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Original Article

The effects of valproic acid on the mRNA expression of Natriuretic Peptide Receptor A and KQT-like subfamily Q-1 in human colon cancer cell lines

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ABSTRACT

Aim and objectives: The histone deacetylase (HDAC) inhibitor, Valproic Acid (VPA), causes growth inhibition and apoptosis in colorectal cancer cells. HDAC inhibition is associated with the transcriptional regulation of Natriuretic Peptide Receptor-A (NPR-A). NPR-A regulates voltage-gated potassium channel, KQT-like subfamily Q, member 1 (KCNQ1). NPR-A and KCNQ1 are also involved in the initiation and propagation of cancer cells. In this study, we investigated the simultaneous expressional changes of NPR-A and KCNQ1 among VPA-treated colon cancer cells.

Materials and methods: Human colorectal cancer cells were cultured and treated with increasing concentrations of VPA at different time points. MTT viability test was conducted to evaluate the growth inhibition. Real Time RT-PCR was used to quantify differential mRNA expression of NPR-A and KCNQ1 genes. Two-way ANOVA and bonferroni post-tests were used to analyze data statistically.

Results: We showed that VPA treatment inhibits the growth of SW-480 cells more efficiently compared to HT-29. NPR-A and KCNQ1 genes were significantly upregulated upon VPA treatment in both cell lines ($P < 0.0001$).

Conclusion: The alteration of NPR-A and KCNQ1 genes were more ordered among SW-480 cancer cells. The expressional changes of KCNQ1 and NPR-A among VPA treated human colon cancer cells follow the same pattern in similar combinations. VPA could regulate the expression of KCNQ1 through altering the mRNA expression of NPR-A.

1. Introduction

Colorectal cancer (CRC) encompasses a heterogeneous and tumor specific complex of diseases resulting from different alterations in genetic and epigenetic molecular pathways.[1] It is the third most common cancer and third leading cause of cancer death in the United States.[1] Modification of histones is an epigenetic process which is involved in gene regulation while inhibitors of Histone deacetylases (HDACs) are introduced as a novel therapeutic class of drugs against different cancer types.[2,3] HDAC inhibitors regulate the expression of genes which are involved in specific biological responses such as apoptosis, immune regulation and angiogenesis.[4–6] Valproic Acid (VPA) is a branched, short-chain fatty acid which acts as a HDAC1 and HDAC2a inhibitor. VPA is an established anti-epileptic drug[7] which is shown to induce differentiation and apoptosis in a variety of carcinoma cells.[8,9] VPA has been also introduced as an antiproliferative compound in CRC, alone or in combination with other therapeutic strategies.[10]

HDAC inhibitors modulate the transcriptional regulation of Natriuretic Peptide Receptor-A (NPR-A).[11,12] NPR-A is overexpressed in numerous human cancer cells including colon adenocarcinoma[13–17] and could be introduced as a novel anticancer target.[15,18] Atrial Natriuretic Peptide (ANP) is the specific ligand for NPR-A[19] which regulates the proliferation of human gastric cancer cells via K+ voltage-gated channel subfamily Q member 1 (KCNQ1; also known as LQT1).[20] KCNQ1 has been also introduced as a novel regulator of cancer cell proliferation and migration in different cancer types.[20–22] Moreover, HDAC inhibitors are reported to be capable of regulating the transcription of ATP sensitive K+ (KATP) channels,[23] which has not been reported among

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human cancer cells previously. In the present study, we investigated the effect of VPA as a HDAC inhibitor on the expression of NPR-A and KCNQ1 and also the possible simultaneous alterations of these genes in response to VPA in colon adenocarcinoma cancer cell lines.

2. Materials and methods

2.1. Cell culture and treatments

HT-29 and SW-480 human colon adenocarcinoma cells were purchased from National Cell Bank of Iran (Pasteur Institute, Iran) and cultured in RPMI 1640 (Gibco, Life Technologies, USA). HCT-116 and Caco-2 colon adenocarcinoma cells were also purchased from National Cell Bank of Iran (Pasteur Institute, Iran) and cultured in DMEM (Gibco, Life Technologies, USA). All cell culture media were supplemented with 10% FBS, 100U/ml penicillin and 100 μg/ml streptomycin. Tissue culture flasks were incubated in a fully humidified atmosphere at 37 °C with 5% CO_2. 0.5×10^6 cells/mL were counted and seeded in each well of 6-well tissue culture plates followed by resuspension in complete growth media and incubated for 24 h in order to keep cells in logarithmic phase growth. HT-29 and SW-480 cells were treated with the most antiproliferative concentrations of VPA (Sigma-Aldrich, USA) (1 mM, 2 mM and 4 mM) with the least cytotoxicity effects on normal cells.[24] Treated and non-treated cells were then incubated for different time points (24, 48 and 72 h). Finally, the cells were detached using 0.025% trypsin-EDTA (Gibco, Life Technologies, USA) for consequent RNA extraction.

2.2. Total RNA extraction and Real Time RT-PCR

Total RNA was extracted using Biozol (Bioer, China) according to the manufacturer's protocol. To remove any genomic DNA contamination, 1 microgram of total RNA was used for DNase I (CinnaGen, Iran) digestion and were reverse transcribed to cDNA using random hexamer primers (Bioron, Germany). Real-time RT-PCR was performed with Bioer Real-time PCR detection system (Bioer Technology, China) and Bioron SYBR green master mix (Bioron, Germany). Human 18S ribosomal RNA (18s rRNA) was used as a consistent internal control for gene expression normalization.[25] Gene specific primers which were designed to span exons for KCNQ1, NPR-A and 18s rRNA are summarized in Table 1. All data are presented as fold changes compared to non-treated cells.

2.3. Cell viability (MTT) assay

The cells were counted, seeded in 96-well plate (10^4 cells/well) and incubated for 24 h in complete culture media. The cells were then treated with different increasing concentrations of VPA (1 mM, 2 mM and 4 mM) at different time intervals (24, 48 and 72 h). After each time point, the complete culture media was removed and a final volume of 80 μl of Phenol Red free RPMI 1640 (Gibco, Life Technologies, USA) with 20 μl of MTT solution (5 mg/mL) were added to each well and incubated for 4 h at 37 °C with 5% CO_2. 100 μl DMSO was then added to each well as a cell lysis solution. Percentage of cell viability was assessed by spectrophotometry at 570 nm using ELx800 Absorbance Reader (Biotek, USA).

Table1
Gene specific primers used for Real Time RT-PCR.

Primer (accession)	Sequence (5′ > 3′)	T_m	Amplicon size (bp)
KCNQ1 (NM_000218.2)	TGTCCACCATCGAGCAGTATG CCGTCCCGAAGAACACCAC	61.5	84
NPRA (NM_000906.3)	GTCAACACAGCCTCAAGA CCTTTGCCCTTCATTTCTAC	60.1	136
18srRNA (M10098)	CAGCCACCCGAGATTGAGCA TAGTAGCGACGGGCGGTGTG	60.7	252

2.4. Statistical analysis

All of the experiments for each sample were repeated in triplicates and data were demonstrated as means ± SE (Standard Error). Statistical software SPSS22.0 and Graphpad Prism 5.04 were used for data analysis. Two-Way ANOVA with Bonferroni post hoc test was used for comparing means of multiple samples. P-values lower than 0.05 were considered as statistically significant.

3. Results

3.1. The mRNA expression level of NPR-A and KCNQ1 among different human colon cancer cell lines

The expression levels of NPR-A and KCNQ1 were quantified among four different human colon adenocarcinoma cell lines including HCT-116, SW-480, HT-29 and Caco-2. NPR-A was overexpressed in SW-480 and HT-29 cells in comparison to HCT-116

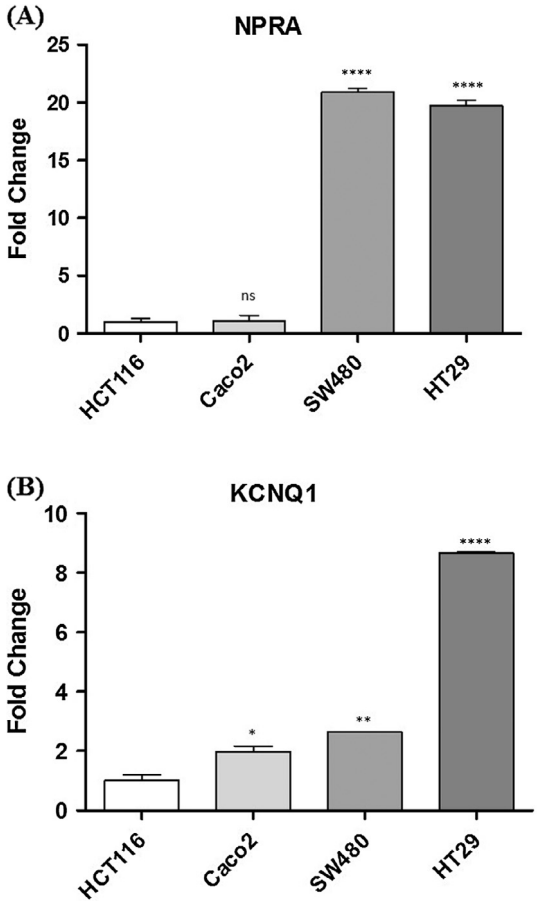

Fig. 1. The mRNA expression level of NPRA and KCNQ1. NPRA and KCNQ1 are overexpressed in HT-29 (A) and SW-480 (B) cell lines in comparison to other human colon cancer cells. Data are expressed relatively to mRNA levels in HCT 116 cell line, arbitrarily set at the value of 1. Data are obtained from experiments in triplicates and presented as means ± SE. Significant differences are evaluated using independent samples *t*-test. P-values lower than 0.05 are considered statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns: not significant.

(Fig. 1A). The expression of NPR-A among Caco-2 cells was not statistically different compared to HCT-116. KCNQ1 was also overexpressed among HT-29 and SW-480 cells (Fig. 1B). Accordingly, HT-29 and SW-480 with the highest expression of NPR-A and KCNQ1 were chosen for the next phase of the study.

3.2. VPA is an effective inhibitor of proliferation in human colon cancer cells

To measure the effects of VPA on cell viability, various increasing concentrations of VPA were applied to cell culture medium of SW-480 and HT-29 cancer cells at different time intervals (Fig. 2A). Percentages of viable cells were measured by MTT Assay. VPA treatment has reduced the viability of HT-29 (Fig. 2B) and SW-480 (Fig. 2C) cells significantly after 24, 48 and 72 h. VPA is a more effective inhibitor of proliferation on SW-480 cancer cells.

3.3. The expression pattern of NPR-A is different among HT-29 and SW-480 cell lines

During the first 24 h, NPR-A was significantly overexpressed in HT-29 cells at 1 mM concentration of VPA. After 48 h, NPR-A was downregulated by increasing the concentrations of VPA. However, no significant difference was observed. However, NPR-A was elevated gently with increasing VPA concentrations after 72 h (Fig. 3A).

Unlike HT-29 cells, NPR-A was overexpressed among SW-480 cells in an ordered manner after 24 and 48 h. SW-480 cells treated with 4 mM VPA showed the highest expression of NPR-A. After 72 h, the expression of NPR-A decreased in all treatments and was the same as non-treated SW-480 cells (Fig. 3B).

3.4. KCNQ1 is overexpressed among HT-29 and SW-480 cells in response to VPA

KCNQ1 was elevated gradually among HT-29 colon cancer cells proportional to the increasing concentrations of VPA after 24 and 48 h. HT-29 cancer cells showed the highest expression level of KCNQ1 at 4 mM VPA treatment after 48 h. KCNQ1 was downregulated after 72 h among VPA treated HT-29 cells except for 4 mM concentration (Fig. 4A). KCNQ1 increased significantly among SW-480 cells only after 48 h of incubation in all concentrations (Fig. 4B).

3.5. Concurrent alterations of KCNQ1 and NPRA in colon cancer cells

In order to estimate the probable simultaneous changes in the expression of NPR-A and KCNQ1 in human colon cancer cell lines, the expression pattern of all concentrations at each time point were adapted to its relevant. There was a correlation between the expressional changes of NPR-A and KCNQ1 among SW-480 cells. In all concentration of VPA and all tested time points, altering the expression of NPR-A directly affected the expression of KCNQ1.

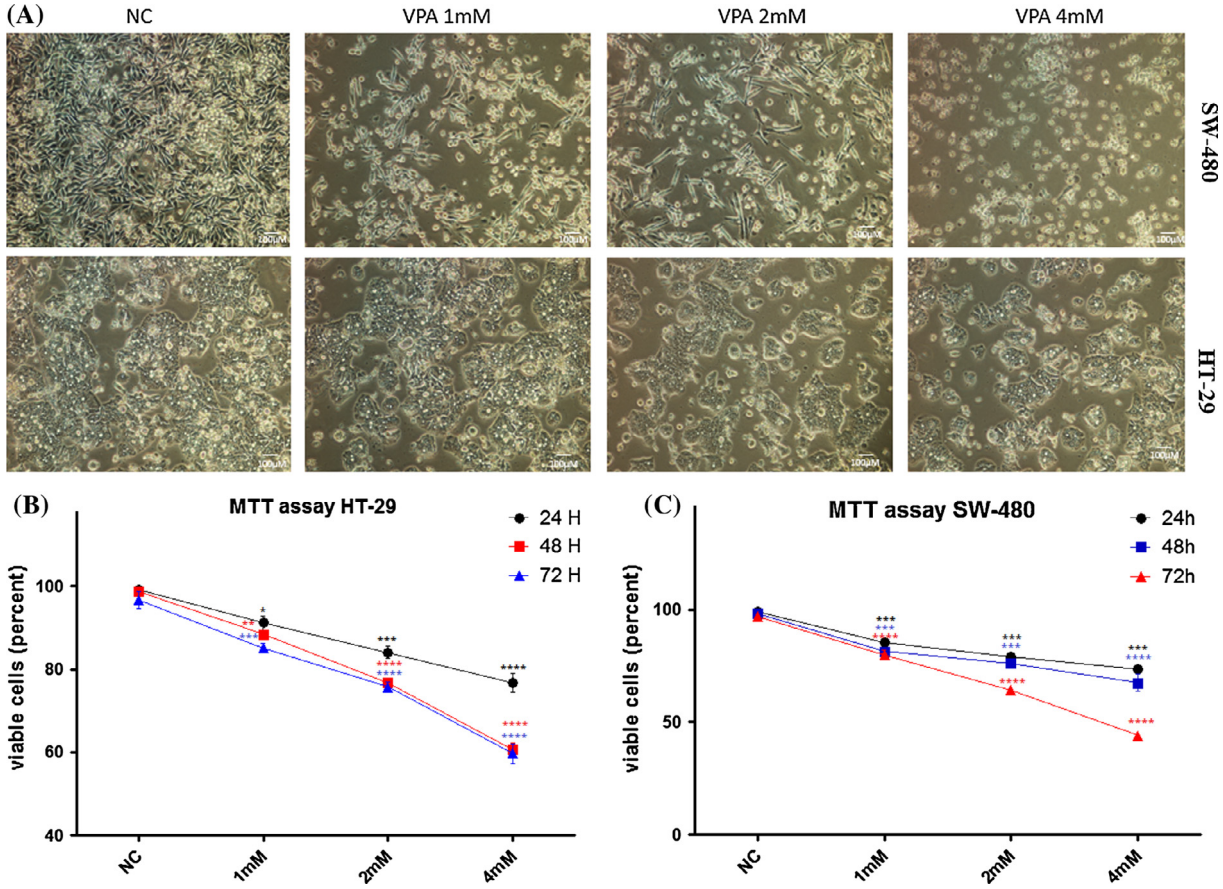

Fig. 2. Effects of VPA on cell proliferation in vitro. (A) Growth inhibition was observed upon VPA treatments (1 mM, 2 mM and 4 mM) at different time points (24 h, 48 h and 72 h) on HT-29 and SW480 cells. MTT assay was performed to determine the proliferation of HT-29 (B) and SW-480 (C) human colon cancer cells. VPA inhibited cell viability of SW480 and HT-29 cells in a concentration and time dependent manner. Data represent the mean ± SE from three independent experiments. Significant differences are evaluated using independent samples *t*-test. P-values lower than 0.05 are considered statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, NC: non-treated cells (mock).

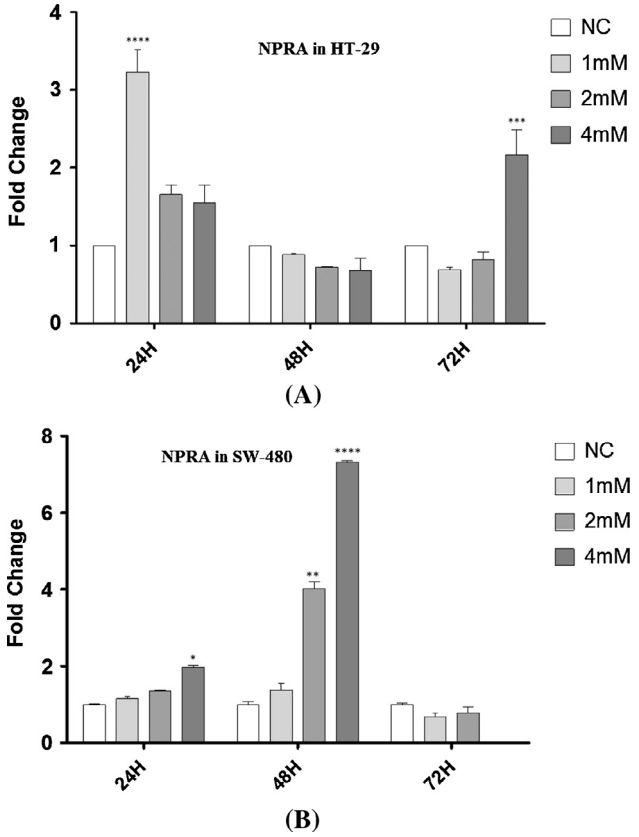

Fig. 3. NPRA expressional alterations. The mRNA expressional changes of NPRA were quantified by Real-Time RT-PCR in each combination representing different patterns among HT-29 (A) and SW-480 (B) human colon cancer cells. Data are expressed relatively to mRNA levels in non-treated (NC) cell lines, arbitrarily set at the value of 1. Data are obtained from experiments in triplicates and presented as means ± SE. Significant differences are evaluated using independent samples *t*-test or one-way ANOVA followed by bonferroni post hoc test while studying multiple comparisons. P-values lower than 0.05 are considered statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, NC: non-treated cells (mock).

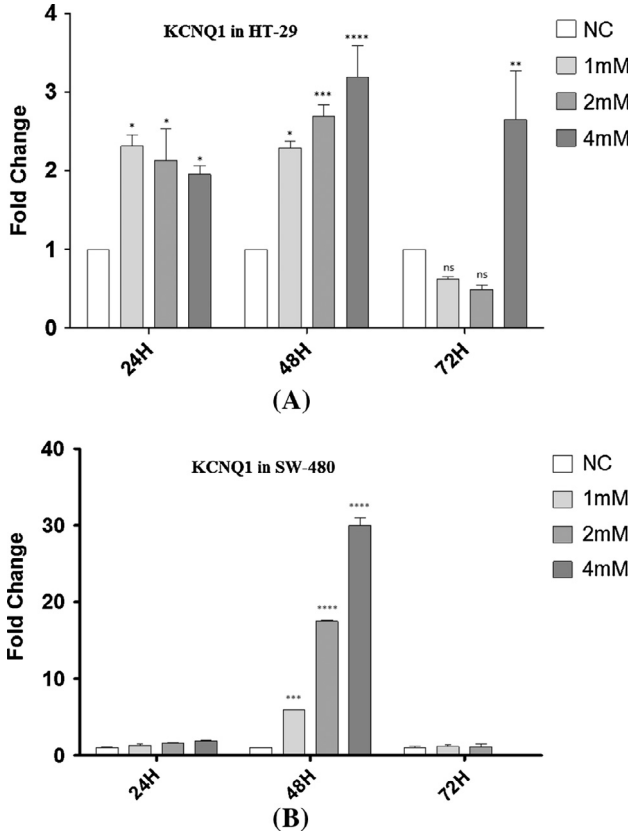

Fig. 4. Overexpression of KCNQ1 upon VPA treatment. The mRNA expression levels of KCNQ1 were quantified by Real-Time RT-PCR in each combination among HT-29 (A) and SW480 (B) human colon cancer cells. KCNQ1 is overexpressed in all VPA treated colon cancer cells. Data are expressed relatively to mRNA levels in non-treated (NC) cell lines, arbitrarily set at the value of 1. Data are obtained from experiments in triplicates and presented as means ± SE. Significant differences are evaluated using independent samples *t*-test or one-way ANOVA followed by bonferroni post hoc test while studying multiple comparisons. P-values lower than 0.05 are considered statistically significant. $^{***}P < 0.001$, $^{****}P < 0.0001$, NC: non-treated cells (mock).

Such a concordance between the expression of NPR-A and KCNQ1 is observed in HT-29 cells except for treatments at 4 mM VPA (Fig. 5).

4. Discussion

Histone deacetylation is an epigenetic modification in gene expression which has diverse effects on gene function, malignant cellular transformation, cell cycle and apoptosis.[5,26,27] These biological and pathological mechanisms are commonly modified by HDAC inhibitors which mostly possess anticancer properties.[2,3,11] VPA is a HDAC1 and HDAC2a inhibitor which has been utilized in the therapy of epilepsy for a long time.[7,8,23] VPA alongside with other HDAC inhibitors have shown promising antiproliferative effects in different cancers including CRC.[28–30] Moreover, the combination of VPA with conventional anticancer therapies has enhanced the effectiveness of current.[30,31]

Here, we conducted MTT viability test to measure the growth inhibitory effects of various concentrations of VPA on SW-480 and HT-29 cancer cells. The antiproliferative effects of VPA have been evaluated in different colon cancer cell lines. According to these reports, SW-480 is one of the most sensitive colon cancer cell lines to VPA treatment.[28] On the other hand, it has been reported that HT-29 as an APC mutant CRC cell line is less sensitive to VPA.[29] We showed that, VPA treatment has reduced the viability of SW-480 and HT-29 cells after each treatment. In accordance with the previous studies, we noted that VPA is a more effective proliferation inhibitor on SW-480 cancer cells compared to HT-29. However, VPA still preserves its growth inhibitory properties against HT-29 cells especially at higher concentrations.

NPR-A which is one of the specific receptors for ANP cardiac hormone is expressed in numerous human cancer cells including CRC.[15,18] NPR-A is involved in different physiological and pathological processes and has been recently introduced as a novel anticancer target.[20,32] However, there is no record available comparing the expression level of NPR-A among different human CRC cell lines.[33] In order to target the CRC cell lines with the overexpression of NPR-A, we examined the expression of NPR-A among four different human CRC cell lines including HCT-116, SW-480, HT-29 and Caco-2. The lowest expression was demonstrated in HCT-116. The expression level of NPR-A was higher among SW-480 and HT-29 cells compared to HCT-116. We also quantified the expression of KCNQ1 gene among all CRC cell lines. Similar to the expression pattern of NPR-A, the mRNA expression of KCNQ1 was higher in SW-480 and HT-29 cells. Therefore, SW-480 and HT-29, with different molecular characteristics, were selected to be studied. These CRC cell lines could be appropriate in vitro models to investigate NPR-A mediated novel anticancer strategies. The expression of NPR-A was not statistically different in Caco-2 compared to HCT-116.

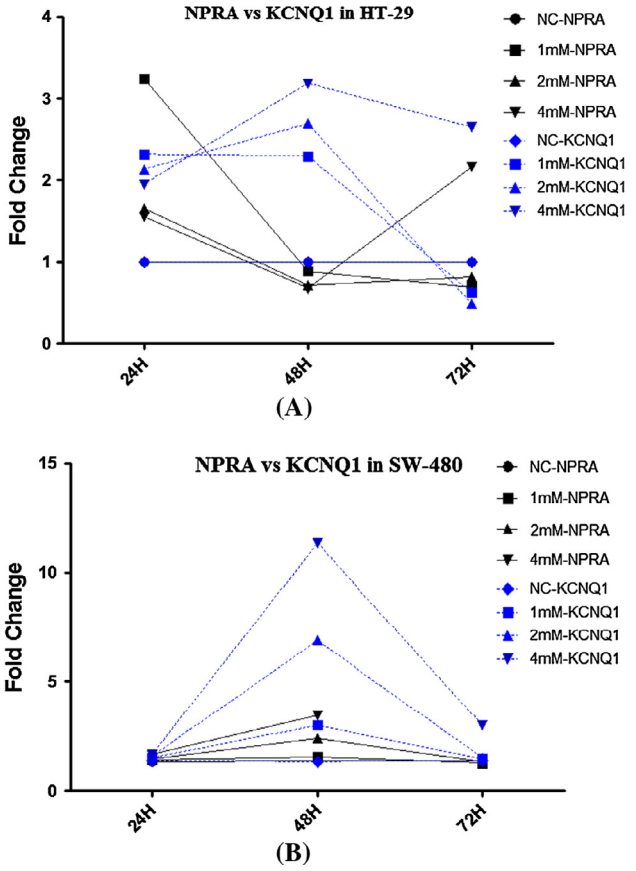

Fig. 5. NPRA and KCNQ1 simultaneous mRNA expression patterns. The quantified mRNA expression of NPRA and KCNQ1 genes were compared in order to derive a possible alteration pattern. We showed that the expressional changes in NPRA and KCNQ1 occur concurrently among SW-480 human colon cancer cells (B) and not HT-29 (A). Data are obtained from experiments in triplicates and presented as means. NC: non-treated cells (mock).

HDAC inhibitors have been shown to enhance NPR-A expression by blocking HDACs and interacting with Sp1, histone acetyltransferase, and acetylated histones.[11] Kumar et al claimed that Trichostatin-A (TSA) as a class I HDAC inhibitor modulates the transcriptional regulation of NPR-A gene.[12] On the other hand, it was revealed that NPR-A knockdown could downregulate the expression of voltage-gated potassium channel, KCNQ1.[20] KCNQ1 channel has been introduced as a novel regulator of cancer cell proliferation in many human cancer cells including CRC. HDAC inhibitors can regulate the ATP sensitive K+(KATP) channels subunit transcription in cardiomyocytes as well,[23] but to date no study has explained the possible effects of HDAC inhibition on the expression of voltage-gated K+ channels including KCNQ1.

We postulated that the effects of VPA might be carried out through regulating the expression of NPR-A and consequently KCNQ1. Therefore, we aimed to investigate the anticancer effects of various concentrations of VPA at different time points on selected CRC cell lines by quantifying the alterations in the expression of NPR-A and KCNQ1 genes and the possible relation between the these two genes.

VPA might have a more transient effect on the expression of NPR-A among HT-29 CRC cancer cells. We noted that, at lower concentrations of VPA which induce the least toxicity, NPR-A is overexpressed but an unclear mechanism (maybe a negative feedback) reverses the expression pattern of NPR-A after 24 h and the expression was reduced. Unlike HT-29 cells, the transcriptional effect of VPA on the expression of NPR-A is more persistent among SW-480 cells. The expression of NPR-A is finally decreased in all treatments and reaches the same as non-treated SW480 cells. Moreover, KCNQ1 is overexpressed among HT-29 and SW-480 colon cancer cells in response to VPA. This overexpression is more vividly observed among HT-29 cells in higher concentrations of VPA at all-time points. The expression level of KCNQ1 among SW-480 cells increased only after 48 h of incubation in all concentrations. The alterations in the expression of NPR-A and KCNQ1 among SW-480 cells occur simultaneously with the same pattern. This pattern was also observed in the expression of NPR-A and KCNQ1 among HT-29 cells with minimal irregularity. Accordingly, VPA alters the expression of NPR-A and KCNQ1 with the same pattern. However, these alterations are more ordered among SW-480 human CRC cells.

5. Conclusion

VPA mediates its effects on human CRC cell lines through altering the expression of NPR-A and KCNQ1 simultaneously. These alteration patterns are more ordered among SW-480 cancer cells. We propose the alterations in the expression of NPR-A and KCNQ1 as a novel molecular mechanism of effect for the effects of VPA in CRC. Our findings need to be investigated in further experiments using more sophisticated in vitro or in vivo examinations.

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