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Suppressive effects of resveratrol on epithelial-mesenchymal transition in the YKG1 glioblastoma cell-line

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Abstract

Glioblastoma multiforme (GBM), is an aggressive tumor type with high mortality and morbidity, which a curative treatment method has not been developed yet. Although Epithelial-mesenchymal transition (EMT) is a known mechanism to be effective in the cancer process, the EMT process seen in these cells has not been fully explained yet. Resveratrol is a flavonoid and has been reported to have positive effects on different types of cancer. In this study, we aimed to compare the effects of resveratrol on EMT in YKG1 GBM cell line. Different doses of resveratrol (50 and 100 µM) were applied to YKG1 glioblastoma cells, and their viability was calculated and immunocytochemical analyzes were evaluated. According to results, it was observed that the 100 µM resveratrol dose significantly reduced the viability compared to the 50 µM resveratrol dose. It was determined that Vimentin and N-Cadherin H scores of 100 µM Resveratrol dose decreased and E-cadherin and Claudin-1 values increased compared to the other groups. The results showed that increasing resveratrol dose was more effective on EMT on glioblastoma cell lines in a dose-dependent manner.

Keywords: Epithelial-mesenchymal transition, Glioblastoma multiforme, Resveratrol, YKG1

Introduction

Glioblastoma multiforme (GBM) is the most common intra-axial primary malignant brain tumor classified as Grade 4 glioma according to the World Health Organization [1]. GBM accounts for about 50% of all gliomas. Current management of GBM includes surgical resection, radiotherapy, and alkylating agent-based therapy such as temozolomide, carmustine, and procarbazine [2]. However, the median overall survival of GBM patients in clinical studies is about 15 months, and 3-year survival is less than 5%. Infiltration and invasion of GBM cells into normal brain tissue is an important cause of treatment failure. Understanding of this phenotype remains a very important goal in the discovery of successful treatment, which is expected [3, 4].

Epithelial-mesenchymal transition (EMT) is a reversible cellular program that transiently places epithelial cells into quasi-mesenchymal cell states [5]. EMT has a very important role in the process of embryo arrangement, wound repairing, and tissue rebuilding. It has been shown that EMT is related to cancer progression, invasion, and metastasis [4]. The EMT process could be demonstrated by the increased expression of EMT-associated transcription factors (EMT-TFs). This may be important markers in the spread of epithelial neoplasms. In addition, it has been shown that expression increases in non-epithelial neoplasms such as melanoma, glioblastoma, and leukemia [6].

The main process in EMT is the reduction or loss of the E-cadherin between epithelial cells. The reason for this is the disruption of the emergence / loss balance of molecules such as SNAI1 / Snail1, ZEB2, TCF3 Twist, Goosecoid in the cells [6, 7]. Similarly, TGF beta, FGF, EGF, etc. factors are among the EMT stimulators. Moreover, E-cadherin, cell adhesion molecule, occludines, claudins, cytokeratins are inhibited, while N-cadherin, vimentin, fibronectin, integrins, and MMPs are expressed during the EMT process [5, 8].

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Resveratrol is a polyphenolic agent that found in grape seed and peel, as well as many fruits and vegetables. Anti-inflammatory, antioxidant, and tissue-protective effects of resveratrol on liver [9], kidney [10], heart-artery [11], and ovary [12] have been shown in many studies to date. Another prominent effect of resveratrol is its anticarcinogenic properties. In a recent study, we showed that resveratrol potentiates the effect of carmustine, which acts on glioblastoma cells [13]. Resveratrol may be beneficial in different stages of treatment by affecting various signal transduction pathways that control cell growth and division, inflammation, apoptosis, metastasis, and angiogenesis in many forms of malignancy [14]. Even fractions of resveratrol have been shown to have an anticarcinogenic activity [15]. Moreover, resveratrol has been reported to cause significant inhibition in glial tumors by inducing apoptosis and inhibiting glioma-induced angiogenesis [8], inhibiting tumor progression by controlling EMT in LN18 and U87 GBM cell lines [4].

The main goal of current study is to determine the effects of resveratrol on EMT in the YKG-1 cell line. In addition, we aimed to demonstrate these EMT effects using different parameters in YKG-1 cell line and to examine its interaction with resveratrol, which is widely used as an antitumor agent today.

Materials and Methods

Cell culture

YKG1 human glioblastoma cell line was provided by RIKEN (RIKEN, BioResource Center, Japan). YKG1 cells were cultured in D-MEM F-12 medium containing heat inactivated %10 FCS, 5 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Resveratrol (CAS 501-36-0, Santa Cruz, USA) was dissolved in culture medium to make stock solution. Then it was added to whole cell culture medium to achieve a working concentration of 50 and 100 µM resveratrol. One group of cells was treated with 50 µM (Res50) and one group with 100 µM resveratrol (Res100) for 48 h; one group of cells was not exposed to resveratrol and was considered the control group.

MTT assay for cell viability analysis

The 3- (4,5-dimethylthiazol2-yl) -2,5-diphenyltetrazolium bromide (MTT) method was used to measure cell viability. 100µl from the cell suspension prepared at 2×10^4 cells / ml was transferred to each well of the 96-well plates. At the same time, resveratrol was added to the cells in the above-mentioned concentrations and incubated at 37°C. At the end of the 48-hour incubation period, the cells in each well were held on at 37°C for 2 hours after addition 5 mg / ml MTT dye. When this period has finished, MTT dye was removed from the cells. The wells were left for ten minutes after adding 200 µl of DMSO. An ELISA plate reader with a wavelength of 540 nm was used for color change detection. Viability rates of experimental cells are expressed as %, assuming control cell viability not treated with the compound as 100%.

Immunocytochemistry

Cells forming the control group and treated with resveratrol were cultured in the 12-well chamber slide. At the end of the 48th hour, the medium was removed with a sterile pipette and 4% paraformaldehyde was used for fixation of cells. After washing with PBS, they were kept on ice for 15 min in 0.1% Triton-X100 solution. The cells were incubated with 3% H_2O_2 for endogenous peroxidase inactivation. After washing with PBS three times for 5 min, it was treated with blocking solution for 10 minutes. Cells were incubated with primary antibodies N-cadherin (1/200, ab18203, abcam), E-cadherin (1/200, ab76055, abcam), vimentin (1/200, ab8978, abcam) and claudin-1 (ready to use, RB9209-R7 Thermo Scientific) for 1 hour at room temperature. Then PBS was used again for 3 washes. After treatment with secondary antibody, it was colored using AEC. Mayer's hematoxylin was used for counter-staining and covered with a water-based sealer.

Cells on stained slides were counted under light microscopy with Image Analysis Program (NIS elements, Japan). 500 different cells were counted at x20 objective magnification. While scoring, the area with the highest score was determined by scoring the staining degree. Scoring was done by a semi-quantitative method (HScore) [16]. Stained cells were evaluated in terms of percentage and staining intensity was taken as a second criteria. There is no, slight, moderate, and severe staining were assessed as 0, 1, 2 and 3, respectively.

Statistical analysis

Data were analyzed by SPSS (Statistical Package for Social Sciences) software version 22.0 (IBM, Armonk, NY, USA). The distribution of data was analyzed by the Kolmogorov-Smirnov test. Continuous variables were expressed as mean ± standard deviation. Student t-test was used, and P-values less than 0.05 were accepted as significant.

Results

Cell viability

The viability test results performed at 48th hour is shown in Table 1. According to these results, it was observed that 100 µM dose of resveratrol had more effective cytotoxic effect against glioblastoma cells compared to 50 µM.

Table 1. Effects of resveratrol treatment on YKG1 glioblastoma cells' viability after 48-hours of treatment

Group	t:48 hour
Control	100
Res50	96
Res100	78

Res50: Resveratrol 50 µM, Res100: Resveratrol 100 µM

Immunocytochemistry

According to the HScore results obtained from the staining, it was observed that vimentin and N-cadherin staining was the highest in the untreated control group, although a slight decrease was detected

in the Res50 group, the staining was less in the Res100 group than in these two groups, and the staining intensity was also decreased. The intensity of staining was quite significant in vimentin staining, while +2 and +3 intensity staining were common in the control group. The intensity of staining was observed to be slightly decreased in the Res50 group compared to the control group, while in the Res100 group the staining was generally observed at +1 intensity (Figs. 1, 2 and 5).

The intensity of E-cadherin and claudin-1 staining was detected at +1 intensity in each group and the least staining was observed in the control group. A slight increase in staining was detected in the Res50 group. Staining in the Res100 group was observed to be increased compared to the other two groups (Figs. 3, 4 and 5).

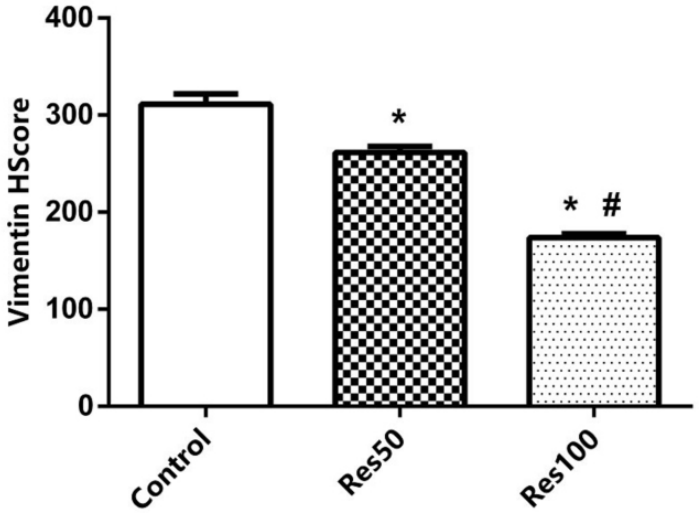

Figure 1. Vimentin HScore results obtained because of immunocytochemical staining. Values are expressed as mean ± SEM, *p<0.05 versus control group and # p<0.05 versus Res50 group

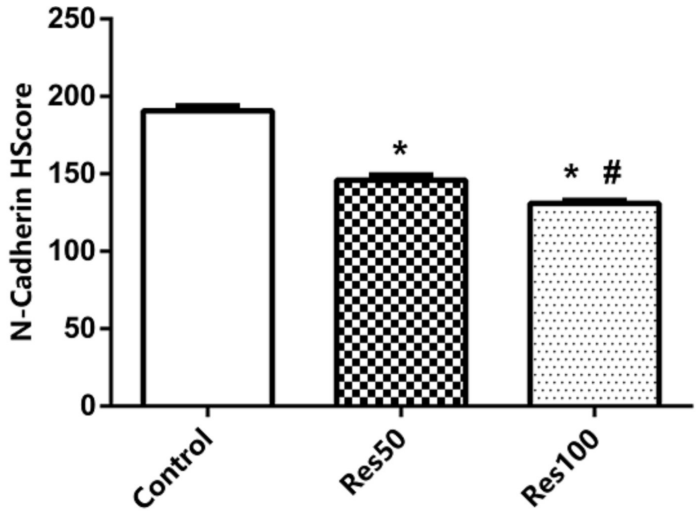

Figure 2. N-Cadherin HScore results obtained because of immunocytochemical staining. Values are expressed as mean ± SEM, *p<0.05 versus control group and # p<0.05 versus Res50 group

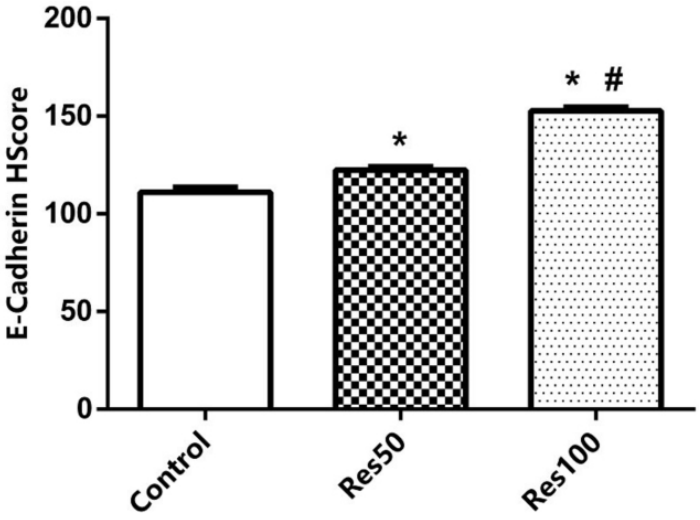

Figure 3. E-Cadherin HScore results obtained because of immunocytochemical staining. Values are expressed as mean ± SEM, *p<0.05 versus control group and # p<0.05 versus Res50 group

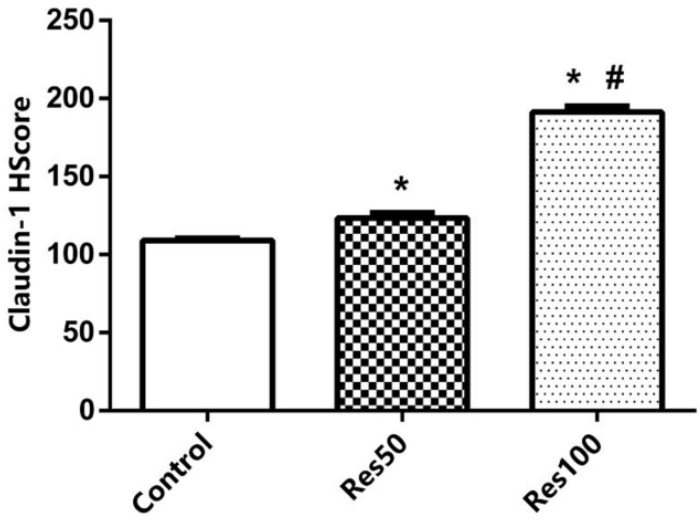

Figure 4. Claudin-1 HScore results obtained because of immunocytochemical staining. Values are expressed as mean ± SEM, *p<0.05 versus control group and # p<0.05 versus Res50 group

Discussion

In this study we present, the cytotoxic effect of resveratrol on GBM cells in a dose-dependent manner is revealed in our immunocytochemical and viability test results support that resveratrol suppresses EMT in GBM cell line.

EMT is a complex process that has been associated with the progression of many types of cancer as well as playing an important role during normal embryogenesis. We know that GBM is a non-epithelial tumor. These processes can also be called epithelium to mesenchymal transition or glial to mesenchyme transition (GMT). However, studies that accept GMT as equivalent to EMT suggest that this situation is due to the recurrence of GBM [17-19].

Cells that transition between epithelial and mesenchymal phenotypes can take an intermediate state with a mixture of both features [20]. EMT is consistently followed by raised expression of mesenchymal markers and declined expression of epithelial

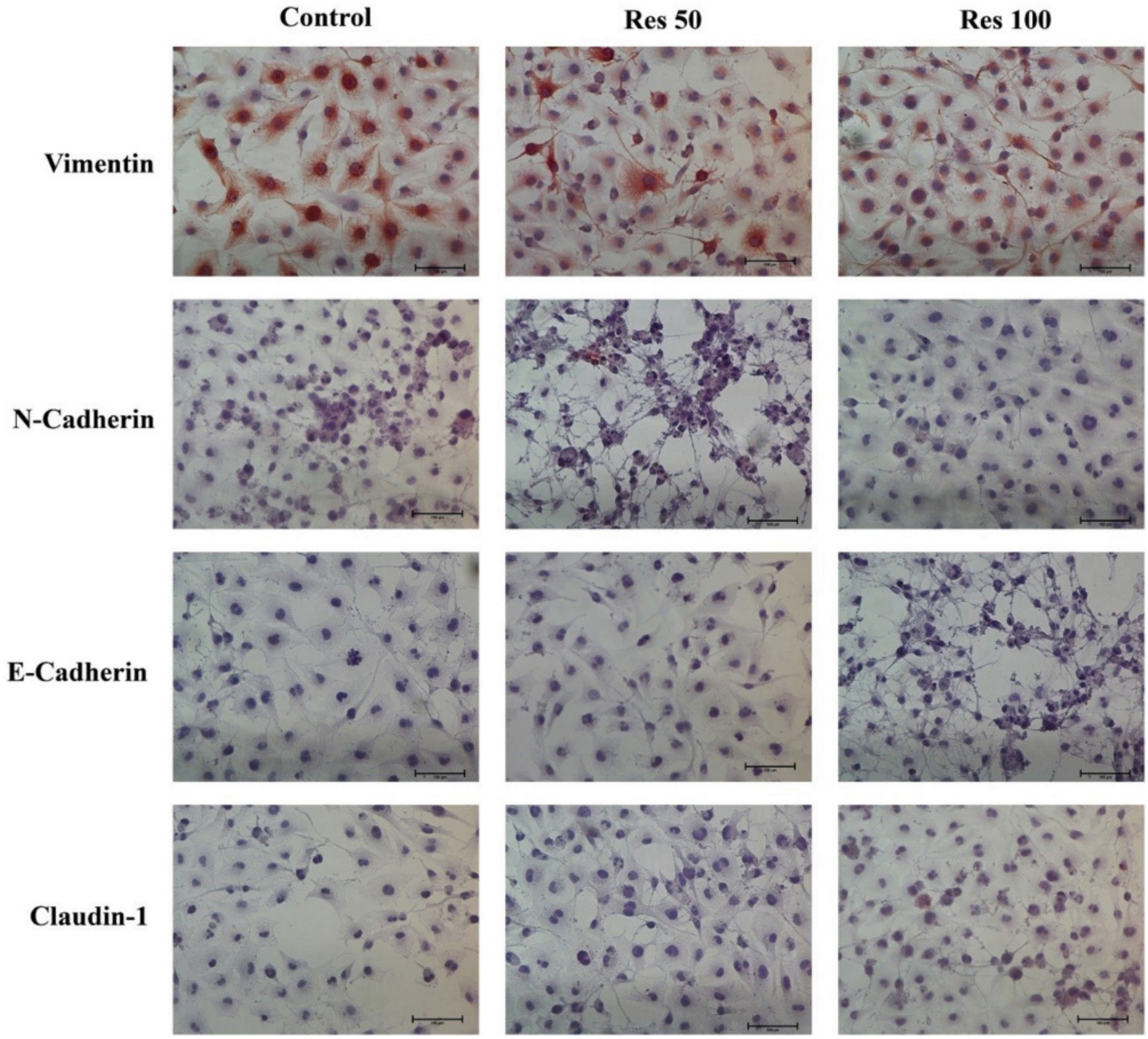

Figure 5. Photomicrographs of immunocytochemical staining's of YKG1cells, which were treated with different doses of resveratrol. The cells were stained with Vimentin, N-Cadherin, E-Cadherin, Claudin-1 antibodies (x 200 magnification)

markers. Some essential regulators either play role as transcription suppressors by inhibiting epithelial-related genes or as activator of mesenchymal genes, hence starting to induction of the mesenchymal phenotype and invasion of cancer cells. According to literature, GBM cells can go through an EMT process that forces to a conversion from a fewer epithelial phenotype to a higher mesenchymal one [4, 20, 21]. Although there are studies showed that resveratrol also inhibits EMT in GBM cells, these studies could not provide sufficient clarification [4]. The EMT process can be demonstrated by the decrease of epithelial markers such as β-catenin, ZO-1, E-cadherin, occludin, claudin, and the increase in the expression of mesenchymal cell markers such as vimentin, ZEB-1, twist, slug, snail [4, 22].

It has been proven that resveratrol can also inhibit GBM cell proliferation and increase cell mortality [23]. In previous studies evaluating the cell viability of resveratrol on GBM cells, it has been reported that resveratrol causes a cytotoxic effect depending on the concentration. Song Y. et al. [4] used 20 μM - 40 μM resveratrol in LN18 and U87 GBM cells in their study and found that doses below 40 μM reduced cell viability to a minimum and effective dose started after 40 μM. In another study, Cilibrasi C. et al. [23] conducted an experiment using 10-50-100-200 μM concentration of resveratrol and found that the dose of 100 μM was the dose that increased the cell death rate the most after 48 hours of treatment. However, this ratio was lower at the dose of 200 μM. Based on these data, 50 μM and 100 μM resveratrol doses were used in our study, too. Cytotoxic effect was observed at Res50 group, and we observed that the Res100 group had a greater cytotoxic effect on YKG1 cells.

Vimentin is a type III intermediate filament protein and is a characteristic biomarker for EMT. Many studies have shown that vimentin is associated with malignant character in brain malignancies [24]. Studies have reported that the increase in vimentin in GBM is an indicator of invasion and malignancy [25-27]. Resveratrol reduced EMT by suppressing the increase of vimentin in LN18 and U87 GBM cells was stated in the study of Song Y. et al. [4]. In our study, the immunoreactivity of vimentin in the YKG1 cell line decreased due to the increase in resveratrol dose, supporting the literature.

While expression of E-cadherin is lost in the EMT process in cancer cells, an increase in N-cadherin expression is observed. While the expression of lost E-cadherin provides connections between epithelial cells, apico-basal cell polarity and disruption of epithelial tissue structure, the increase in N-cadherin expression causes cells to separate from the tissue and gain invasive capacity. This event is called "cadherin-switching" and is associated with tumor invasiveness and poor prognosis [28]. GBM is also an invasive cancer with a poor prognosis, and many studies have shown that while E-cadherin expression decreases in GBM, N-cadherin expression increases [28, 29]. Reversing the change of fate is the main goal in cancer treatment. It has been emphasized in previous studies that resveratrol suppresses EMT in cancer cells in this way [30, 31]. In our study, while N-cadherin immunoreactivity decreased in YKG1 cell line due to resveratrol dose increase, E-cadherin immunoreactivity increased. Resveratrol increases E-cadherin expression in GBM cells while decreasing N-cadherin expression, suggesting it is a promising agent in therapy.

In conclusion, our findings indicate that resveratrol has a suppressive effect on EMT on the increased dose of resveratrol is more effective on EMT on glioblastoma cell lines in a dose-dependent manner. However, further studies are necessary to clarify the EMT mechanism of action of resveratrol.

Conflict of interests
The authors declare that they have no competing interests.

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