Effect of Valproic Acid on miRNAs Affecting Histone Deacetylase in a Model of Anaplastic Thyroid Cancer

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Abstract

Thyroid cancer is the most common malignant tumor of the endocrine system seen in the thyroid gland. More than 90% of thyroid cancers comprise papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC). Although anaplastic thyroid carcinoma (ATC) accounts for less than 2% of thyroid cancer. But patients' lifespan after diagnosis is about 6 months. Surgical interventions, radioactive iodine use, and chemotherapy are not sufficient in the treatment of ATC, so alternative therapies are needed. The WST-1 assay test was performed to evaluate the anti-proliferative effects of Valproic acid (VPA). Also, Effect of VPA on miRNAs affecting histone deacetylase were determined by Quantitative RT-PCR. In BRAFV600E / mut SW1736 (SW1736) cell line IC50 dose for VPA found 1.6 mg/ml. in our study, the level of oncogenic genes expression in cells treated with VPA, including miR-184, miR-222-5p, miR-124-3p, and miR-328-3p, decreased. Also, the expression of tumor inhibitory genes is miR-323-5p, miR-182-5p, miR-138-5p, miR-217, miR-15a-5p, miR-29b-3p, miR-324-5p and miR-101- 5p increased significantly. VPA can adjust countless gene expression patterns, including microRNAs (miRNAs), by targeting histone deacetylase (HDAC). We got very promising results with VPA.

Introduction

Thyroid cancer is the most common endocrine malignancy. Different types of cancer include papillary cancer (PTC), follicular cancer (FTC), medullary cancer (MTC), and anaplastic cancer (ATC), of which papillary cancer is the more common and the most dangerous type of anaplastic [1]. ATC has a poor prognosis because of the lack of early detection, its highly aggressive nature, as well as its failure with surgical interventions, radioactive iodine use and chemotherapy [2]. So that the survival rate is less than 25 weeks. Therefore, success in therapeutic approaches for thyroid cancer is crucial for patient survival [3].

MicroRNAs (miRNAs) are a group of regulators of gene expression. Which are endogenously synthesized and can affect the expression of genes. These molecules are single-stranded and noncoding RNAs so that in functional mode the length of these miRNAs does not exceed approximately 23 nucleotides [4]. Important point is that a miRNA can target multiple mRNAs and thereby show a regulatory effect on different genes. Besides, studies have shown that one mRNA is regulated by several miRNAs [5]. MiRNA expression differs in tissues. This difference in expression is also seen in a particularly healthy cell and its cancer cell, which may indicate the importance of miRNAs involved in the pathogenesis process [6]. Some miRNAs act as tumor suppressors or oncogenes by targeting cancer-related genes. They also play important roles in apoptosis, proliferation, migration, and cell invasion [7, 8]. MiRNAs decrease the expression of the target gene by reducing mRNA stability and/or preventing its translation [5, 9]. By reducing the abundance of specific proteins in this way, miRNAs exert management effects on many physiological processes. It is not surprising that their dysregulated expression may affect the neoplastic disease [10, 11].

Cancer is associated with many genetic and epigenetic changes. Genetic changes are seen in the number and order of nucleotides of DNA, while epigenetic changes occur in the structural proteins of DNA (histones). Epigenetic modifications can alter the transcription and translation of target genes and increase or decrease their functional level [12]. Histone deacetylase (HDACs) enzymes remove acetyl groups from histone lysine units and reduce gene expression by altering DNA density [13]. These histone modifications can regulate the expression status of cancer-related genes [14]. Studies have shown that overexpression of HMGA2 enhances the activity of transcription factors and growth signals. The result of this increase is the expression of tumor cell proliferation [15, 16]. Findings have shown that silencing LAMC2 in ATC cells reduces growth, migration, and cell invasion [17]. Use of HDAC inhibitors induces overexpression of Let 7 family miRNAs. This increase in expression by affecting HMGA2 leads to cell cycle blockage and cell death in cancer cells [18]. Therefore, HDACs inhibitors can be used as a treatment for cancer [19]. Valproic acid (VPA) is an anticonvulsant drug and inhibits HDAC [20]. It has been suggested that VPA binds to the catalytic site, inhibiting class I and IIa HDACs [21]. Because it is well tolerated and induces differentiation and apoptosis of carcinoma cells, it has recently been tested in clinical trials as a potential chemotherapeutic agent for a variety of cancers [22, 23]. VPA as an HDAC inhibitor changes the expression profile of miRNA in the ATC model. We examined the changes in miRNA expression that cause cancer treatment. The results showed that VPA could be a promising treatment for these patients.

Materials And Methods

Cell line and culture conditions. In our study, the effect of Valproic acid as an HDAC inhibitor on the BRAF^{V600E / mut} SW1736 (SW1736) cell line was evaluated by cell culture assays. These cells were commercially prepared as cancer cells (ATCC). For cell lines, RPMI 1640 (Biological industries) medium with L-Glutamine supported by %10 fetal bovine serum (FBS) (Biological industries) containing 2mM L-Glutamine (Biological industries), 1% penicillin-streptomycin (Biological industries) was used; incubated at 37°C, 95% of humidity and 5% CO₂ until adequate cell density was obtained; and follow-ups were carried out using “inverted” microscope.

Cytotoxicity calculation. Individual effects of the active agents on cells were specified using Colorimetric WST-8 Assay Kits. This procedure lasted for three days and three replications were performed for the control group and different treated doses. For cytotoxicity experiments, (10x10⁴) cell/ml was used for each well. Model cells were planted into 96 well plates; after the 24, 48, and 72-hour incubations following the addition of active agent solutions of appropriate concentrations, 10 µL/well WST-1 solution was added and incubated for 1 hour. Quantitative measurement of the formazan dye produced after the incubation was performed on a microplate reader (Multiskan FC, Thermo) every 15 minutes at 450 nm absorbance 620 nm reference range. IC₅₀ doses which are defined as the inhibitor concentrations causing a 50% decrease in cell proliferation were determined for Valproic acid on SW1736 cell lines was measured using Graph Pad Prism v8.0.2 (Graph Pad Software) software dependent on time and dose.

Determination of Gene Expression Changes. The expression changes produced by the IC₅₀ doses of active agents were determined with real time PCR in the 48 hours. For expression experiments, (5x10⁵) cell/ ml were plated into 6-well plates the specified dose was applied with dilutions suitable for the cells. The Active agent was not applied to the cells that were accepted as the control group. MiRNA isolation was obtained from each cell line with miRNeasy mini Kit (qiagen) and cDNA synthesis was performed using RT² First Strand Kit (miScript II RT kit). The expression changes of miRNAs were evaluated using the MIHS-108ZF plate (giagen).

Data Analyses. The statistical analysis of the cytotoxic effect of Valproic acid and change of gene expression in SW1736 cells was performed by using Graph pad Prism v8.0.2 and xCELLigence® RTCA (Roche) software, respectively. The $\Delta\Delta CT$ method was used for the miRNA fold change calculations.

Results

Evaluation of the Effects of Valproic acid on Cell Viability on the SW1736 Cell Lines via WST-1. In order to specify the Valproic acid IC₅₀ value, Valproic acid dilutions of (10⁻³- 10 mg/ml) were evaluated with WST-1 test in the 24th, 48th, and 72nd hours for SW1736 cell lines. Valproic acid IC₅₀ values on SW1736 cell lines were found as 1.6 mg/ml in the 48th hour (Fig. 1).

Gene Expression Changes caused by Valproic acid in SW1736. When the gene changes of the SW1736 cells exposed to the IC₅₀ dose of (1.6 mg/μM) Valproic acid in the 48th hour were compared to the control group, it was determined that Valproic acid increased miR-323-5p, miR-182-5p, miR-138-5p, miR-217, miR-15a-5p, miR-29b-3p, miR-324-5p, miR-101-5p, expressions 4.51 fold, 3.51 fold, 3.23 fold, 3.20 fold, 2.91 fold, 2.45 fold, 2.45 fold, 2.40 fold respectively while decreasing miR-184, miR-222-5p, miR-124-3p, miR-328-3p expressions 0.24 fold, 0.25 fold, 0.31 fold, 0.40 fold respectively in SW1736 cell (Fig. 2).

Discussion

The number of patients with ATC makes up a small fraction of thyroid cancer, but the survival rate is often less than one year. Therefore, appropriate treatments are desperately needed. Mutations in BRAF are commonly seen in thyroid carcinoma [24]. Understanding the role of epigenetic changes in thyroid cancer can provide valuable opportunities to identify molecular mechanisms to provide novel therapeutic approaches. Epigenetic changes can affect miRNA expression [25]. MiRNAs can have regulatory effects on tumorigenesis, cancer cell proliferation, and other physiological processes [26]. They can act as tumor suppressors or oncogenes in different types of cancer because they can target different genes [7, 8]. Based on the findings, we examined the expression of mRNAs in the SW1736 cell line using microarrays.

In our study, increased expression of miR-217 in VPA-treated SW1736 cells showed that it could increase apoptosis of cells in anaplastic thyroid cancer. VPA increased the expression level of miR-217 by inhibiting HDAC. In a study by Zhu L and colleagues, it was found that MAPK1 is a target of miR-217. MiR-217 inhibits MAPK1 and reduces cell viability, migration, and invasion while enhancing cell

apoptosis. They have introduced miR-217 as a tumor suppressor gene in cervical cancer [27]. Another study found that miR-217 expression was different in normal pancreatic cells compared with pancreatic cancer cells. And the increased expression of miR-217 inhibits cell growth and invasion of cancer cells, in contrast to the effect on E2F3 that induces apoptosis [28]. Other studies have shown that miR-217 binds to its target mRNA and acts as a tumor suppressor gene in gastric and liver cancers [29, 30].

Studies have shown that miR-138 is less expression in thyroid cancer than in normal thyroid tissue [31]. Low expression of miR-138 is associated with increased cell migration. MiR-138-5p has been shown to directly target Vim (vimentin) in cancer cells. These changes allow cancer cells to migrate [32]. In our study, expression of this miRNA increased after treatment with VPA, indicating a positive effect of VPA on our studied cell line. And introduced miR-138 as a tumor suppressor gene.

The expression level of miR-15a in PTC tissue is lower than in normal tissue. Overexpression of miR-15a prevents the proliferation and invasion of PTC cells and induces apoptosis. Overexpression of miR-15a by targeting RET and AKT inhibits tumor growth [33]. In another study, increased expression of miR-15a-5p inhibited proliferation of HepG2 or SNU-182 cancer cells in vitro [34]. In our study, expression of miR-15a-5p was also increased after the treatment of the studied cell line. In fact, VPA increased miR-15a-5p expression by inhibiting HDACs. The increased expression of miR-15a-5p prevented the proliferation of the SW1736 cell line, in fact, this miRNA activated the apoptotic mechanisms in these cells.

The results of our study showed that miR-323a-5p expression was induced by VPA on the SW1736 cell line which induced apoptosis of cells. And showed that VPA has antitumor activity. In other words, VPA by inhibiting HDAC could increase 323-miR expression. This increase in expression suppresses the tumor. One group showed that miR-323a-5p has a tumor suppressor function in neuroblastoma cells. And increased levels of miR-323a-5p decrease the proliferation of neuroblastoma cells [35]. A study by Men Y and colleagues found that miR-323a-3p expression was lower in esophageal cancer cell lines than in healthy esophageal epithelial cells and inhibits cell proliferation. Besides, miR-323a-3p prevented cell migration and invasion in some of these cell lines. These results further revealed the tumor suppressor status for miR-323a-3p [36].

In contrast, some miRNAs act as oncogenes. The expression of these miRNAs in cancer cells is higher than in healthy cells. These miRNAs induce cell proliferation and suppress apoptosis in the cell, leading the cells to become cancerous. MiR-184 has an oncogenic role in SCC squamous cell carcinoma. In a study by Wong TS and colleagues, plasma miR-184 levels were significantly higher in patients with SCC compared to normal subjects. They induced apoptosis of these cells by inhibiting miR-184 in cancer cells of the tongue [37]. Another study showed that miRNA-184 levels were higher in the EGFRm-BM group than in the control group [38]. A team of researchers showed that miR-184 enhances the biological behavior of cancer by targeting FIH-1 in the human glioma cell line A172 [38]. In microarray data analysis we found that expression of this miRNA in our SW1736 cell line was higher than in the control group. Expression of miR-184 decreased after treatment with VPA. By targeting other genes, VPA decreases miR-184 expression. By reducing this miRNA activates the mechanisms that induce apoptosis.

The cell cycle is controlled by a variety of factors. One of these factors is p27, which suppresses the cell cycle. These factors involved in the cell cycle are regulated by miRNAs. The observed miR-222 targeting p27 enhances cell proliferation. And acts as an oncogene [39, 40]. Some studies confirmed the effects of increased miR-222 expression on cancer cell metastasis [41, 42]. Inhibition of miR-222 results in PTEN rearrangement. As a result, cell proliferation and invasion are significantly suppressed and apoptosis is increased [43]. In our study, this miRNA was also increased in the studied cell lines and was identified as an oncogene in line with previous studies. Our drug was able to reduce the expression of miR-222. We have shown that VPA inhibits miR-222 by epigenetic changes and induces apoptosis in cells.

The level of miR-328-3p in ovarian cancer stem cells (CSC) is very high and may play an important role in the metastasis of these cells. Inhibition of miR-328 in rat ovarian xenografts inhibited tumor growth and prevented tumor metastasis [44]. In our study, a decrease in miR-328 was observed with Valproic acid. Our data showed that our drug reduced the expression of this miRNA and had antitumor effects.

One study found that treatment with imatinib alone was not able to grow anaplastic cells in thyroid cancer cells, but that combination therapy (imatinib and VPA) had a cytostatic effect. This effect is mediated by stunted G1 growth and acts through p21 expression and impaired AKT phosphorylation [45]. So combination therapies can be very helpful.

Conclusion

The effective role of miRNA in targeting the numerous genes that reason cancer, is unknown, so further studies are needed. Many miRNAs have shown different expression levels in response to treatment with HDAC inhibitors. Some of them can enhance or decrease the antitumor response. However, further studies are needed to identify the precise mechanisms of miRNAs for antitumor activity. It is difficult to accurately identify clinical miRNAs because of their low stability, which is why it needs accurate verification. We suggest that the effect of VPA on miRNA and its association with the cell cycle, apoptosis, be considered in future studies. However, VPA is well tolerated and can induce tumor suppressor miRNAs such as mir-323a, mir-15-a, mir-318, mir-217 by inhibiting HDAC in ATC cells.

Declarations

Compliance with ethical standards

Conflict of interest

All authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Satisfaction with publication

All authors agree to publish the data of this study.

Authorship contribution statement

Nur Selvi Günel: Writing - original draft. **Nihal Birden:** Methodology. **Cansu Çalışkan Kurt:** Methodology, Data curation. **Bakiye Göker Bağca:** Data curation, Methodology. **Çığır Biray Avcı:** Conceptualization, Methodology, Writing - review & editing. **Behrouz Shademan:** Conceptualization, Writing - original draft. **Fatma Soğutlu:** Methodology. **Neslihan Pinar Ozates:** Methodology.

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Figures

Figure 1

IC50 doses of Valproic acid in the 24h (A) 48h (B) 72h (C) on SW1736 cells. IC50 dose of Valproic acid was determined as 1.6 mg/ml in the 48h hour on SW1736.

Figure 2

Changes of miRNAs expression in the SW1736 cell line after treatment with Valproic acid (A). Fold-Change is the normalized miRNA expression in each Test Sample divided the normalized miRNA expression in the Control Sample. The miRNAs affecting histone deacetylase in RT2 Profiler PCR Array gene table used in the study (B). The heat map of the gene expression changes of in (C).