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Therapeutic potential of 4-OH-coumarin in neuroblastoma: cellular, molecular, and epigenetic insights

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ABSTRACT

The effect of 4-OH-Coumarin, a warfarin derivate, on the cellular characteristics and metastasis of SH-SY5Y neuroblastoma cells and HUVEC cells was aimed to determine. After IC50 concentrations were detected wound healing and hematoxylin-eosin assays were performed on both cell lines. Ki-67, hTERT, PI3K, AKT, mTOR, HIF-1α, PINK1, Parkin, Cyt C, p53 gene expressions and piR-651, piR-823, miR-126 expressions were determined by RT-PCR. The proliferation, wound closure and survival decreased after 4-OH-Coumarin treatment (p<0.001). Ki-67, hTERT, PI3K, mTOR, HIF-1α, PINK1, Parkin, Cyt C and piR-823 expressions were decreased, while AKT, p53, piR-651, and miR-126 increased on SH-SY5Y (p<0.001). AKT (p<0.05), Parkin, and piR-651 expressions increased on only HUVEC cells (p<0.001). We believe that the study of various molecules that are secondary metabolites such as 4-OH-Coumarin may provide valuable data to observe the effects and mechanisms of new therapeutics that have the potential to be used for cancer treatment.

Keywords: 4-OH-Coumarin; Hematoxylin/Eosin; Migration; Neuroblastoma; Proliferation

INTRODUCTION

Coumarins, first isolated from *Dipteryx odorata Willd*, are secondary metabolites found naturally in various plant families and essential oils [1]. Clinically, coumarin derivatives are well-known agents in a variety of natural and synthetic drugs [2]. In addition to their use in the treatment of prostate cancer, renal cell carcinoma and leukemia, coumarins are being investigated as therapeutic candidates for different cancer types thanks to their anti-cancer activity [3]. The mechanism of coumarin derivatives is telomerase enzyme inhibition, protein kinase inhibition and downregulation of oncogene expression, or caspase-9-mediated apoptosis [4-7]. However, it has been emphasized in various previous studies that coumarin derivatives can stop the cell cycle of cancer cells in G0/G1 and G2/M phases by affecting p-glycoprotein [5, 8]. Hydroxycoumarins, which cause oxidative stress by triggering free radical formation, exhibit important roles in the inhibition of cancer proliferation [9]. Studies have shown that various

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hydroxycoumarin species have antiproliferative or cytotoxic activity in various cell lines [10, 11]. 4-OH Coumarin, a type of coumarin with a hydroxy group at the 4th position, is derived from vanilla and cinnamon and comprises a large class of phenolic compounds [12]. 4-OH Coumarin may be a potential therapeutic agent for liver cancer [13]. Compounds synthesized from 4-OH-Coumarin have inhibitory effects on the motility of lung cancer cells [14].

The PI3K/AKT/mTOR signaling pathway, which plays a role in processes such as proliferation, differentiation, angiogenesis, transcription and translation, is upregulated in many cancer types [15]. AKT protein, also known as protein serine/threonine kinase B, is pathologically active in neuroblastoma and this activation is associated with poor prognosis [16]. In addition to PI3K gene expression in neuroblastoma, the increased levels of phosphorylated AKT and phosphorylated mTOR [17, 18].

Parkin is a tumor suppressor protein with E3 ubiquitin ligase activity that is involved in the mitophagy pathway, the process of removing damaged mitochondria from the cell, and has been associated with various cancers [19, 20]. Parkin is activated by PTEN-induced kinase 1 (PINK1) before Parkin marks damaged mitochondria with ubiquitin. PINK1 searches for damaged mitochondria in the cell, accumulates on damaged mitochondria and stimulates their activation by phosphorylating outer mitochondrial membrane proteins and Parkin. PINK1 is a damage sensor in the mitophagy process, while Parkin acts as an effector protein in the process [21]. Parkin causes DNA damage and activation of Ataxia telangiectasia mutation (ATM), which in turn leads to activation of p53, a tumor suppressor protein [20]. Cytochrome C (Cyt C), released from mitochondria and stimulating the formation of the apaptosome complex, causes cells to death. The transcription factor HIF-1α, which enables cells to adapt to low oxygen conditions, increases cancer cell survival [22]. A feature of cancer cells is the maintenance of chromosomal telomere length, and the hTERT gene encodes the enzyme telomerase reverse transcriptase, which is involved in this process [23]. Ki-67 is used as a marker of proliferation in various cancers, especially breast cancer. In addition, a parallel expression profile with hTERT is observed [13]. The possible mechanism of Ki-67 is to suppress p53 and inhibit the expression of DNA repair genes, leading to the emergence of various oncogenic properties, especially proliferation [13].

Small non-coding RNAs are a family of epigenetic regulators that cause cellular changes at the mRNA level. PIWI Interacting RNA (piRNA) is a family of small non-coding RNAs that, although mainly involved in transposon silencing, are also transcriptionally active. Micro RNAs (miRNAs) are also small non-coding RNAs that alter gene expression both transcriptionally and post-transcriptionally. miRNAs and piRNAs are epigenetic factors that play important roles in carcinogenesis; while piR-651 is effective on metastatic characters, piR-823 and miR-126 are more involved in proliferation [24]. piR-651 was initially found to be effective in the proliferation, cell cycle and metastasis of gastric cancers [25], and it has been found to be more effective on metastasis with research on various cancers [26-29]. piR-823 exhibits different expression profiles (such as oncogenic or tumor suppressor) in different cancer types. piR-823 suppresses PINK1-Parkin-mediated mitophagy to some extent in colorectal cancer [30]. miR-126 is particularly effective in breast cancer and metastasis; while it increases the proliferation of benign breast cancer cells via PI3K/AKT/mTOR pathway, it has a short non-coding RNA profile that is effective in triggering metastasis and invasion characteristics in malignant breast cancer cells [31]. In this study, we aimed to observe the changes induced by 4-OH-Coumarin, a novel coumarin derivative, on the proliferation and cellular characteristics of SH-SY5Y neuroblas-toma cells in comparison with healthy human umbilical epithelial (HUVEC) cells.

MATERIALS AND METHODS

Cell Culture and 4-OH-Coumarin Treatment: Neuroblastoma cell line (SH-SY5Y; ATCC, USA) and healthy human umbilical vein endothelial cell line (HUVEC; ATCC, USA) were grown at 37°C in a 5% CO_2 incubator under appropriate medium conditions [10% Fetal

Bovine Serum (FBS; Gibco, USA) and Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) containing 1% Penicillin/streptomycin (Biowest, USA)] and passaged to 80% confluent. Before the experiments, they were removed with trypsin (Gibco; USA) and seeded on appropriate plates according to the experiment. 4-OH-Coumarin (Sigma, USA) was dissolved in dimethylsulfoxide (DMSO; Sigma, USA) and 20 µM, 10 µM, 5 µM, 2.5 µM and 1 µM 4-OH-Coumarin was applied to both SH-SY5Y cells and HUVEC cells, respectively. Furthermore, a sham group containing DMSO-containing medium was designed to observe the effect of solvent. Cells were incubated for the appropriate time according to the experiment to be performed.

Determination of IC50 Value of 4-OH-Coumarin in HUVEC and SH-SY5Y Cells: Incubated HUVEC and SH-SY5Y cells were seeded and incubated in each well of 96-well plate with 7×10^3 cells after being removed with trypsin. HUVEC and SH-SY5Y cells were treated with 4-OH-Coumarin at concentrations of 20 µM, 10 µM, 5 µM, 2.5 µM and 1 µM 24 hours after seeding (n=7 per each concentration group). We designed 7 groups: a sham group, and a control group, in which we did not perform any manipulation. The inhibitory concentration (IC50) in 4-OH-Coumarin treated cells was measured at the 24th, 48th and 72nd hours using the XTT (Biological Industries, Israel) method at 450 nm on a microplate reader (BioTek, Korea).

Hematoxylin-Eosin Staining: For Hematoxylin-Eosin staining, HUVEC and SH-SY5Y cells were seeded on chamber slides with $2x10^4$ cells per well (n=3 per assay). 4-OH-Coumarin at the optimal time and concentrations determined in the IC50 were then applied to HUVEC (24 h, 2.5 µM 4-OH-Coumarin) and SH-SY5Y (24 h, 10 µM 4-OH-Coumarin) cells and incubated. After the incubation period, for fifteen minutes, the slides were fixed in a 37% formaldehyde solution (Sigma Aldrich, Canada). Following fixation, the samples were dripped with hematoxylin (Sigma Aldrich, Canada) dye, incubated for ten minutes, and then rinsed with tap water. The slide was then dripped with eosin stain (Sigma Aldrich, Canada), incubated for one minute, and then rinsed with tap water. Lastly, the preparations were photographed and viewed at 10X magnification using an Olympus light microscope (Germany).

Total RNA Isolation, Reverse-Transcription to cDNA and Real Time Polymerase Chain Reaction (RT-qPCR): 4-OH-Coumarin treated HUVEC (2.5 µM) and SH-SY5Y (10 µM) cells were seeding in 6-well plates with 5×10^5 cells in each well. Total RNA was isolated from the cells (AnalitikJena, Germany) and translated into cDNA at 42°C for 60 min and 95°C for 5 min following the kit protocol (Nucleogene, Turkey). Ki-67, hTERT, PI3K, AKT, mTOR, HIF-1α, PINK1, Parkin, Cyt C and p53 (Supplementary Table 1; Oligomer, Turkey) gene expressions were determined by Real-Time PCR (Kogene Biotech, South Korea) using 5 min of pre-denaturation at 95°C, 40 cycles of denaturation at 95°C for 10 s, and 30 s of annealing/extension at 60°C (n=7 per each gene expression). By applying the same RT-qPCR conditions, the expressions of piR-651, piR-823 [32] and miR-126 [31], which are considered as epigenetic proliferation markers, were determined in both cell lines. ΔΔCT values were calculated with the data obtained [33]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Oligomer, Turkey) was used to calculate ΔΔCT values as an internal control (n=7 per each gene expression).

Wound Healing Assay: HUVEC and SH-SY5Y cells were seeded at 5×10^5 cells using 2×35 mm tissue culture petri dishes. After the cells were 80% confluent, the wound was created and optimal 4-OH-Coumarin concentrations previously determined for both cell lines in IC50 assay were applied. For each petri, the wound width was measured under a microscope with a micrometer scale ruler and recorded. Wound width of each group (n=7 for each group) was measured at the 0, 24th, 48th and 72nd hours (Turgut Cosan et al., 2016). At the same time, the image of each group was recorded during the measurement. The measurements obtained were calculated by substituting them in the formula below.

% MI (Motility Index) = 1 – [Wt (24/48/72 wound width determined in hours) / W0 (Initial wound width)] MI=1 full healing; MI=0 there is no healing.

Statistical Evaluations: Normal distribution of continuous variables was ensured using the Kolmogorov-Smirnov compatibility test. Comparisons between normally distributed variable groups were evaluated using one-way analysis of variance (ANOVA). Comparisons between variable groups that did not show normal distribution were evaluated using Kruskal-Wallis test. Sidak test was used for multiple comparisons. All analyses were performed using IBM SPSS Statistics 21.0 software package. The data obtained were expressed as mean ± standard deviation (SD). The correlation analysis was performed by using Pearson's Correlation test.

RESULTS

It was observed that 20, 10, 5, 2.5 and 1 µM 4-OH-Coumarin concentrations treated to HUVEC cells caused a decrease in cell proliferation at the 24th, 48th and 72nd hours compared to the control group (Fig. 1; $p < 0.001$). The IC50 value determined for HUVEC cells was determined as 2.5 µM 4-OH-Coumarin at the 24th hour (Fig. 1; $p < 0.001$). This optimal time and concentration were used for HUVEC cells in other experiments.

4-OH-Coumarin concentrations of 20, 10, 5, 2.5 and 1 µM applied to SH-SY5Y cells caused a decrease in cell proliferation at the 24th, 48th and 72nd hours compared to the control group. (Fig. 1, $p < 0.001$). At the 24th hour, proliferation in SH-SY5Y cells treated with 10 µM 4-OH-Coumarin (16955 ± 231) was determined as 50% inhibitory concentration in SH-SY5Y cells compared to the control group (29246 ± 517; Fig. 1A; $p < 0.001$). The other concentrations applied also caused a certain amount of cell death at the 24th, 48th and 72nd hours, but approximately 50% death was not more effective than 10 µM 4-OH-Coumarin at the 24th hour (Fig. 1A; $p < 0.001$). Therefore, this optimal time and concentration was used for SH-SY5Y cells in further experiments.

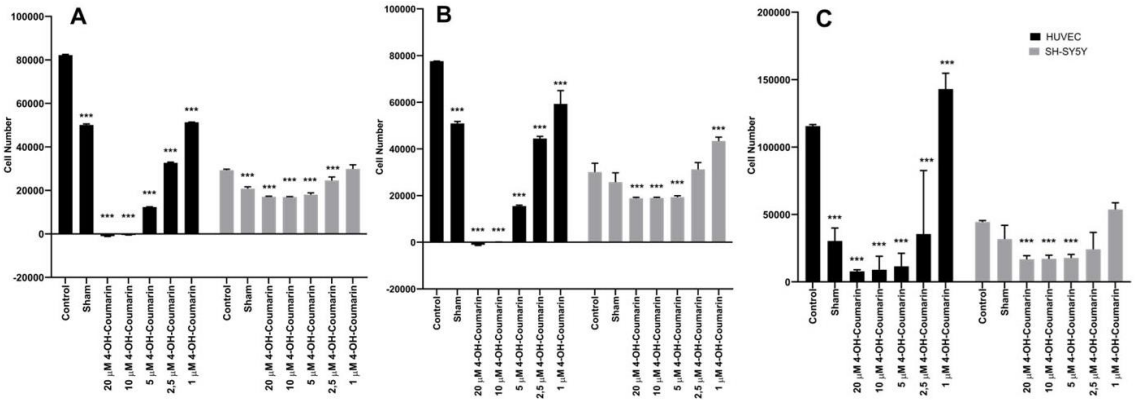

Figure 1: IC50 values measured after 24 (**A**), 48 (**B**), and 72 (**C**) hours of treatment with control, sham, 10 µM, 5 µM, 2.5 µM and1 µM 4-OH-Coumarin in HUVEC and SH-SY5Y cells (p<0.001).

The morphological and number of HUVEC cells in all groups did not show any differences. Furthermore, there weren't any morpholigical and number in control and sham groups of SH-SY5Y cells. However, we detected the decreased number and degenerated morphology after 4-OH-Coumarin treatment to SH-SY5Y cells (Fig. 2).

Ki-67 gene expression of 4-OH-Coumarin group applied to both HUVEC (1.681 ± 0.018) and SH-SY5Y cells (0.029 ± 0.003) was significantly decreased compared to the control group (9.228 ± 0.018; 6.653 ± 0.012; Fig. 3A; $p < 0.001$). hTERT gene expression was significantly downregulated in both HUVEC cells treated with 4-OH-Coumarin (1.142 ± 0.007) and treated

SH-SY5Y cells (0.031 ± 0.002) compared to the control group (3.117 ± 0.006; 0.245 ± 0.012; Fig. 3B; $p < 0.001$).

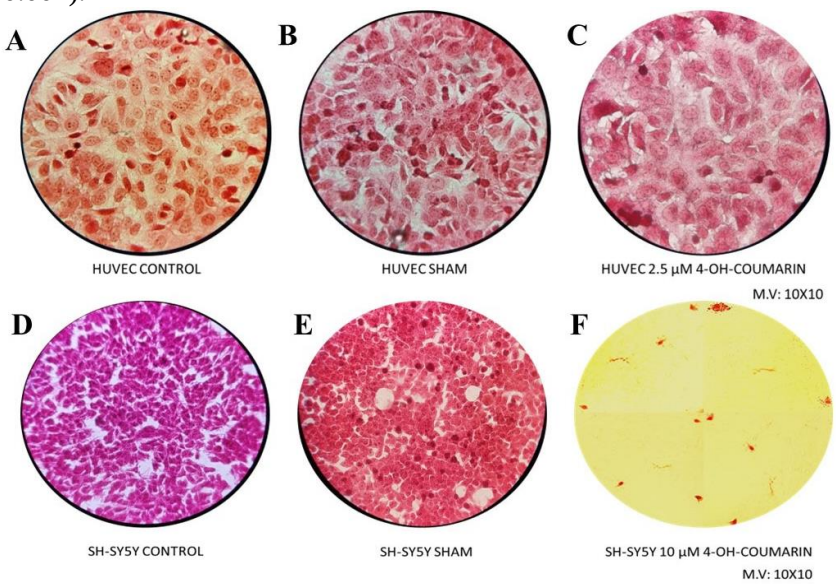

Figure 2: Hematoxylin-Eosin staining of HUVEC and SH-SY5Y cells. **A)** The number and the morphology of hematoxylin-eosin-stained Control HUVEC cells. **B)** The number and the morphology of hematoxylin-eosin-stained Sham HUVEC cells. **C)** The number and the morphology of hematoxylin-eosin-stained 4-OH-Coumarin Treated HUVEC cells. **D)** The number and the morphology of hematoxylin-eosin-stained Control SH-SY5Y cells. **E)** The number and the morphology of hematoxylin-eosin-stained Sham SH-SY5Y cells. **F)** The number and the morphology of hematoxylin-eosin-stained 4-OH-Coumarin Treated SH-SY5Y cells.

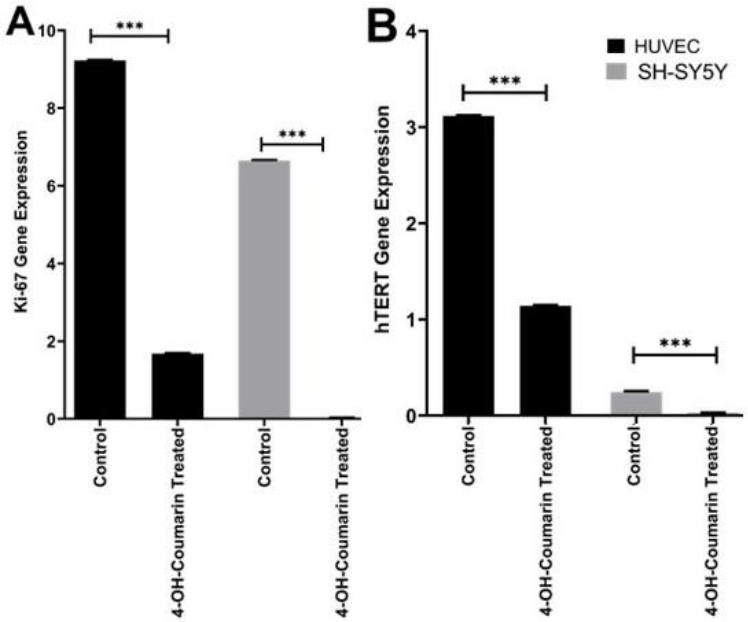

Figure 3: The gene expressions of Ki-67 and hTERT. **A)** Ki-67- Gene expressions in HUVEC and SH-SY5Y cells. **B)** hTERT Gene expressions in HUVEC and SH-SY5Y cells.

In both HUVEC and SH-SY5Y cells, PI3K gene expression was downregulated in 4-OH-Coumarin treated group (99.187 ± 1.262; 0.22 ± 0.019) compared to the control group (220.403 ± 0.359; 9.639 ± 0.58; Fig. 4A; $p < 0.001$). AKT gene expression was significantly upregulated in both HUVEC cells treated with 4-OH-Coumarin (7.062 ± 0.604; $p < 0.05$) and treated SH-SY5Y cells (0.417 ± 0.022) compared to the control group (5.918 ± 0.437; 0.147 ± 0.015; Fig. 4B; $p < 0.001$). Furthermore, mTOR gene expression was significantly upregulated in HUVEC

cells treated with 2.5 µM 4-OH-Coumarin (21.18 ± 0.274) compared to the control group (13.215 ± 0.847; Fig. 4C; $p < 0.001$). in SH-SY5Y cells treated with 10 µM 4-OH-Coumarin (0.001 ± 0.0001), mTOR gene expression was significantly downregulated compared to the control group (0.971 ± 0.01; Fig. 4C; $p < 0.001$).

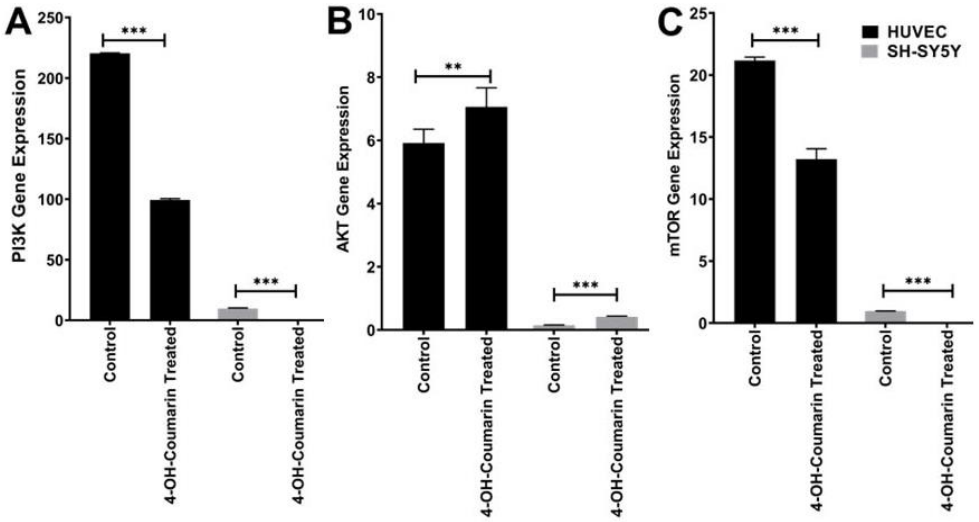

Figure 4: The gene expressions of PI3K, AKT and mTOR**. A)** PI3K Gene expressions in HUVEC and SH-SY5Y cells. **B)** AKT Gene expressions in HUVEC and SH-SY5Y cells. **C)** mTOR Gene expressions in HUVEC and SH-SY5Y cells.

HIF-1α gene expression was significantly decreased in both HUVEC group treated with 4-OH-Coumarin (0.734 ± 0.022) and treated SH-SY5Y cells (2.92 ± 0.017) compared to the control group (1.741 ± 0.012; 12.951 ± 0.007; Fig. 5A; $p < 0.001$). Parkin gene expression increased in 4-OH-Coumarin treated HUVEC cells (1.709 ± 0.101) compared to the control group (0.973 ± 0.06; Fig. 5B; $p < 0.001$) while a decrease in SH-SY5Y cells treated with 4-OH-Coumarin (0.0001 ± 0.00001) compared to the control group (0.057 ± 0.008; Fig. 5B; $p < 0.001$). PINK1 gene expression decreased in both 4-OH-Coumarin-treated HUVEC cells (99.182 ± 3.508) and in SH-SY5Y group (0.218 ± 0.011) treated with 4-OH-Coumarin compared to the control group (220.403 ± 1.94; 9.64 ± 0.023; Fig. 5C; $p < 0.001$).

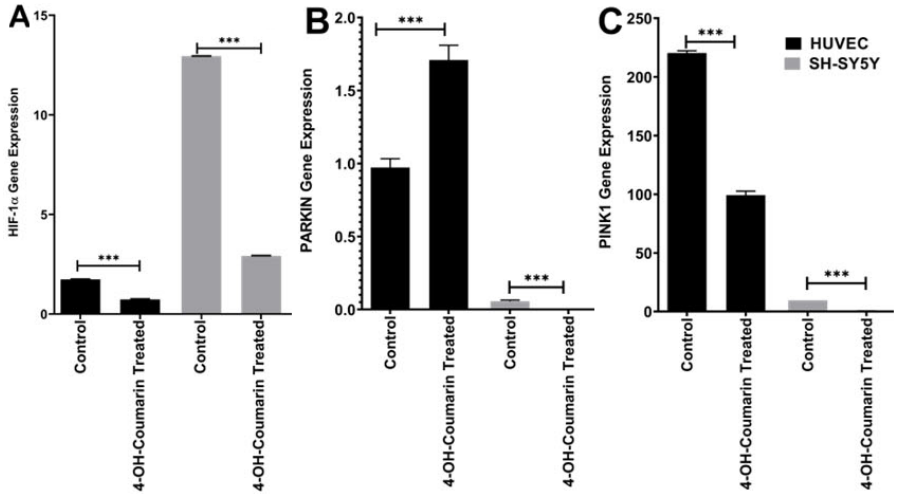

Figure 5: The gene expressions of HIF-1α, Parkin and PINK1. **A)** HIF-1α Gene expressions in HUVEC and SH-SY5Y cells. **B)** Parkin Gene expressions in HUVEC and SH-SY5Y cells. **C)** PINK1 Gene expressions in HUVEC and SH-SY5Y cells.

At the 24th hour, Cyt C gene expression decreased in both HUVEC cells treated with 4-OH-Coumarin (1.126 ± 0.039) and treated SH-SY5Y cells (0.545 ± 0.015) compared to the control

groups (2.912 ± 0.02; 0.792 ± 0.018; Fig. 6A; $p < 0.001$). p53 gene expression decreased in HUVEC cells treated with 2.5 µM 4-OH-Coumarin (4.432 ± 0.304) compared to the control group (40.899 ± 0.871) at the 24th hour (Fig. 6B; $p < 0.001$). An increased p53 gene expression was observed at the 24th hour in SH-SY5Y cells treated with 10 µM 4-OH-Coumarin (0.039±0.008) compared to the control group (0.016 ± 0.003; Fig. 6B; $p< 0.001$).

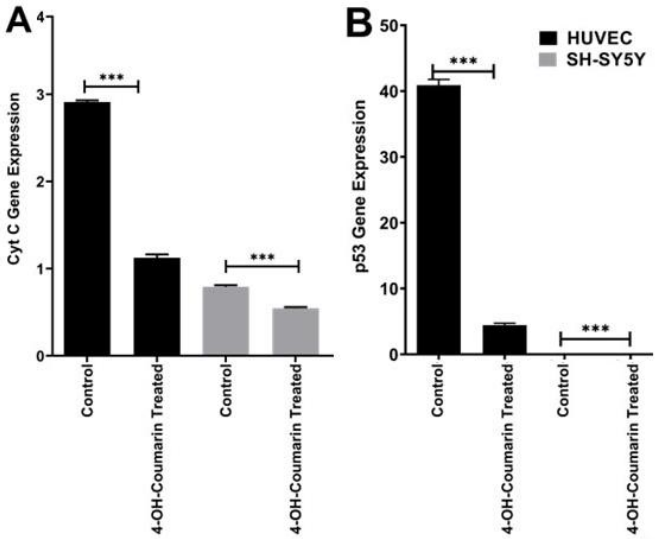

Figure 6: The gene expressions of Cyt C and p53. **A.** Cyt C Gene expressions in HUVEC and SH-SY5Y cells. **B.** p53 Gene expressions in HUVEC and SH-SY5Y cells.

piR-651 expression increased in both HUVEC cells treated with 2.5 µM 4-OH-Coumarin (9.507 ± 0.03) and SH-SY5Y cells treated with 10 µM 4-OH-Coumarin (13.965±0.387) compared to the control group (6.351 ± 0.024; 15.504 ± 0.058) at the 24th hour (Fig. 7A; $p < 0.001$). Obtained data showed that piR-823 expression decreased in both HUVEC cells treated with 2.5 µM 4-OH-Coumarin (0.074 ± 0.006) and SH-SY5Y cells treated with 10 µM 4-OH-Coumarin (4.443 ± 0.123) compared to the control groups (0.327 ± 0.018; 5.192 ± 0.132) at the optimal timeline (Fig. 7B; $p < 0.001$). Decreased expression of miR-126 was determined in HUVEC cells treated with 2.5 µM 4-OH-Coumarin (0.441 ± 0.032) and increased expression of miR-126 was detected in 10 µM 4-OH-Coumarin (0.675 ± 0.017) compared to the control groups (0.975 ± 0.014; 0.164 ± 0.013) at the 24th hour (Fig. 7C; $p < 0.001$).

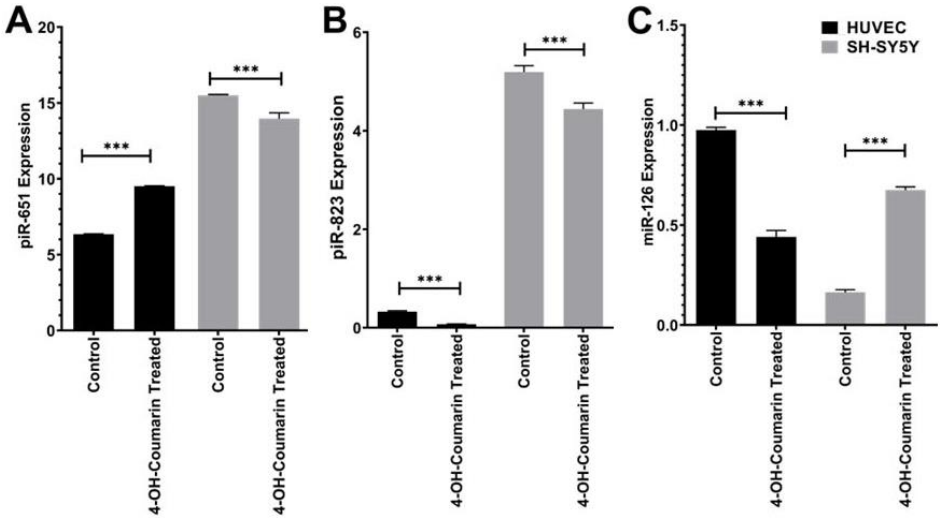

Figure 7: The expressions of piR-651, piR-823 and miR-126. **A)** piR-651 expressions in HUVEC and SH-SY5Y cells. **B)** piR-823 expressions in HUVEC and SH-SY5Y cells. **C)** miR-126 expressions in HUVEC and SH-SY5Y cells.

In HUVEC control group, piR-651 and piR-823 indicated a strong positive correlation (r = 0.820; $p < 0.05$; Fig. 8A). Furthermore, there was a weak negative correlation observed between piR-823 and miR-126 (r = -0.193; $p > 0.05$; Fig. 8A). Moreover, a weak negative correlation was detected between piR-651 and miR-126 (r = -0.296; $p < 0.01$; Fig. 8A). In 4-OH-Coumarin treated HUVEC cells, strongly positive correlation between piR-651 and piR-823 increased, indicating stronger co-expression after 4-OH-Coumarin treatment (r = 0.851; $p < 0.05$, Figure 8B). Furthermore, the negative correlation between piR-823 and miR-126 became more pronounced, suggesting treatment enhances the inverse relationship (r = 0.044; $p > 0.05$; Fig. 8B). Moreover, the correlation between piR-651 and miR-126 remained moderate negative (r = 0.468; $p < 0.05$; Fig. 8B).

In SH-SY5Y control group, piR-651 and piR-823 indicated a moderate positive correlation (r = 0.415; $p < 0.05$, Fig. 8C). Although there was a weak negative correlation observed between piR-823 and miR-126 (r = 0.021; $p > 0.05$; Fig. 8C) while there was a strong correlation between piR-651 and miR-126 (r = 0.885; $p < 0.05$; Fig. 8C). In 4-OH-Coumarin treated SH-SY5Y group, the correlation of piR-651 and piR-823 indicated a moderate positive correlation (r = 0.379, $p < 0.01$; Fig. 8D). Furthermore, the correlation between piR-823 and miR-126 persisted with a weak positive correlation (r = 0.204), when there was a strong positive correlation (r = 0.914, $p < 0.05$) between piR-651 and miR-126 (Fig. 8D).

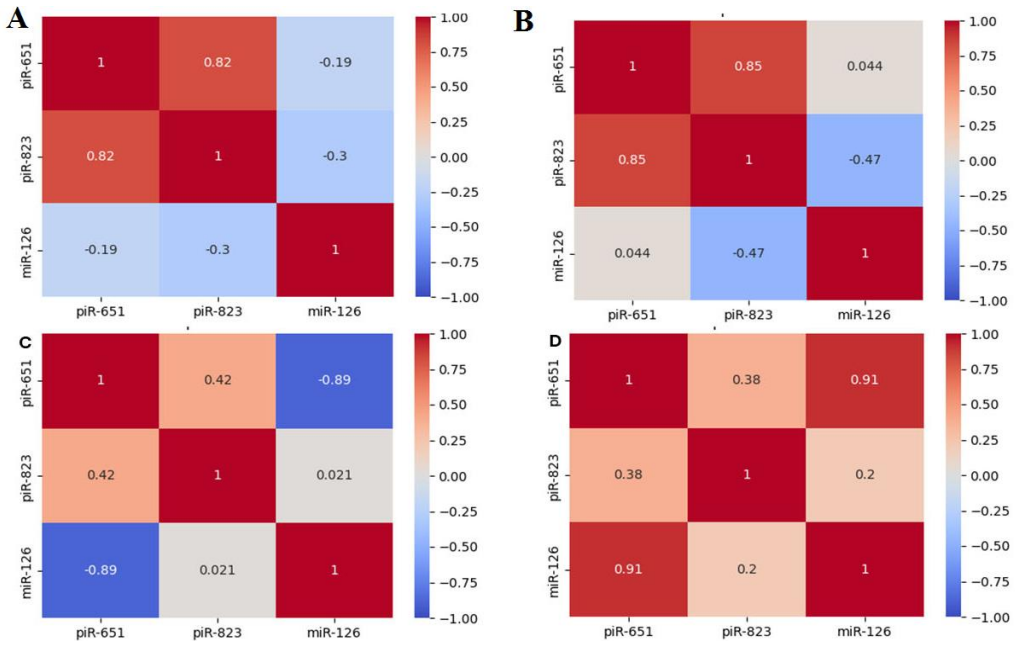

Figure 8: Correlation Matrix Heatmap of piR-651, piR-823, and miR-126 Between Groups. Red shades indicate positive correlation (values close to +1); Blue shades show negative correlation (values close to -1), and White/light shades indicate weak or no correlation (values near 0). In HUVEC cells, 4-OH-Coumarin treatment strengthens some correlations and increases negative associations. Furthermore, 4-OH-Coumarin treatment strengthens the relationship between piR-651 and piR-823 and increases the negative association between piR-823 and miR-126 in HUVEC cells. This may reflect a shift in regulatory networks or co-expression patterns due to treatment. In SH-SY5Y cells, 4-OH-Coumarin treatment disrupts previously strong correlations. In SH-SY5Y cells, treatment disrupts the strong positive relationship between piR-651 and piR-823, suggesting a cell-type specific effect of 4-OH-Coumarin on RNA interactions.

A statistically significant decrease in the motility of HUVEC cells treated with 2.5 µM 4-OH-Coumarin was observed only at the 24th hour compared to the control group (Fig. 9A; $p < 0.001$). Complete wound closure was observed at the other timelines and no statistically significant difference was observed compared to the control group (Fig. 9B; $p > 0.05$). In SH-SY5Y cells, 10 µM 4-OH-Coumarin treatment (-0.417 ± 0.012; -0.237 ± 0.018; -0.345 ± 0.019)

decreased in all three time points compared to the control group (0.269 ± 0.013; 0.607 ± 0.013; 0.576 ± 0.011; Fig. 9A and 9B; $p < 0.001$).

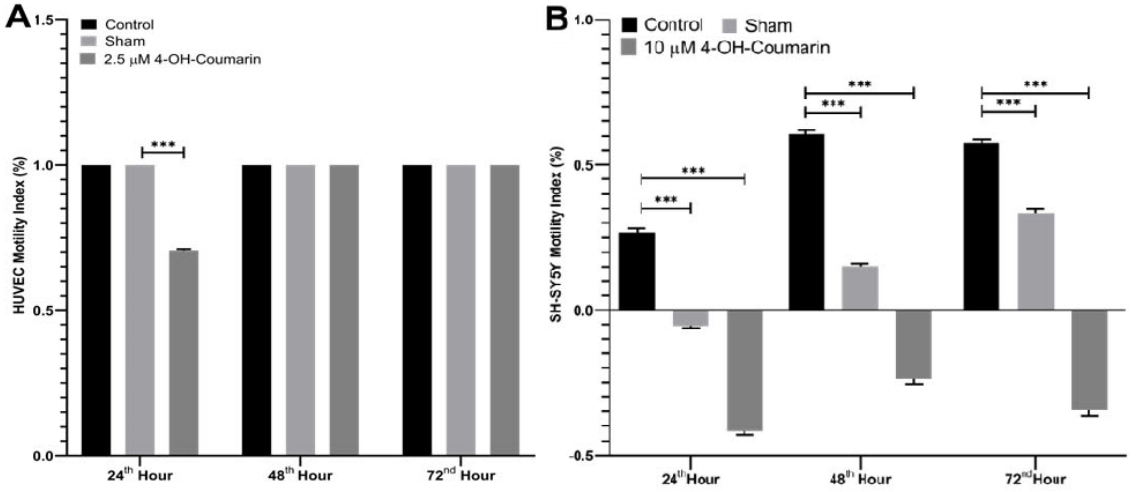

Figure 9: The % Motility Indexes of HUVEC and SH-SY5Y cells. **A)** % Motility Index of HUVEC Cells. **B)** % Motility Index of HUVEC Cells.

DISCUSSION

Neuroblastomas are solid tumors that can exhibit mild, moderate and severe prognoses, and the difficulty in treating high-risk neuroblastoma highlights the need to investigate therapeutic agents for the disease [34]. Coumarins and coumarin derivatives are compounds that may exhibit anti-cancer activity through molecular pathways such as inhibition of microtubule polymerization, inhibition of DNA topoisomerase and induction of apoptosis [35-37].

Since the IC50 value is a concentration that causes 50% cell death, it is supported by the decrease in the expression of the relevant genetic and epigenetic regions examined in our research, which causes a decrease in proliferation and deterioration of morphology in both cell lines. Ki-67, an antigen whose function is closely related to mitosis, was included in our study because it is an accepted marker for the proliferation and growth of cancer cells (Wang et al., 2024). Furthermore, hTERT, whose increased expression leads to increased cell proliferation, is a reverse transcriptase that is not actively expressed in normal adult somatic cells and protects genome integrity by preventing the shortening of telomere sequences at the ends of chromosomes as a result of cell division [38]. hTERT, which is also highly expressed in cancer patients, plays important roles especially in cancer initiation and progression [39]. Liu et al. (2010) reported that a specially designed coumarin derivative containing a 4,5-dihydropyrazole moiety increased telomerase inhibition in SGC-7901 gastric cancer cells, leading to shortening of telomeres and suppression of cell proliferation [40]. According to our obtained data, 2.5 µM 4-OH-Coumarin treated to HUVEC cells decreased Ki-67 and hTERT gene expressions because the treated dose is the dose that inhibits the 50% proliferation of the cells, which is an expected result. On the other hand, the decrease in Ki-67 and hTERT gene expressions in SH-SY5Y cells treated with 4-OH-Coumarin indicates that 4-OH-Coumarin has an inhibitory effect on the proliferation of neuroblastoma cells, which is also confirmed from a genetic point of view. Decreased hTERT gene expression after 4-OH-Coumarin administration suggests that coumarins may be involved in the intervention of coumarins in cancer processes through the mechanism of telomerase inhibition. According to the hematoxylin-eosin results, 4-OH-Coumarin had no effect on the morphology of HUVEC cells, whereas it affected the viability, number and morphology of SH-SY5Y cells. In particular, the numerical decrease in SH-SY5Y cells supports the Ki-67 and hTERT gene expression results and emphasizes once again the

potential of 4-OH-Coumarin as an anti-cancer agent for neuroblastomas. Another proliferation parameter for this research is PI3K/AKT/mTOR pathway. In cancer development, it is well known that high expression of this pathway components was observed. However, PI3K and mTOR gene expressions decreased in both cell lines, AKT gene expression upregulated after 4-OH-Coumarin treatment. This is an unexpected result. The decrease in PI3K and mTOR gene expression may be the main cause of the decrease in proliferation in another pathway not via PI3K/AKT direction because of the upregulation of AKT after 4-OH-Coumarin treatment in both cell lines. Moreover, our results show that 4-OH-Coumarin doesn't have an impact on PI3K/AKT/mTOR pathway.

Mitophagy, the process of removing damaged mitochondria from the cell through autophagy to maintain cellular function, is among the important physiological pathways [41]. There are two main pathways if a cell goes mitophagy because of the mitochondria dysfunction. The first pathway is PINK1/Parkin pathway. In 4-OH-Coumarin treated HUVEC cells, Parkin gene expression increased, while PINK1 gene expression decreased. These genes are also used as degeneration markers. According to the degeneration sight, we should evaluate these two genes independently. PINK1 is the key molecule of degeneration because of this reason, the downregulation of PINK1 causes cells to escape degeneration. Our hematoxylin/eosin results support this hypothesis. The upregulation of Parkin may be a result of the number of dead cells because of the effect of inhibitory concentration of 4-OH-Coumarin on HUVEC cells. On the other hand, 4-OH-Coumarin treatment to neuroblastoma cells decreased the amount of mitophagy via Parkin and PINK1, cells escaped from mitophagy. The second mitophagy pathway ise HIF-1α/ BNIP3 pathway. Furthermore, HIF-1α is a transcription factor that responds to hypoxic conditions and plays a role in cancer pathophysiology by contributing to the processes of cancer metastasis, invasion and migration [42]. Activation of hypoxia-induced transcription factors (HIF) is a known mechanism for the adaptation of solid tumors to hypoxic conditions. Significant increases in the expression of transcription factors such as HIF-1α and HIF-2α have been reported in clinical neuroblastoma samples. [43]. HIF-1α, which is overexpressed in cancer cells, suggests that 4-OH Coumarin may be involved in the reduction of cancer cell proliferation through mitophagic pathways. HIF-1α downregulation due to decreased proliferation in HUVEC cells suggests that 4-OH-Coumarin may affect mitophagy through the HIF-1α/BNIP3 pathway if it had an impact on mitophagy.

Boonyarat et al. (2022) showed that Nordentatine, a coumarin derivative, inhibited proliferation and migration in SH-SY5Y cells by inhibiting the phosphorylation of GSK-3, which controls an anti-apoptotic protein (Mcl-1) expression, and consequently induced apoptosis by activation of caspase-3 [44]. Sargolzaei et al. (2020) reported that prenyl-hydroxy-Coumarin derivatives induced apoptotic pathway and exhibited anti-cancer activity in mouse neuroblastoma cell line N2A [45]. In another study, a coumarin derivative RKS262 induces caspase-3-mediated apoptosis via ROS production in neuroblastoma cells [46]. Furthermore, coumarin-based derivative caused increased expression of caspase 9 and caspase 7 and decreased expression of anti-apoptotic Bcl-XL in MGC-803 gastric cancer cells [47]. To determine the impact of 4-OH-Coumarin on HUVEC and SH-SY5Y cells, we focus on Cyt C gene expression. If Cyt C is released from mitochondria to cytosol, the apoptosis starts. Released Cyt C from mitochondria in the caspase-dependent apoptotic pathway forms the apaptosome (Apaf1/procaspase 9) complex and triggers cell death by activating the apoptotic pathway as a result of activation of caspase 9 [48]. It was observed that 4-OH-Coumarin applied to HUVEC cells caused an increase in Cyt C and a decrease in p53, a tumor suppressor, gene expression levels because it killed the cells by 50%. 4-OH-Coumarin treatment of SH-SY5Y neuroblastoma cells decreased Cyt C and increased p53 gene expressions. Accordingly, the results suggest that 4-OH-Coumarin does not interfere with Cyt C-dependent apoptotic processes but interferes with p53 in SH-SY5Y cells. This increase suggests that neuroblastoma cells activate a cell death mechanism through p53 activation and consequently reduce proliferation. It was observed that 4-OH-Coumarin was effective in SH-SY5Y cells both in

apoptotic markers such as p53, Cyt C and in the expression of survival and proliferation genes such as Ki-67 and hTERT.

piR-651 and piR-823 are known to be epigenetic regulators that are effective in both proliferation and motility [49]. piR-651 is observed to be more effective in metastatic mechanisms [50], while piR-823 is more effective in proliferation mechanisms [51-53]. miR-126 is known to be effective in cell proliferation, especially Ras/Raf/MAPK or PI3K/AKT/mTOR [31]. According to the obtained data, considering that HUVEC cells can form veins and SH-SY5Y cells have active metastasis and angiogenesis mechanisms, increased expression of piR-651, which promotes metastasis, is an expected result; however, it is possible that genetic markers may give contrary results. Similar results were observed in miR-126 expressions and it was observed that 4-OH-Coumarin did not affect the expression profiles of these two small non-coding RNAs epigenetically. On the other hand, the expression of piR-823, which regulates proliferation, decreased in both cell lines in correlation with the proliferation results and indicating that piR-823 expressions is epigenetically affected by 4-OH-Coumarin. The strongest and statistically significant relationships are observed between piR-651 and piR-823 in both HUVECs, and between piR-651 and miR-126 in SH-SY5Y groups (negative in control, positive in treated). Most other correlations are weak or moderate and not statistically significant, as indicated by higher p-values. These results suggest that 4-OH-Coumarin treatment and cell type can influence the relationships between these markers, with some pairs showing strong co-regulation or inverse regulation depending on the group (Figure 8A.-D.).

Zhou et al. (2022) found that 4-OH-Coumarin derivatives significantly reduced lung cancer cell motility in lung cancer cell lines compared to the control group. [14]. Another study by Tian et al. (2024) found that a coumarin-based derivative significantly reduced the migration of MGC-803 gastric cancer cells [47]. In HUVEC cells, wound closure was not complete in the 2.5 µM 4-OH-Coumarin treated group, although wound closure was observed in the control and Sham groups in the first 24^{th} hour. At the 48^{th} and 72^{nd} hours following 24^{th} hour, HUVEC cells were observed to close the wound in all groups. Significant decreases in the motility of SH-SY5Y cells treated with 10 µM 4-OH-Coumarin were detected, and it was observed that the wound gradually opened after 24 hours. It is thought that the reason for this opening is that 4-OH-Coumarin applied to neuroblastoma cells kills the cells. It was observed that 4-OH-Coumarin treated to SH-SY5Y neuroblastoma cells effectively decreased cell motility and caused the wound to open, although in HUVEC cells the IC50 concentrations haven't affected the motility and survival.

These data provide important preliminary data for future *in vivo* and *in vitro* cancer treatment studies. In the light of the literature, when the results obtained are evaluated together, it can be said that 4-OH-Coumarin is a promising therapeutic agent for neuroblastomas. We believe that further experiments will confirm that 4-OH-Coumarin may be a good therapeutic agent for the treatment of neuroblastoma.

Conflict of Interest: All authors declare their conflict of interest.

Authors' Contribution: ÇÖ; Corresponding Author, Project administration, Funding acquisition, Conceptualization, Methodology, Software, Validation, Investigation, Visualization, Supervision, Writing- Reviewing and Editing. HEK; Project administration, Funding acquisition, Conceptualization, Methodology, Software, Validation, Investigation, Visualization, Supervision, Writing- Reviewing and Editing. DK; Conceptualization, Methodology, Software, Validation, Investigation, Visualization, Supervision, Writing- Reviewing and Editing. EÇ; Data curation, Formal analysis, Writing- Original draft preparation.

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