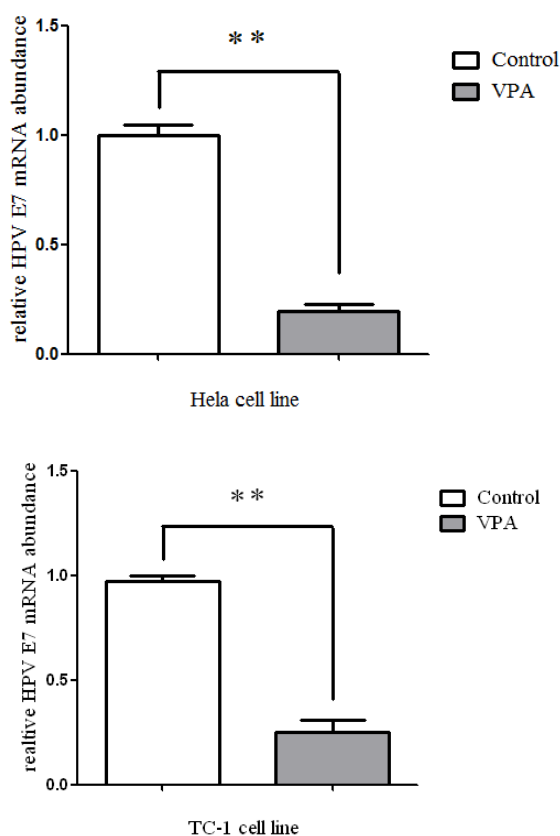

Figure 4. Valproic acid down-regulated HPV E7 expression in human papillomavirus positive cell lines. Hela and TC_1 cell lines were treated by 2 mM VPA. After 48 hr of treatment, cells were harvested and fold change of HPV E7 expression was analyzed by real time PCR using the $2^{-\Delta\Delta Ct}$ method. **(A)** HPV E7 expression in Hela cell line treated by VPA. **(B)** HPV E7 expression level in TC_1 cell line treated by VPA. Each experiment was done three times (n=3). Error bars represent SEM. * P< 0.05, ** P< 0.01

MDCK cells, and Caberg *et al* demonstrated that suppression of HPV E7 using siRNA in HPV_{16} transformed human keratinocytes, restores the E-cadherin expression in these cells (30, 31). So, we set out to test the VPA effects on HPV E7 expression in HeLa and TC_1 cell lines. Each experiment was done three times (n=3). The results showed that HPV E7 expression is decreased 5.2 and 4 times in HeLa and TC_1 cell lines with P-value= 0.0058 and P-value= 0.0069, respectively (Figure 4A, B).

Discussion

Epigenetic is composed inducible genetic changes that regulates gene expression. DNA methylation and histone acetylation are two most common mechanisms that induce epigenetic changes (32). DNA methylation occurs at cytosine within CpG islands and it is catalyzed by DNA methyltransferases (DNMTs) protein family (33). DNA methylation directly interferes with transcription factors binding to recognition regions in promoter sites, and causes transcriptional suppression (34). Interaction of DNMTs with other chromatin

modifying enzymes such as HDACs and histone methyltransferase brings these proteins in the vicinity of promoter sites and alters gene expression (35-38).

Positive charge at lysine residues neutralized by histone acetylation, loosen the chromatin structure that finally causes transcriptionally active chromatin (9, 10). DNMTs bring HDACs in the vicinity of promoter regions, cause local histone deacetylation, and condense chromatin structure that reduce gene expression levels by limiting accessibility of transcription factors to this sites (39). HDACs include 18 genes that are divided into 4 classes (13).

HDACIs have emerged as the hopeful clinical drugs for cancer (11, 12). VPA has proven as one of the most encouraging useful therapeutics with anticancer properties. In comparison with other HDACIs such as trapoxin and trichostatin A that have toxic side effects, VPA is relatively nontoxic and safe *in vivo* (13). VPA inhibits invasion and migration of tumor cells by several mechanisms (16-19).

During epithelial to mesenchymal transition (EMT) process, a malignant cell can break away from the cancerous tumor (40). A critical molecular feature of EMT is the downregulation of E-cadherin (41). E-cadherin acts as a tumor suppressor protein and inhibits invasion and metastasis. (42).

EMT provides molecular mechanisms for cervical cancer metastasis, and decreased or aberrant expression of E-cadherin have been reported in cervical carcinoma (43-45).

Carcinoma of the cervix is the second most common cancer in women worldwide (1). Development of this cancer is caused by the integration of hrHPVs in the host genome (4 HPV E6 and E7 viral proteins may induce EMT in cervical cancer (46, 47), so in this study we analyzed valproic acid effects on E-cadherin expression in HPV positive cell lines and HPV negative cell lines.

Our results showed that E-cadherin expression is increased in HPV positive cell lines (HeLa and TC_1) and HPV negative cell lines (MKN_{45} and HCT_{116}); this result is consistent with previous studies. The expression of E-cadherin is up-regulated by VPA in LNCaP, DU_{145}, PC_3, and luminescent PC_3 prostate cancer cell lines and prostate cancer xenograft mouse models (48). The VPA effects on ovarian cancer using human ovarian cancer cell line $SKOV_3$ and a human ovarian cancer model transplanted subcutaneously in nude mice showed increased expression of E-cadherin and reduced matrix metalloproteinase-9 and VEGF *in vitro* and *in vivo* (49). VPA and SAHA treatment, in combination with all-trans retinoic acid (ATRA), restore E-cadherin, TSGs $RARb_2$, P_{53}, and $P_{21}CIP_1$ expression in human cervical cancer cells (50). It has been also reported that S-valproate reduces transcriptional suppressor of E-cadherin proteins vimentin and ZEB_1 and increases E-cadherin protein expression in non-small cell lung cancer (51). Based on previous studies, VPA treatment increases the expression of E-cadherin in RM_1 and PC_3 cell lines, and might be useful to inhibit E-cadherin-mediated migration in prostate cancer cells (52, 53).

The effects of VPA on cervical cancer cell lines have been also investigated before. VPA has shown a growth inhibitory effect on HeLa, SiHa, and CaSki cervical cancer cell lines (54, 55). VPA increases p53 transcription and protects it from degradation by E6 (54). Evaluating the effects of VPA on cervical cancer cell lines HeLa, SiHa, and CaSki and *in vivo* cancer models demonstrated increased p21 expression and histone H_3 acetylation. Chronic treatment of VPA significantly decreased tumor growth and improved survival in tumor xenograft studies (56). Another study revealed that VPA treatment suppresses cell growth and induces cell cycle arrest in human cervical cancer HeLa cells, and also increases the tumor suppressor, $Notch_1$ (57).

According to our findings, E-cadherin augmentation in HPV positive cell lines, HeLa and TC-1, is higher than HPV negative cell lines, MKN_{45} and HCT_{116}. Previous studies have showed the effect of HPV E7 oncoprotein on E-cadherin expression, one study revealed that the expression of the HPV_{16} E7 in MDCK cells reduces the expression of E-cadherin (30), and suppression of HPV E7 using siRNA in HPV_{16} transformed human keratinocytes restores the E-cadherin expression in this cell (31). We studied the effects of VPA on HPV E7 expression in HeLa and TC_1 cell lines; VPA significantly decreased HPV E7 mRNA level in both cell lines. This reduction of HPV E7 expression by VPA might lead to more augmentation of E-cadherin in HPV positive cell lines.

Conclusion

VPA increases E-cadherin expression in HPV positive cell lines and HPV negative cell lines; however, E-cadherin augmentation in HPV positive cell lines is higher than HPV negative cell lines. VPA decreases HPV E7 mRNA level in HeLa and TC-1 cell lines, which might lead to augmentation of E-cadherin in HPV positive cell lines. VPA showed anticancer properties and could potentially be a promising candidate for further cervical cancer treatment.

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