Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 607480, 10 pages
doi:10.1155/2011/607480

Research Article

Does Valproic Acid Induce Neuroendocrine Differentiation in Prostate Cancer?

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Received 3 June 2010; Revised 28 September 2010; Accepted 30 September 2010

Academic Editor: Christian Seiser

Valproic Acid (VPA) is a histone deacetylase inhibitor that holds promise for cancer therapy. Here, we investigate whether VPA treatment induces neuroendocrine differentiation of Prostate Cancer (PCa). A tissue microarray of VPA-treated and untreated tumor xenografts and cell lines of human PCa (LNCaP, C4-2, DU145, and PC-3) were generated and were analyzed by immunohistochemical analysis (IHC) for NE markers chromogranin A (CgA), synaptophysin, and NCAM (neural cell adhesion molecule). Western blot analysis for CgA was performed to confirm the results of the TMA. IHC analysis did not reveal any induction of CgA, synaptophysin, or NCAM in any xenograft after VPA treatment *in vivo*. *In vitro*, VPA treatment induced little synaptophysin expression in C4-2 and PC-3 cells and NCAM expression in LNCaP and PC-3 cells. In the case of CgA, VPA treatment decreased its expression *in vitro* in a dose-dependent manner, as determined by western blot analysis. Thus our data demonstrates that VPA does not induce NE differentiation of PCa cells in the physiologically relevant *in vivo* setting.

1. Introduction

Histone acetylation and deacetylation by histone acetyl transferases and histone deacetylases is involved in the epigenetic regulation in human cells [1, 2]. Recently, this post-translational modification has become a popular molecular target for cancer therapy. HDAC inhibitors (HDACIs) have demonstrated significant antitumor activity by hyperacetylation of nucleosomal histones resulting in reexpression of repressed genes that produce growth arrest, terminal differentiation, and/or apoptosis in carcinoma cells [3]. Valproic Acid (VPA), an HDACI and an antiepileptic agent, causes marked decrease in proliferation of Prostate Cancer (PCa) cells *in vitro* and significant reduction in tumor volume *in vivo* [4, 5]. Multiple pathways including cell cycle arrest, apoptosis, angiogenesis, and senescence contribute to the antitumor effects of VPA.

Neuroendocrine (NE) cells are the third and minor epithelial cell type in prostate, in addition to the more abundant luminal secretory cells and basal cells [6]. NE cells have dual properties of neurons and endocrine cells and are believed to be involved in the regulation, secretion and differentiation of other prostatic cells [7]. Conventional adenocarcinoma with focal NE cells represents the most common type of PCa. Small cell PCa and prostatic carcinoid are relatively rare and are considered pure NE tumors with a poor prognosis [8]. Neuroendocrine differentiation thus has been suggested as a poor prognostic sign by some authors, but the exact role of NE differentiation of the prostate remains unclear, and its prognostic importance

in prostate cancer still remains controversial [7, 9]. The characteristics of NE differentiation in PCa are very much similar to those seen in patients who develop this histologic phenotype in non-small-cell lung cancer [10]. NE cells in prostate express NE markers such as Chromogranin A (CgA), synaptophysin, B-tubulin, neural cell adhesion molecule (NCAM or CD56), neuron specific enolase (NSE), and so forth. NE cells can be generally identified by electron microscopy or immunohistochemical (IHC) staining with antibodies for NE markers [11].

Recently, some studies have documented increased neuroendocrine markers after *in vitro* treatment of prostate cancer cell lines with HDACIs [9, 12] indicating neuroendocrine transdifferentiation. In contrast, studies done in neuroendocrine tumors such as carcinoid, pheochromocytoma, and small cell lung cancers have shown VPA and other HDACIs to exert antitumor effects [13–15]. VPA has been shown to promote apoptosis, reduce NE phenotype and expression of NE markers, and is suggested as a promising therapy for these tumors [16, 17]. Thus the role of HDACI's in neuroendocrine differentiation still remains unclear and has thus warranted further investigation.

The goal of this study is to carefully determine whether VPA induces NE differentiation in the PCa cell lines, *in vivo* and *in vitro,* by studying a variety of markers associated with NE differentiation in numerous PCa cell and tumor models. Markers including CgA, synaptophysin, and NCAM were quantified by IHC in a tissue microarray (TMA) format from several VPA-treated human PCa cells grown *in vitro* and *in vivo* as tumor xenografts in nude mice.

2. Materials and Methods

2.1. Tumor Cell Lines. Human prostate cancer cell lines LNCaP, PC-3, and DU145 were obtained from American Type Culture Collection (Manassas, VA), and C4-2 line was a gift from Dr. Leland Chung (Emory University, Atlanta, GA). All the cells were grown in RPMI 1640 with L-glutamine (Cellgro, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Inc., Carlsbad, CA), 5 μg/mL ciprofloxacin hydrochloride (U.S. Biological, Swampscott, MA), and 50 μg/mL gentamicin (Quality Biological, Inc., Gaithersburg, MD). Cells were allowed to grow until 80% to 90% confluent and harvested with 0.05% trypsin/0.53 mmol/L EDTA (Cellgro, Herndon, VA) before each subsequent passage.

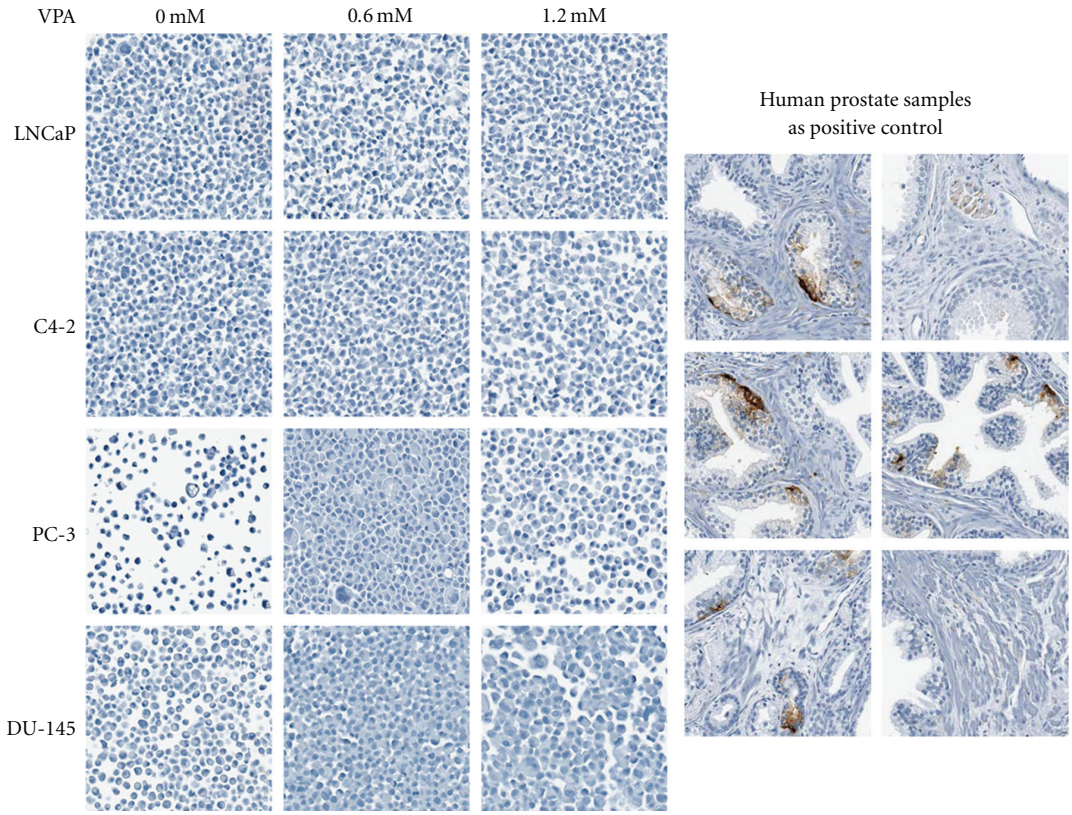
2.2. Establishment of Tumor Xenografts. Cell lines were grown to 80% to 90% confluent and harvested. Cells were resuspended in 1x phosphate-buffered saline (pH 7.4; BioSource, Rockville, MD), mixed 1x with Matrigel (BD Biosciences, Palo Alto, California), and injected (1×10^6 per injection) subcutaneously into the lateral flanks of male athymic *nu/nu* mice. Once palpable tumors were established, animals were randomized into control and treatment arms.

2.3. Valproic Acid Treatment. VPA (1 mol/L; VPA sodium salt; Sigma, St. Louis, MO) stock was made in PBS and filters sterilized through a 0.22 μm filter. For in vitro experiments cell lines were treated with 0, 0.6, and 1.2 mM VPA for 14 days. Medium was replaced every 48 hours with fresh medium containing VPA. For in vivo experiments, animals received 0.4% w/v VPA in drinking water. This has been shown to produce blood levels in mouse [4] comparable to FDA approved levels in humans [18]. Animals in treatment arm were treated for 35 days before excision of tumors. In *in vivo* experiments, chronic treatment implies to long-term treatment with regards to life span. We considered 35 days of treatment in nude mice (with life span of 1 year approx. in our experience) as long-term treatment. This period would correspond to years of treatment in humans.

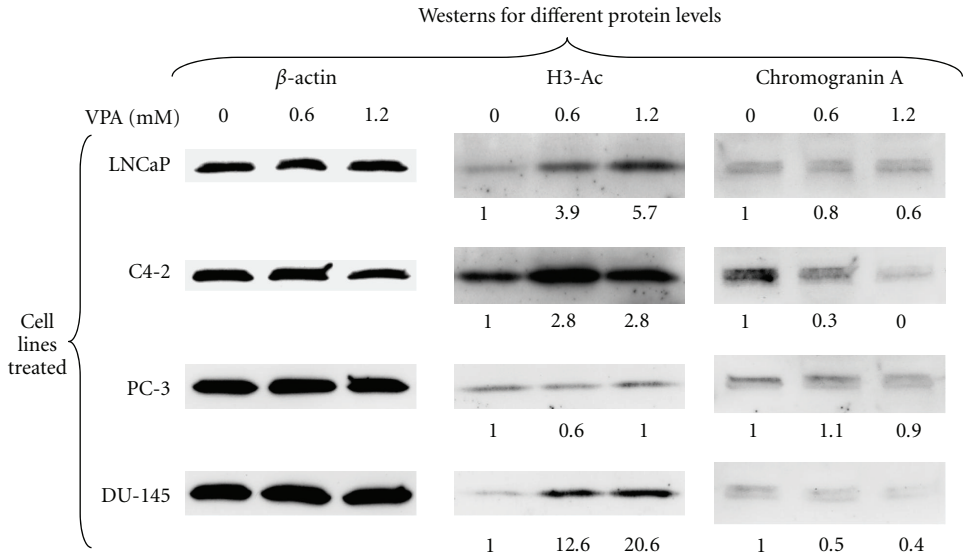
2.4. Western Immunoblotting. Cells treated with different doses of VPA were harvested by trypsinization and resuspended with 5 volumes of cold lysis buffer (RIPA buffer, Cat# R0278, Sigma, St. Louis, MO) and supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Equal amounts of proteins were separated by SDS-PAGE and the resolved proteins transferred to nitrocellulose membrane. The membrane was blocked for an hour in blocking buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20] with 5% nonfat dry milk and then incubated with rabbit antiacetylated histone H3 (Upstate, Charlottesville, VA) overnight followed by antirabbit IgG peroxidase conjugate (Sigma, St. Louis, MO) for 1.5 hours at room temperature. Immunoreactive bands were detected using the enhanced chemiluminescence plus western blotting detection system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Anti-Cip1/WAF1/p21 mouse monoclonal IgG (Upstate, Charlottesville, VA), CgA (LK2H10) mouse monoclonal antibody (Santa Cruz Biotechnology, INC., Santa Cruz, CA), and antimouse IgG peroxidase (Sigma, St. Louis, MO) were used to test p21 and CgA expression separately. Monoclonal anti-β-actin in mouse (Sigma, St. Louis, MO) and antimouse IgG-peroxidase (Sigma, St. Louis, MO) were used to normalize protein loading.

2.5. TMAs: Construction and IHC Staining. For *in vitro* models, cells were harvested and washed in PBS. Resulting cell pellets were incubated for 1-2 hr in Bouin's fixative (75% saturated picric acid, 20% formalin (40%), 5% acetic acid, rinsed with 70% ethanol, and dehydrated according to standard procedures with ethanol and xylene. Cell pellets > 5 mm were split in order to achieve sufficient dehydration. Cells were embedded in paraffin following 90 min of incubation in liquid paraffin at 60°C. For *in vivo models,* tumors were excised and portioned on day 35. Portions were either immediately frozen in liquid nitrogen and stored at −80°C or fixed in buffered formalin and subsequently embedded in paraffin.

A tissue microarray (TMA) of the paraffin embedded materials was generated as described previously [19]. Each array block also contained control normal human prostate tissues and animal xenograft tissues such as bladder, kidney, lung and spleen. Immunohistochemical stains for



(a)



(b)

Figure 1: Chromogranin A staining in prostate cancer cells treated with VPA *in vitro*. (a): Representative images of CgA staining for cell pellet sections from control and VPA-treated groups: LNCaP, C4-2, PC-3, and DU-145 (Scanned at 20X magnification using the APERIO imaging system). The tissue specimens of normal human prostate were positive internal controls for CgA staining. (b) Western blot analysis of cells treated *in vitro*. LNCaP, C4-2, DU-145, and PC-3 cells treated with VPA show induction of acetyl-H3, verifying HDACI activity, and downregulation of the NE marker CgA.

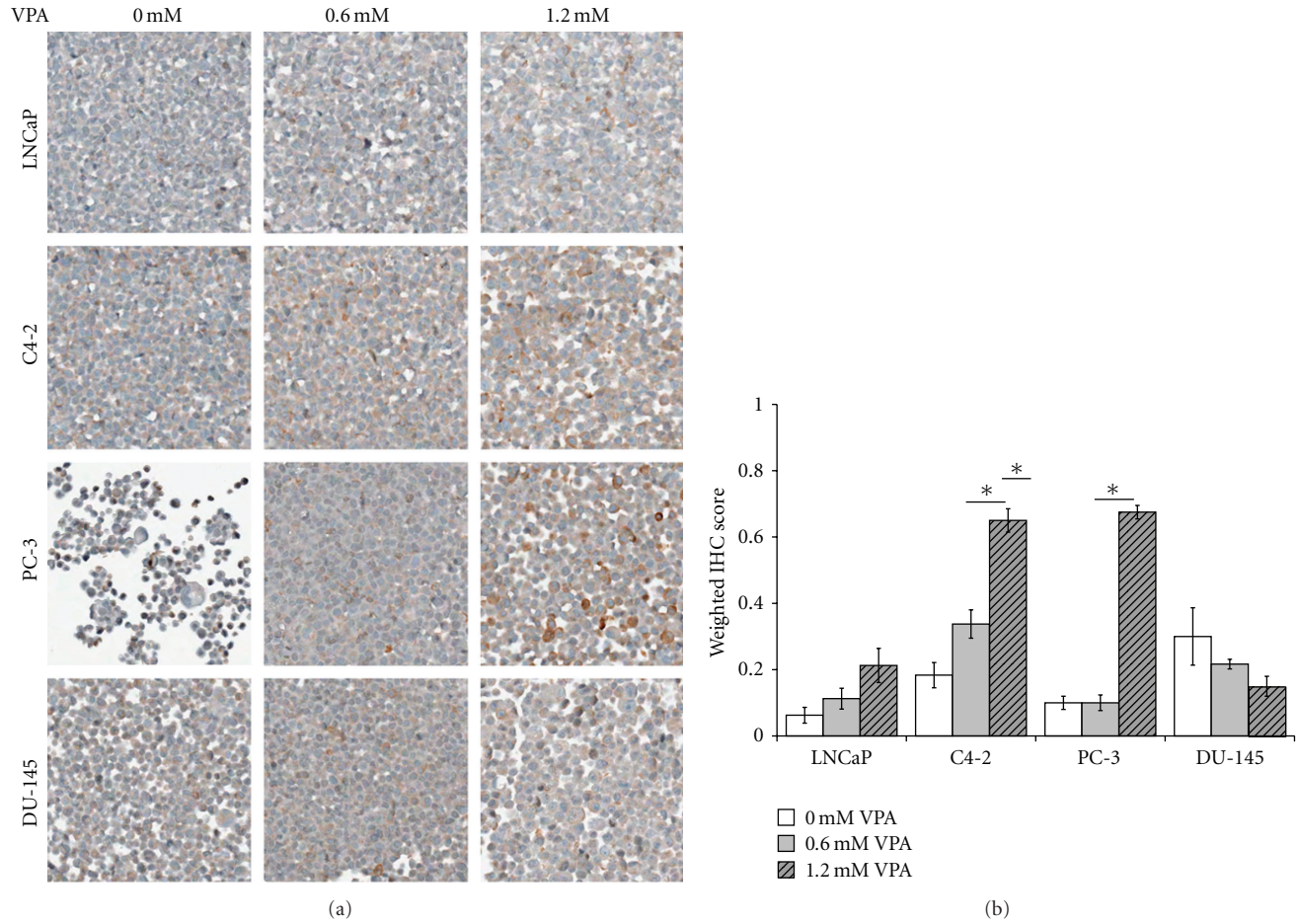


Figure 2: Synaptophysin staining in prostate cancer cells treated with VPA *in vitro*. (a): Representative images of synaptophysin staining for cell pellet sections from control and VPA-treated groups: LNCaP, C4-2, PC-3, and DU-145 (Scanned at 20X magnification using the APERIO imaging system). (b) Weighted scoring of IHC staining for synaptophysin (* indicates $P < .05$).

chromogranin A (Clone LK2H10, Ventana, Tucson, AZ), synaptophysin (Polyclonal, Cell Marque, Rocklin, CA), and NCAM/CD56 (123C3.D5, Cell Marque, Rocklin, CA) were performed separately on sections cut from the TMA. Stained TMA slides were scanned (at 20x magnification setting) using the APERIO imaging system and the images were uploaded and viewed using TMAJ [20, 21]. Each array spot was then formed into a composite image for viewing and scoring on a personal computer monitor.

2.6. Scoring of IHC Staining. IHC specimens were provided to pathologist for scoring. The identity of sample and treatment was blinded to the scorer. The specimens showed a varying degree of staining intensity and percentage of cells staining. Therefore, a combined intensity and percentage positive scoring method was used [22]. Strong intensity staining was scored as 3, moderate as 2, weak as 1, and negative as 0. For each intensity score, the percentage of cells with that score was estimated visually. A combined weighted score consisting of the sum of the percentage of cells staining at each intensity level was calculated for each sample, for example, a case with 50% strong staining, 20% moderate staining, and 10% weak staining would receive a score as follows: $(50 \times 3 + 20 \times 2 + 10 \times 1) = 200$. The maximum score is 300.

2.7. Statistical Analysis. Analysis was done using GraphPad Prism 4.0. Data is plotted as means(SE). One way ANOVA with post-hoc testing was done to evalauate differences in mean staining score between different groups.

3. Results

3.1. In Vitro. The most reliable method to assess NE differentiation in PCa is the detection of CgA in tumor cells. IHC staining for CgA and two other neuroendocrine markers synaptophysin and NCAM were done.

3.1.1. Chronic VPA Treatment Reduces CgA Expression. The IHC staining of sections constructed of cell lines treated for 14 days with VPA was not able to detect any expression of CgA (Figure 1(a)). In order to verify the absence of CgA by

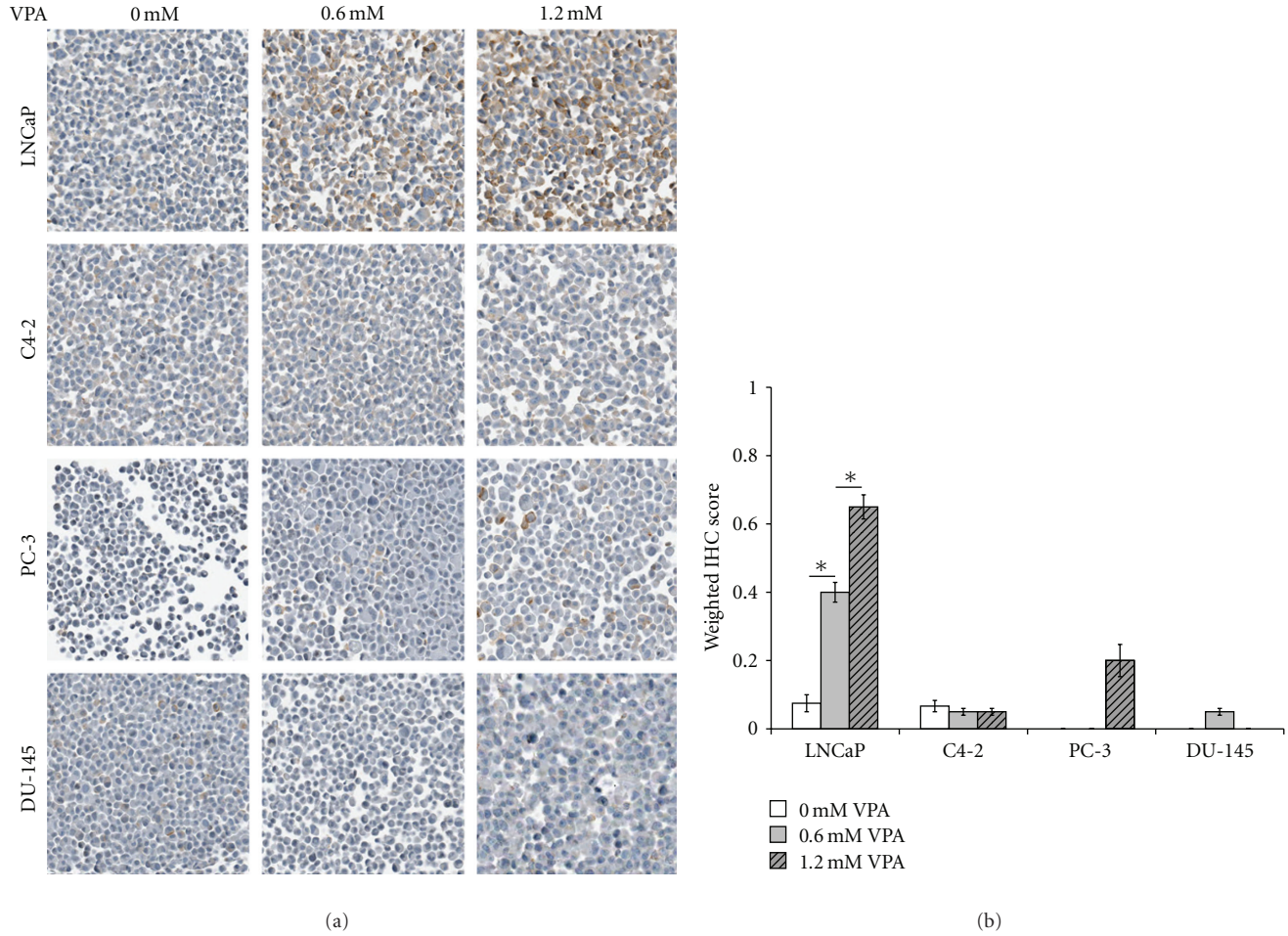


Figure 3: NCAM staining in prostate cancer cells treated with VPA *in vitro.* (a): Representative images of NCAM staining for cell pellet sections from control and VPA-treated groups: LNCaP, C4-2, PC-3, and DU-145 (scanned at 20X magnification using the APERIO imaging system). (b) Weighted scoring of IHC staining for NCAM (* indicates $P < .05$).

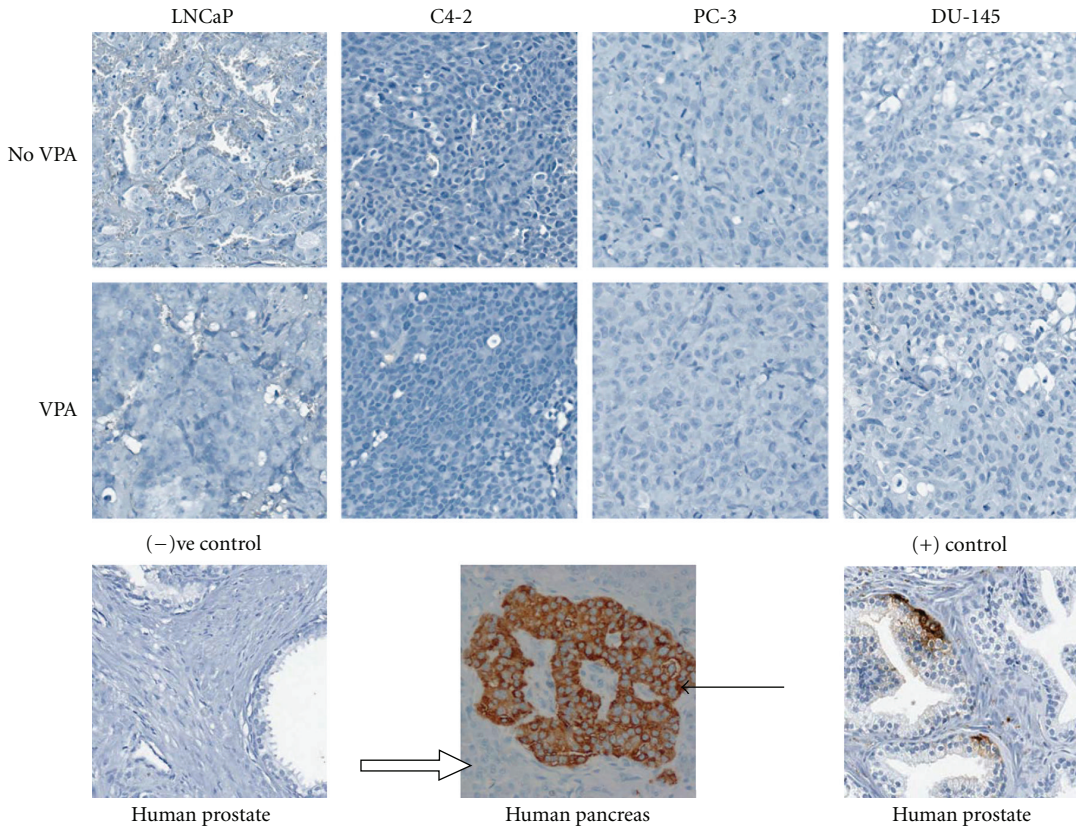
the IHC in all the cell lines tested, we performed Western blot analysis (Figure 1(b)). Results revealed CgA expression in these cell lines; however, CGA protein levels were reduced in a dose-dependent manner after VPA treatment. Histone 3 acetylation confirmed VPA activity.

3.1.2. Chronic VPA Treatment and Synaptophysin and NCAM Expression. Chronic *in vitro* treatment of C4-2 cells with VPA resulted in increased synaptophysin expression (mean weighted score 65(±4) at 1.2 mM versus 34(±4) at 0.6 mM ($P = .002$) and 18(±4) at 0 mM ($P < .001$)). Significantly increased expression was also found in PC-3 cells treated at 1.2 mM (mean weighted score 68(±2) versus 10(±2) in other two groups, $P < .001$). However, synaptophysin expression was not altered in LnCap and DU145 cell lines following VPA treatment (Figure 2).

Chronic *in vitro* treatment of VPA increased the expression of NCAM in LNCaP (weighted score 8(±3) at 0 mM versus 40(±3) at 0.6 mM, ($P < .001$); 40(±3) at 0.6 mM versus 65(±4) at 1.2 mM, ($P = .002$). In PC-3 cells, no expression of NCAM was seen at 0 and 0.6 mM VPA, but slight expression was seen at 1.2 mM (mean weighted score 20(±5)). None or very little NCAM staining was seen in C4-2 and DU145 at either dose (Figure 3).

3.2. In Vivo

3.2.1. Chronic VPA Treatment In Vivo Does Not Induce the Expression of CgA, Synaptophysin, or NCAM. Unilateral tumor xenografts were established in 20 animals each for every cell line. Half of the animals were randomized to receive 0.4% VPA in drinking water. We have shown previously that administration of 0.4% VPA in mouse drinking water can achieve plasma VPA levels similar to the levels obtained in human patients [4]. VPA treatment at these levels was shown to induce acetyl H3K9, p21, and reduce tumor volume, thus confirming the pharmacologic activity of VPA [4]. Animals were sacrificed, and tumors were harvested on day 35. To investigate the effects of VPA on NE markers of PCa tumors *in vivo*, we evaluated expression of NE markers by IHC on the excised tumors. IHC did not reveal any CgA



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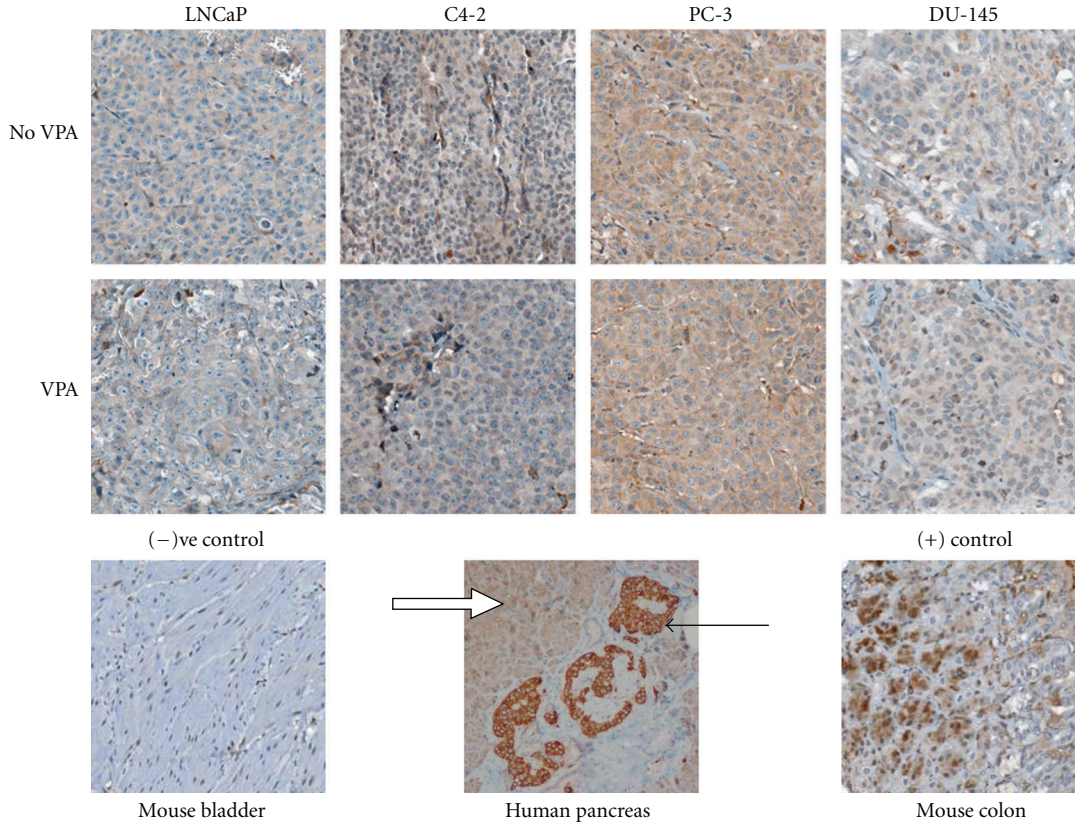

(b)

Figure 4: Continued.

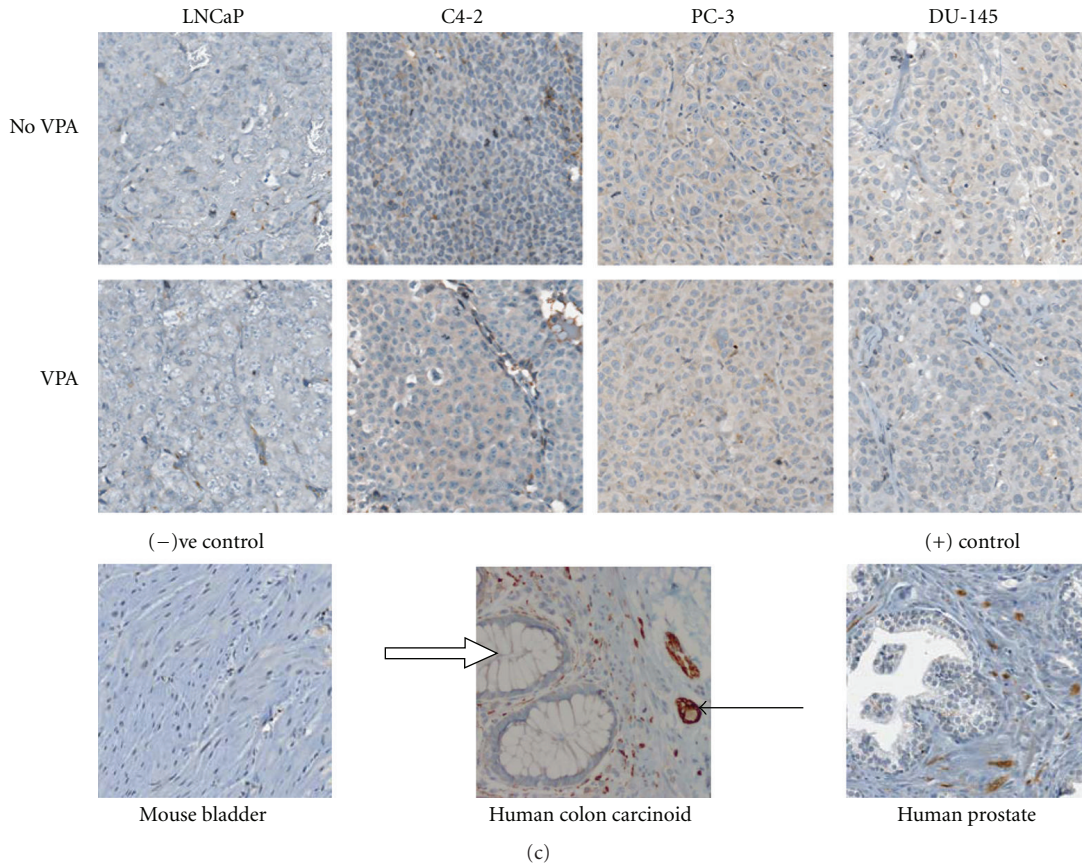

Figure 4: Chromogranin A staining in prostate xenografts animals treated with or without VPA. (a): Representative images of CgA staining for xenograft sections from control and VPA-treated groups: LNCaP, C4-2, PC-3, and DU-145 (Scanned at 20X magnification using the APERIO imaging system). The tissue specimens of human pancreas and human prostate were positive internal controls for CGA staining, while another human prostate section served as a negative control. (b) Synaptophysin staining in prostate xenografts animals treated with or without VPA. A: Representative images of synaptophysin staining for xenograft sections from control and VPA-treated groups: LNCaP, C4-2, PC-3, and DU-145 (Scanned at 20X magnification using the APERIO imaging system). The tissue specimens of human pancreas and mouse colon were positive internal controls for synaptophysin staining, while mouse bladder as negative internal controls. (c) NCAM staining in prostate xenografts animals treated with or without VPA. (a): Representative images of CD56 staining for xenograft sections from control and VPA-treated groups: LNCaP, C4-2, PC-3, and DU-145 (scanned at 20X magnification using the APERIO imaging system). The tissue specimens of human colon carcinoid and normal human prostate were positive internal controls for NCAM staining, while mouse bladder was a negative control.

staining in either treatment or control arms in all cell lines (Figure 4(a)). TMAs from C4-2 tumors revealed decreased expression of both synaptophysin (mean weighted score 47(±10) versus 15(±5), $P < .001$) and NCAM (44(9) versus 5(6), $P = .002$) in treatment arms (Figure 4(b)). None of the other arms revealed any significant staining (weighted scores less than 30) for NCAM or synaptophysin (Figures 4(b) and 4(c)). Thus VPA does not induce any NE markers in the physiologically relevant *in vivo* setting.

4. Discussion

NE cells are considered to be derived from local stem cells and are an example of normal, terminally-differentiated cells without proliferative activity [6]. NE cells in tumor lesions are phenotypically similar to NE cells in normal prostate epithelium in terms of expression of neuropeptides and biogenic amines. Furthermore, dual epithelial characteristics such as prostatic acid phosphatase and/or PSA production and NE marker expression, such as CgA, are frequently coexpressed in the malignant phenotype of NE cells [23]. Studies evaluating the role of focal NE differentiation in PCa prognosis have reported varied results: some reports indicate a negative correlation with prognosis while some show little or no relationship to prognosis [10, 23–28].

Histone deacetylase inhibitors are a promising new class of cancer therapy which have antiproliferative and prodifferentiation properties. For prostate cancer, it was recently reported that HDAC gene expression is elevated in tumors. Moreover, high expression levels of HDAC2 were associated with poor prognosis [29]. Thus VPA, which is capable of inhibiting HDACs classes I and IIa, may be a good

option for PCa therapy. In preclinical models, VPA treatment leads to proliferation arrest and differentiation and apoptosis of cancer cells of various tissue origin, while nominal effects were reported in normal cells [2, 4, 5]. However, Valentini et al. reported VPA to cause an increase in the secretion of NSE in LNCaP cells (*in vitro*) which may indicate an NE differentiation [12]. In order to better understand the role of VPA in possibly stimulating NE differentiation in PCa cell models, we selected the clinically recommended panel of antibodies for the IHC investigation of NE cells in multiple PCa cell lines and xenograft tumors.

Chromogranin A or parathyroid secretory protein 1 is a member of the chromogranin-secretogranin family and forms the major constituent in neurosecretory peptide containing dense core granules in NE cells. CgA is highly expressed by cells of neuroendocrine origin, both normal and tumoral, functional and nonfunctional. While Neuron Specific Enolase (NSE) has also been used as an NE marker, it is known to be expressed in a variety of non-NE cells and tumors, which has led researchers to question its specificity [30, 31]. Serum CgA levels, on the other hand, have been reported to be better predictors of neuroendcocrine differentiation than NSE [32, 33]. Thus, CgA now is widely regarded as an excellent and more specific marker of NE differentiation. In our study, CgA expression was not detected by IHC in either control or treated groups in human prostate cell lines of LNCaP, C4-2, PC-3, and DU145 *in vitro* or *in vivo.* Western blotting, being more sensitive, revealed low CgA expression in these cell lines; which reduced further with VPA treatment in a dose-dependent manner. Histone acetylation and p21 induction (data not shown) confirmed that active VPA doses were achieved as we have previously demonstrated [5, 34]. Our results are further corroborated by similar reduction in NE markers and NE morphology in NE tumors after treatment with HDACIs [13, 14, 16]. These studies in fact report apoptotic effects of VPA on neuroendocrine cells. Yu et al. have previously demonstrated CgA to be an important neuropeptide promoting the growth of prostate cancer cells and its suppression leading to programmed cell death in multiple prostate cell lines [35]. Gong et al. later found antiapoptotic effects of CgA to be dependent on a Protein Kinase B/Akt (an antiapoptotic protein or prosurvival factor) mediated pathway [36]. Also HDACIs have been known to downregulate Akt phosphorylation in prostate cancer cells [37] Taken together, it suggests a link between HDACI's-mediated apoptosis and CgA inhibition. Further studies will be required to determine the contribution of CgA and Akt to the VPA therapeutic effect.

Synaptophysin and NCAM are other specific and fairly sensitive markers for NE differentiation [38, 39]. In *in vitro* experiments, these markers showed varying trends (increased synaptophysin staining in C4-2 and PC-3 cells but unaltered in LnCaP and DU145; increased NCAM in LnCAP and PC-3 cells but unaltered in C4-2 and DU145), and no consistent pattern was seen. In *in vivo* experiments, the staining did not reveal any significant expression of these markers in any of the xenografts except in C4-2 tumors where it revealed a downward trend on treatment.

5. Conclusion

The findings in our study do not support any neuroendocrine differentiating role of VPA. On the contrary, CgA, a very specific marker, was reduced in all studied cell lines, following chronic VPA treatment. Synaptophysin and NCAM showed some inconsistent induction following VPA treatment in some cell lines but, *in vivo*, VPA treatment did not induce any significant expression of any NE markers. 0.4% VPA in mouse drinking water can achieve plasma VPA levels similar to the therapeutic levels obtained in human patients. TMAs from xenografts of different cell lines either did not stain for NE markers or had very little staining without any induction on VPA treatment. Thus, our data clearly demonstrates that VPA does not induce NE differentiation of PCa cells in the physiologically relevant *in vivo* setting.

Acknowledgment

A. Sidana and M. Wang contributed equally to the work.

References

[1] M. Dokmanovic, C. Clarke, and P. A. Marks, "Histone deacetylase inhibitors: overview and perspectives," *Molecular Cancer Research*, vol. 5, no. 10, pp. 981–989, 2007.

[2] P. A. Konstantinopoulos, M. V. Karamouzis, and A. G. Papavassiliou, "Focus on acetylation: the role of histone deacetylase inhibitors in cancer therapy and beyond," *Expert Opinion on Investigational Drugs*, vol. 16, no. 5, pp. 569–571, 2007.

[3] K. B. Glaser, "HDAC inhibitors: clinical update and mechanism-based potential," *Biochemical Pharmacology*, vol. 74, no. 5, pp. 659–671, 2007.

[4] S. Shabbeer, M. S. Q. Kortenhorst, S. Kachhap, N. Galloway, R. Rodriguez, and M. A. Carducci, "Multiple molecular pathways explain the anti-proliferative effect of valproic acid on Prostate cancer cells in vitro and in vivo," *The Prostate*, vol. 67, no. 10, pp. 1099–1110, 2007.

[5] Q. Xia, J. Sung, W. Chowdhury et al., "Chronic administration of valproic acid inhibits Prostate cancer cell growth in vitro and in vivo," *Cancer Research*, vol. 66, no. 14, pp. 7237–7244, 2006.

[6] P. A. di Sant'Agnese, K. L. de Mesy Jensen, C. J. Churukian, and M. M. Agarwal, "Human prostatic endocrine-paracrine (APUD) cells. Distributional analysis with a comparison of serotonin and neuron-specific enolase immunoreactivity and silver stains," *Archives of Pathology and Laboratory Medicine*, vol. 109, no. 7, pp. 607–612, 1985.

[7] A. Komiya, H. Suzuki, T. Imamoto et al., "Neuroendocrine differentiation in the progression of Prostate cancer," *International Journal of Urology*, vol. 16, no. 1, pp. 37–44, 2009.

[8] P. A. di Sant'Agnese, "Neuroendocrine differentiation in carcinoma of the Prostate: diagnostic, prognostic, and therapeutic implications," *Cancer*, vol. 70, no. 1, pp. 254–268, 1992.

[9] D. E. Frigo and D. P. McDonnell, "Differential effects of Prostate cancer therapeutics on neuroendocrine transdifferentiation," *Molecular Cancer Therapeutics*, vol. 7, no. 3, pp. 659–669, 2008.

[10] S. F. Slovin, "Neuroendocrine differentiation in Prostate cancer: a sheep in wolf's clothing?" *Nature Clinical Practice Urology*, vol. 3, no. 3, pp. 138–144, 2006.

[11] D. Hirano, T. Jike, Y. Okada et al., "Immunohistochemical and ultrastructural features of neuroendocrine differentiated carcinomas of the Prostate: an immunoelectron microscopic study," *Ultrastructural Pathology*, vol. 29, no. 5, pp. 367–375, 2005.

[12] A. Valentini, M. Biancolella, F. Amati et al., "Valproic acid induces neuroendocrine differentiation and UGT2B7 up-regulation in human Prostate carcinoma cell line," *Drug Metabolism and Disposition*, vol. 35, no. 6, pp. 968–972, 2007.

[13] J. T. Adler, D. G. Hottinger, M. Kunnimalaiyaan, and H. Chen, "Histone deacetylase inhibitors upregulate Notch-1 and inhibit growth in pheochromocytoma cells," *Surgery*, vol. 144, no. 6, pp. 956–962, 2008.

[14] D. Y. Greenblatt, A. M. Vaccaro, R. Jaskula-Sztul et al., "Valproic acid activates Notch-1 signaling and regulates the neuroendocrine phenotype in carcinoid cancer cells," *Oncologist*, vol. 12, no. 8, pp. 942–951, 2007.

[15] C. S. Platta, D. Y. Greenblatt, M. Kunnimalaiyaan, and H. Chen, "Valproic acid induces Notch1 signaling in small cell lung cancer cells," *Journal of Surgical Research*, vol. 148, no. 1, pp. 31–37, 2008.

[16] J. T. Adler, D. G. Hottinger, M. Kunnimalaiyaan, and H. Chen, "Combination therapy with histone deacetylase inhibitors and lithium chloride: a novel treatment for carcinoid tumors," *Annals of Surgical Oncology*, vol. 16, no. 2, pp. 481–486, 2009.

[17] J. T. Adler, D. G. Hottinger, M. Kunnimalaiyaan, and H. Chen, "Inhibition of growth in medullary thyroid cancer cells with histone deacetylase inhibitors and lithium chloride," *Journal of Surgical Research*, vol. 159, no. 2, pp. 640–644, 2010.

[18] D. M. Turnbull, M. D. Rawlins, D. Weightman, and D. W. Chadwick, "Plasma concentrations of sodium valproate: their clinical value," *Annals of Neurology*, vol. 14, no. 1, pp. 38–42, 1983.

[19] S. Zha, W. R. Gage, J. Sauvageot et al., "Cyclooxygenase-2 is up-regulated in proliferative inflammatory atrophy of the Prostate, but not in Prostate carcinoma," *Cancer Research*, vol. 61, no. 24, pp. 8617–8623, 2001.

[20] S. Manley, N. R. Mucci, A. M. de Marzo, and M. A. Rubin, "Relational database structure to manage high-density tissue microarray data and images for pathology studies focusing on clinical outcome the Prostate specialized program of research excellence model," *American Journal of Pathology*, vol. 159, no. 3, pp. 837–843, 2001.

[21] D. A. Faith, W. B. Isaacs, J. D. Morgan et al., "Trefoil factor 3 overexpression in prostatic carcinoma: prognostic importance using tissue microarrays," *The Prostate*, vol. 61, no. 3, pp. 215–227, 2004.

[22] A. M. de Marzo, B. Knudsen, K. Chan-Tack, and J. I. Epstein, "E-cadherin expression as a marker of tumor aggressiveness in routinely processed radical Prostatectomy specimens," *Urology*, vol. 53, no. 4, pp. 707–713, 1999.

[23] J. Huang, C. Wu, P. A. di Sant'Agnese, J. L. Yao, L. Cheng, and Y. Na, "Function and molecular mechanisms of neuroendocrine cells in Prostate cancer," *Analytical and Quantitative Cytology and Histology*, vol. 29, no. 3, pp. 128–138, 2007.

[24] H. Bonkhoff, "Neuroendocrine differentiation in human Prostate cancer. Morphogenesis, proliferation and androgen receptor status," *Annals of Oncology*, vol. 12, supplement 2, pp. S141–S144, 2001.

[25] E. F. Tamas and J. I. Epstein, "Prognostic significance of Paneth cell-like neuroendocrine differentiation in adenocarcinoma of the Prostate," *American Journal of Surgical Pathology*, vol. 30, no. 8, pp. 980–985, 2006.

[26] S. Shimizu, J. Kumagai, Y. Eishi et al., "Frequency and number of neuroendocrine tumor cells in Prostate cancer: no difference between radical Prostatectomy specimens from patients with and without neoadjuvant hormonal therapy," *The Prostate*, vol. 67, no. 6, pp. 645–652, 2007.

[27] Y. Yamada, K. Nakamura, S. Aoki et al., "Is neuroendocrine cell differentiation detected using chromogranin A from patients with bone metastatic Prostate cancer a prognostic factor for outcome?" *Oncology reports.*, vol. 15, no. 5, pp. 1309–1313, 2006.

[28] E. Ishida, M. Nakamura, K. Shimada, M. Tasaki, and N. Konishi, "Immunohistochemical analysis of neuroendocrine differentiation in Prostate cancer," *Pathobiology*, vol. 76, no. 1, pp. 30–38, 2009.

[29] W. Weichert, A. Röske, V. Gekeler et al., "Histone deacetylases 1, 2 and 3 are highly expressed in Prostate cancer and HDAC2 expression is associated with shorter PSA relapse time after radical Prostatectomy," *British Journal of Cancer*, vol. 98, no. 3, pp. 604–610, 2008.

[30] S. Pahlman, T. Esscher, and K. Nilsson, "Expression of γ-subunit of enolase, neuron-specific enolase, in human non-neuroendocrine tumors and derived cell lines," *Laboratory Investigation*, vol. 54, no. 5, pp. 554–560, 1986.

[31] H. Haimoto, Y. Takahashi, T. Koshikawa, H. Nagura, and K. Kato, "Immunohistochemical localization of γ-enolase in normal human tissues other than nervous and neuroendocrine tissues," *Laboratory Investigation*, vol. 52, no. 3, pp. 257–263, 1985.

[32] A. Angelsen, U. Syversen, O. A. Haugen, M. Stridsberg, O. KR. Mjølnerød, and H. L. Waldum, "Neuroendocrine differentiation in carcinomas of the Prostate: do neuroendocrine serum markers reflect immunohistochemical findings?" *The Prostate*, vol. 30, no. 1, pp. 1–6, 1997.

[33] N. Kamiya, H. Suzuki, K. Kawamura et al., "Neuroendocrine differentiation in stage D2 Prostate cancers," *International Journal of Urology*, vol. 15, no. 5, pp. 423–428, 2008.

[34] M. S. Q. Kortenhorst, S. Isharwal, P. J. van Diest et al., "Valproic acid causes dose- and time-dependent changes in nuclear structure in Prostate cancer cells in vitro and in vivo," *Molecular Cancer Therapeutics*, vol. 8, no. 4, pp. 802–808, 2009.

[35] D.-S. Yu, D.-S. Hsieh, and S.-Y. Chang, "Modulation of Prostate carcinoma cell growth and apoptosis by chromogranin A," *Journal of Urology*, vol. 170, no. 5, pp. 2031–2035, 2003.

[36] J. Gong, J. Lee, H. Akio, P. N. Schlegel, and R. Shen, "Attenuation of apoptosis by chromogranin A-induced Akt and survivin pathways in Prostate cancer cells," *Endocrinology*, vol. 148, no. 9, pp. 4489–4499, 2007.

[37] C.-S. Chen, S.-C. Weng, P.-H. Tseng, H.-P. Lin, and C.-S. Chen, "Histone acetylation-independent effect of histone deacetylase inhibitors on Akt through the reshuffling of protein phosphatase 1 complexes," *Journal of Biological Chemistry*, vol. 280, no. 46, pp. 38879–38887, 2005.

[38] S. M. Jensen, A. F. Gazdar, F. Cuttitta, E. K. Russell, and R. I. Linnoila, "A comparison of synaptophysin, chromogranin, and L-dopa decarboxylase as markers for neuroendocrine differentiation in lung cancer cell lines," *Cancer Research*, vol. 50, no. 18, pp. 6068–6074, 1990.

[39] K. Takamatsu, B. Auerbach, R. Gerardy-Schahn, M. Eckhardt, G. Jaques, and N. Madry, "Characterization of tumor-associated neural cell adhesion molecule in human serum," *Cancer Research*, vol. 54, no. 10, pp. 2593–2603, 1994.