Combining Ellagic Acid with Temozolomide Mediates the Cadherin Switch and Angiogenesis in a Glioblastoma Model

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■ OBJECTIVE: **We aimed to evaluate the combined effect of ellagic acid (EA) and temozolomide (TEM) on the cadherin switch and angiogenesis in the C6 glioma cell line.**

■ METHODS: **A total of 100 μM EA and 100 μM TEM were applied to rat C6 glioma cells for 24, 48, and 72 hours. Cell proliferation was detected by 5-bromo-2′-deoxyuridine immunohistochemistry. The messenger RNA and protein levels of E-cadherin, N-cadherin, and vascular endothelial growth factor (VEGF) were determined by real-time polymerase chain reaction and their immunohistochemistry, respectively, subsequent to EA treatment combined with TEM.**

■ RESULTS: **EA in combination with TEM conspicuously reduced the viability of C6 glioma cells at all incubation times ($P < 0.001$). EA upregulated the expression of E-cadherin at the gene and protein levels in a time-independent manner ($P < 0.05$ and $P < 0.001$, respectively). By the presence of TEM, the increase was exaggerated at 24-hour incubation ($P < 0.01$). Conversely, EA reduced N-cadherin expression and immunoreactivity in a time-independent manner ($P < 0.05$ and $P < 0.001$, respectively), and combination with TEM enhanced this effect at the 24th hour ($P < 0.001$). Combination also downregulated the gene expression ($P < 0.001$) and immunoreactivity of VEGF only at 72 hours ($P < 0.001$).**

■ CONCLUSIONS: **A successful therapeutic efficacy of EA combined with TEM is suggested probably by inhibiting the cadherin switch and angiogenesis.**

INTRODUCTION

Glioblastoma (GBM) is a common malignant and aggressive brain tumor of the central nervous system with a poor prognosis in children and adults.[1,2] The Stupp protocol has become standard care for the treatment of GBM since 2005, consisting of radiotherapy and concomitant chemotherapy with temozolomide (TEM), an alkylating agent. The surgery followed by chemotherapy with TEM and radiotherapy is a risky option for managing GBM.[1,3,4] Therefore, patients with GBM have a low median survival (i.e., only 14.6 months with radiotherapy plus TEM and 12.1 months with radiotherapy alone).[4] The heterogeneity of the tumor,[5] the poor capacity of therapeutics to penetrate the blood–brain barrier,[6] and the variation in the sensitivity of tumors to TEM resistance complicate the resistant nature of GBMs against TEM treatments.[7] Therefore, the development of innovative therapeutic strategies, such as new combinations of potent agents with basic chemotherapeutics, is urgently required to prevent recurrence, prolong patient survival, and improve quality of life.[8]

Several studies have shown that combination therapies including phytochemical compounds, such as phenolic acids extracted from fruits and vegetables, exhibit various cytotoxic and antiproliferative effects similar to those of chemotherapeutics.[8-11] Ellagic acid (EA) is a natural dietary polyphenol compound found in plants and fruits[9] that exhibits antitumor pharmacologic properties, such as inhibition of tumor formation and growth via cell cycle arrest, induction of apoptosis,[12] and suppression of angiogenesis.[13] EA has antioxidant, antifibrotic, and chemopreventative effects.[14] However, the effect of EA combined with common chemotherapeutics in patients with glioma is poorly understood.

Key words
- Cancer therapy
- Ellagic acid
- Glioblastoma
- Temozolomide

Abbreviations and Acronyms
ASA: Acetylsalicylic acid
Br-dU: 5-Bromo-2′-deoxyuridine
EA: Ellagic acid
EMT: Epithelial-to-mesenchymal transition
GBM: Glioblastoma
qPCR: Quantitative polymerase chain reaction
TEM: Temozolomide
VEGF: Vascular endothelial growth factor

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Epithelial-to-mesenchymal transition (EMT) plays a distinctive role in changing the cellular phenotype by the switch of an epithelial cell to a mesenchymal cell, resulting in higher migratory potential, higher resistance to apoptosis, and more capacity of invasion.[15] This phenomenon can ensue by the downregulation of epithelial markers, such as E-cadherin, and the upregulation of mesenchymal markers, such as N-cadherin and vimentin, resulting in other genetic and phenotypic changes.[16] Most GBMs do not express E-cadherin,[17] which might support the EMT phenotype occurring in these aggressive tumors. However, in vitro studies using C6 glioma cells have shown that E-cadherin expression was increased, whereas the invasive ability of the cells was decreased, which is advanced proof for the crucial role of cadherins in gliomas.[18]

The other common therapeutic target of cancer treatment is the angiogenesis in GBM cells. Because the invasive and aggressive nature of this type of cancer needs triggering factors for angiogenesis to supply food and oxygen to invading cells, vascular endothelial growth factor (VEGF) has a well-known role in angiogenesis. However, the expression profile of VEGF, which may be altered by the combination therapies with TEM, needs to be elucidated specifically in GBM cells.

The exact action mechanism of TEM on the cadherin switch and the combined effect with EA have not been demonstrated in gliomas. To identify the mechanism of the possible synergistic activity of TEM with EA, we examined the combined effect of EA and TEM on cadherin profiles and VEGF expression in the C6 glioma cell line.

MATERIALS AND METHODS

Cell Culture

C6 glioma cells obtained from ATCC (Manassas, Virginia, USA) were cultured at 37°C in 95% humidified air with 5% carbon dioxide in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were subcultured every 3 days using trypsin. Concentrations of 1, 10, and 100 μM EA (E2250-10G [Sigma Aldrich, St. Louis, Missouri, USA]) and 1, 10, and 100 μM TEM (Temozolid 20 mg [Dem İlaç, Istanbul, Turkey]) were selected according to those used in previous studies.[19,20] The cells were divided into 4 groups: the control group, TEM (100 μM) group, EA (100 μM) group, and a combined TEM and EA group. The cells were incubated with each treatment for 24, 48, or 72 hours. All experiments were repeated at least 3 times.

5-Bromo-2′-Deoxyuridine Cell Proliferation Assay

Cell proliferation was analyzed by 5-bromo-2′-deoxyuridine (Br-dU) immunohistochemistry according to a previous study.[21] Br-dU (SC-32323 [Santa Cruz Biotechnology, Santa Cruz, California, USA]) and the Histostain-Plus Bulk Kit (SensiTek, ScyTek Laboratories, Logan, Utah, USA) were used. Mouse monoclonal anti–Br-dU (Bu20A, SC-20045 [Santa Cruz Biotechnology]) was used as the primary antibody (1:200, overnight), and 3-Amino-9-Ethylcarbazole (SensiTek, ScyTek Laboratories) was applied as the chromogen. Br-dU labeling was assessed by 2 researchers, and the proliferation index was calculated by evaluating at least 3000 cells and scored as the number of positively stained cells/total number of cells counted.[21]

Immunohistochemistry

C6 glioma cells cultured on cover slips were incubated for 24 hours, and the EA and TEM groups were established. After 24, 48, or 72 hours of treatment, the experiments were terminated and repeated 3 times. The cells were fixed in cold methanol for 5 minutes and immunostained using the indirect streptavidin immunoperoxidase method with an antipolyvalent HRP Kit (SensiTek, ScyTek Laboratories) to detect E-cadherin, N-cadherin, and VEGF proteins. The cover slips were incubated overnight at 4°C with the following primary antibodies: anti–E-cadherin (sc-7870 [Santa Cruz Biotechnology]), anti–N-cadherin (orb11100 [Biorbyt, Cambridge, United Kingdom]), and anti-VEGF (ab46154 [Abcam, Cambridge, United Kingdom]). The antigen–antibody complex was visualized using the 3-Amino-9-Ethylcarbazole Substrate Detection System (SensiTek, ScyTek Laboratories). The intensity of immunoreactivity was evaluated semiquantitatively by the H-score analysis as described previously.[21]

Expression Analysis

Total RNA was extracted using the Total RNA Purification Kit (Jena Bioscience, Jena, Germany) according to the manufacturer's protocol. Complementary DNA was reverse-transcribed using the SCRIPT cDNA Synthesis Kit (Jena Bioscience) according to the manufacturer's instructions. The real-time quantitative polymerase chain reaction (qPCR) was conducted in a CFX96 Touch (Bio-Rad, Hercules, California, USA) machine using the qPCR GreenMaster UNG (Jena Bioscience). The primer pairs are as follows: E-cadherin, F: 5′-CTCTACTCTCATGCTGTGTCATC-3′ and R: 5′-CTCTGGCCTGTTGTCATTCT-3′; N-cadherin, F: 5′-GAGAGGAAGACCAGGACTATGA-3′ and R: 5′-TCTCGTCTAGCCGTCTGATT-3′; VEGF, F: 5′-CACTTCCAGAAACACGACAAAC-3′ and R: 5′-CTGGTCGGAACCAGAATCTTTA-3′; and glyceraldehyde 3-phosphate dehydrogenase, F: 5′-GCAAGGATACTGAGAGCAAGAG-3′ and R: 5′-GGATGGAATTGTGAGGGAGATG-3′.

After normalization to glyceraldehyde 3-phosphate dehydrogenase expression, the relative expression levels of E-cadherin, N-cadherin, and VEGF transcripts in treated cells compared with control cells were calculated as mean ± standard error of the mean.

Statistical Analysis

Semiquantitative and quantitative data from all groups were evaluated statistically using GraphPad Instat version 3.06 (GraphPad Inc., La Jolla, California, USA). All data are presented as mean ± standard error of the mean. The means of continuous variables were compared by 1-way analysis of variance, and differences between groups were compared using the Tukey-Kramer multiple comparison test. $P < 0.05$ was considered significant.

RESULTS

Treatment with EA and TEM Synergistically Inhibits Cell Proliferation

To determine whether EA potentiated the inhibitory effects of TEM on the proliferation of C6 glioma cells, we semiquantitatively analyzed the effects of EA with or without TEM on cell proliferation using the Br-dU proliferation assay. As shown in Table 1, treatment with EA alone time-dependently suppressed cell proliferation significantly compared with the control group ($P < 0.001$). Moreover, the EA and TEM combined treatment inhibited the cell proliferation more significantly compared with TEM alone ($P < 0.001$).

EA in Combination with TEM Regulates E-Cadherin and N-Cadherin Expression at the Gene and Protein Levels

To determine the regulatory effects of EA on the cadherin profile in glioma cells, E-cadherin and N-cadherin messenger RNA and protein levels were analyzed by qPCR (Figure 1) and immunohistochemistry (Figures 2 and 3), respectively. EA alone significantly upregulated the expression ($P < 0.001$) at 48 and 72 hours of the treatment, but not at 24 hours' incubation. The immunoreactivity of E-cadherin in C6 glioma cells was significantly higher than the control group at every time point. Interestingly, EA combined with TEM upregulated E-cadherin expression significantly ($P < 0.001$), especially at 48 and 72 hours' incubation, but not at 24 hours (Figure 3). However, TEM alone failed to upregulate E-cadherin expression but increased the immunoreactivity at all incubation time points ($P < 0.05$ for 24 hours, $P < 0.01$ for 48 hours, and $P < 0.001$ for 72 hours). EA combined with TEM distinctly elevated the immunoreactivity of E-cadherin at all time points compared with the control group ($P < 0.01$ for 24 hours and $P < 0.001$ for 48 and 72 hours), and this effect was higher than the TEM alone group ($P < 0.05$).

EA treatment downregulated the levels of N-cadherin significantly at 48 and 72 hours of incubation ($P < 0.001$), regardless of the presence of TEM, whereas TEM alone failed to downregulate N-cadherin expression at 24 and 48 hours of incubation, compared with the control group (Figure 3). Immunohistochemical analysis showed that EA in combination with TEM decreased N-cadherin immunoreactivity dramatically at 24 and 72 hours ($P < 0.01$), and also significantly at 48 hours ($P < 0.05$), but TEM alone failed to reduce the protein levels at 24 and 48 hours' incubation, suggesting a time-independent modulatory effect of EA in combination with TEM on the cadherin switch in glioma cells.

Long-Term Exposure to EA Downregulates the Expression of VEGF at the Gene and Protein Levels Independently of TEM

Figure 4 represents VEGF expression in C6 glioma cells treated with EA and TEM, whereas Figure 5 gives the immunohistochemical analysis of VEGF. As a clinical antiangiogenesis agent, EA alone downregulated VEGF expression at the gene and protein levels in glioma cells at 48 hours ($P < 0.001$ for gene and $P < 0.05$ for protein levels) and 72 hours of incubation ($P < 0.001$ for both), whereas TEM alone can reduce the expression at only 72 hours compared with the control group ($P < 0.001$ for both). Similarly, EA in combination with TEM reduced both the expression level and immunoreactivity of VEGF significantly only at 72 hours ($P < 0.001$ for both), suggesting a potentiating effect of EA on inhibition of angiogenesis during long-term exposure, independently of the presence of TEM.

DISCUSSION

GB is a disease characterized by a considerable proliferative and invasive capacity, which is well known to be resistant to traditional surgical resections after chemotherapy or radiotherapy. Unfortunately, patients with GBM have a poor prognosis.[22] Alkylating agents such as TEM can easily cross the blood—brain barrier, allowing a useful cytotoxic effect on GBM.[23] TEM is known to induce DNA lesion O6-methylguanine, activating the repair pathways in return but also inducing the drug resistance mechanisms.[24] Therefore, recent studies have focused on combining phytochemical compounds with TEM, which eventually becomes ineffective on the overall survival of patients with glioma because of its resistant nature gained by time.[12,25,26] These combined approaches may be more advantageous over traditional single-agent chemotherapy regimens for brain tumors.[26] In the present study, the effects of combined EA and TEM therapy were investigated on the expression of E- and N-cadherin, which are EMT markers, and VEGF, a common angiogenic marker, in an in vitro model of C6 glioma cells. Expectedly, in vitro EA treatment with TEM selectively increased E-cadherin but decreased N-cadherin expression in a time-independent manner, which might be correlated with a mesenchymal—epithelial transition in glioma cells. However, long-term application of EA with or without TEM reduced VEGF expression, suggesting an inhibitory role of EA on angiogenesis in glioma cells.

The EMT promotes the migratory and invasive abilities of tumor cells without a decrease in proliferation or viability of cells.[15] The EMT process is a prominent activator of the phenotype of cancer stem cells.[27] In vitro studies have reported that glioma cells expressing these stem cell markers are considerably invasive to

Table 1. Proliferation Indexes of C6 Glioma Cells in the Control, Ellagic Acid Applied, Temozolomide Applied, and Combination Groups

Incubation Time	Control Group	EA Group	TEM Group	EA + TEM Group
24 hours	84.87 ± 2.25	54.52 ± 4.69*	49.42 ± 1.47†,‡	7.94 ± 1.30*
48 hours	88.48 ± 2.37	19.75 ± 3.99‡	54.92 ± 2.57‡	6.94 ± 0.93‡
72 hours	86.10 ± 1.65	8.76 ± 1.5‡,§	45.78 ± 0.31‡	4.86 ± 1.65‡,§

EA, ellagic acid; TEM, temozolomide; EA + TEM, ellagic acid and temozolomide.
*$P < 0.001$ versus all groups.
†$P < 0.01$ versus EA group.
‡$P < 0.001$ versus control group.
§$P < 0.001$ versus TEM group.

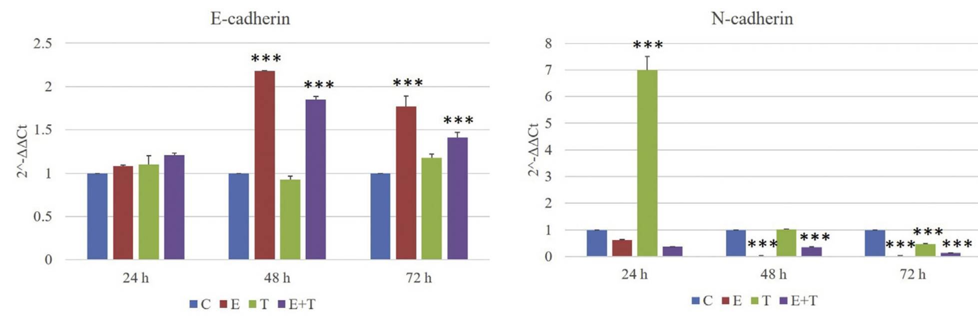

Figure 1. E-cadherin and N-cadherin expression in C6 glioma cells treated with ellagic acid (EA) and temozolomide (TEM). 2^-ΔΔCt, fold difference in ΔCt treated relative to ΔCt untreated (control); C, control group; E, EA group; E+T, TEM and EA group; T, TEM group. $***P < 0.001$ versus all groups.

neighboring tissues and resistant to chemo- and radiotherapy.[28,29] Several downstream pathways of the EMT essentially involve an E-cadherin to N-cadherin switch, and both of these molecules are pertinent to the cancer mechanisms of invasion and metastasis and to the therapeutic resistance of gliomas. This cadherin switch is a possible therapeutic target of combined therapies for incurable cancers.[30] Noh et al.[31] evaluated the expression of E-cadherin and N-cadherin by correlating with the survival rates in patients with glioma and reported that the EMT process may be exacerbated by high levels of N-cadherin expression, resulting in adverse prognosis in gliomas. In the present study, EA significantly reversed the E-cadherin to N-cadherin switch by downregulating N-cadherin and upregulating E-cadherin expression, suggesting an antiproliferative and cytotoxic effect of EA by interfering with the cadherin shift in glioma cells. EA combined with TEM also exerted a similar and sufficient effect on cells as that reported for EA treatment.

GBMs with mesenchymal features presenting a shorter overall survival and progression-free prognosis are associated with severe aggressiveness and therapeutic resistance of cancer against the traditional radiotherapy and chemotherapy.[14] In fact, the clinical reflection of invasive features is obviously induced by the EMT. Kahlert et al.[30] suggested that a small subgroup of glioma cells undergoes molecular processes related to cytoskeletal reorganization and resistance to apoptosis. Hence, the tumor cells become excessively motile and invasive and shortly thereafter gain treatment-resistant features.[30] To overcome resistance in gliomas, we suggest promoting the chemotherapeutic effects of TEM by combining it with EA, which can be used safely with drugs, especially for aggressive and persistent cancers.

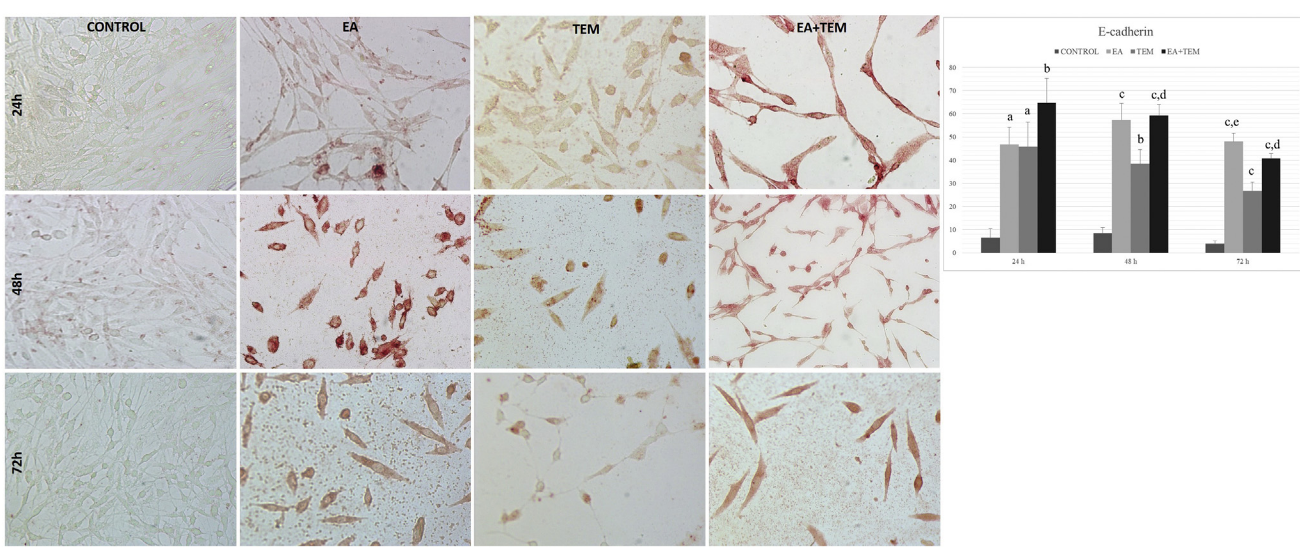

Figure 2. Immunohistochemical analysis of E-cadherin in C6 glioma cells treated with EA and TEM. Magnification: 400×. EA, ellagic acid; TEM, temozolomide. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ versus control group; $^{d}P < 0.05$ versus TEM group; $^{e}P < 0.01$ versus TEM group.

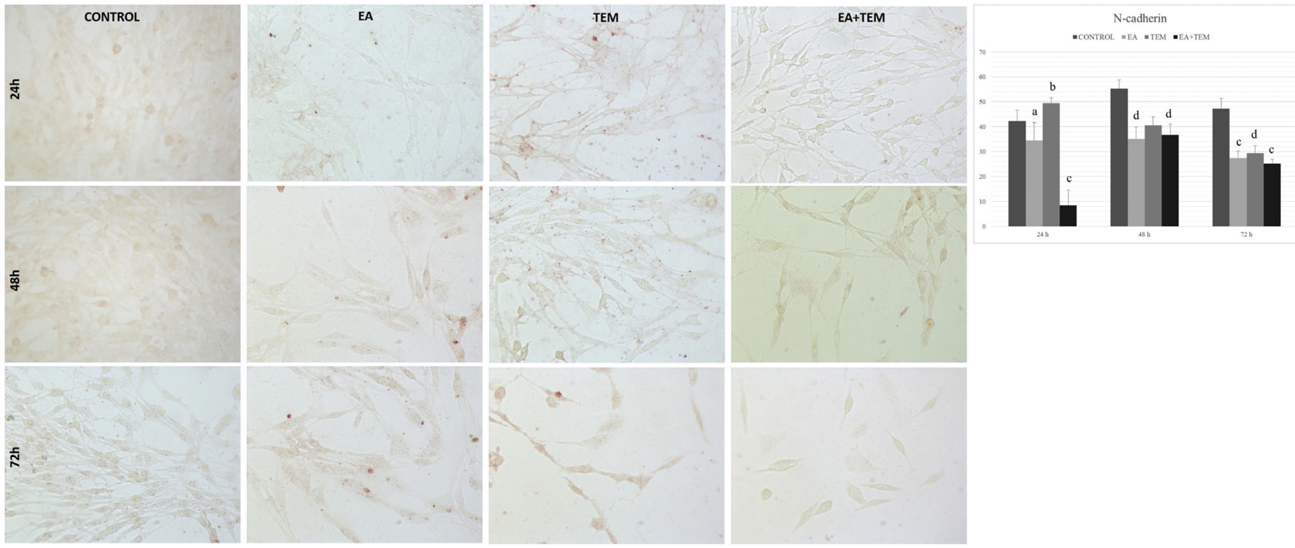

Figure 3. Immunohistochemical analysis of N-cadherin in C6 glioma cells treated with EA and TEM. Magnification: 400×. EA, ellagic acid; TEM, temozolomide. $^{a}P < 0.05$, $^{b}P < 0.001$ vresus EA+TEM; $^{c}P < 0.01$, $^{d}P < 0.05$ versus control group.

Angiogenesis has a fundamental role in GBM progression, supported by the abnormal existence of various proangiogenic factors, such as VEGF, and by the unusual hypoxic microenvironment characterizing GBM.[32] Recent studies have shown that combination therapies could reduce the incidence of GBM and relevant mortality by targeting VEGF and its cellular receptors.[33-35] Previously, the vascular compartment of the human brain tumors was defined and the drug response of GBM endothelial cells was well-characterized. Acetylsalicylic acid (ASA) (aspirin) with or without stimulation with VEGF was reported to inhibit the angiogenesis and metastasis in GBM cells by inhibiting tube-like structure formation and cell migration.[34] Interestingly, aspirin also proved its impact in combination with TEM, enhancing its effect on GBM. Aspirin application was also shown to result in a slight decrease of gene expression of VEGF, whereas its strongest effect was noted in combination with TEM. In fact, the tumor induces the collateral signaling pathways to overcome angiogenesis inhibition produced by TEM, in a feedback mechanism that mediates vascular formation and maintains the dynamic stability of the vascularity.[35] Driven by these promising effects of combination therapies, we investigated in vitro effects of EA in combination with TEM on VEGF expression. According to the results, long-term application of EA with TEM downregulated the gene expression and immunoreactivity of VEGF. Reminding that VEGF is a key mediator in angiogenesis, our results draw attention to using EA combined with TEM in the treatment of GBM.

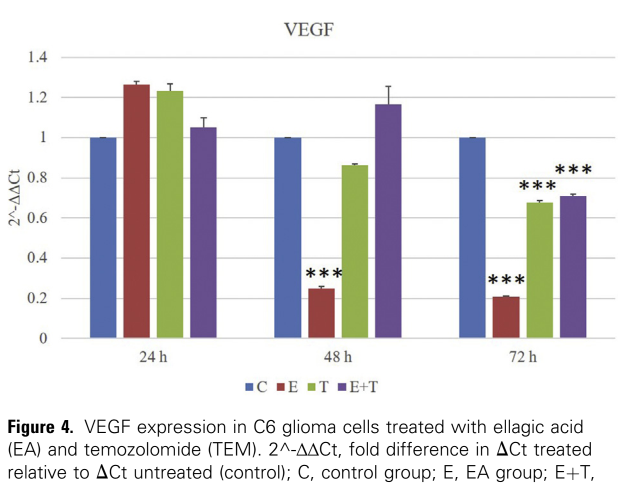

Figure 4. VEGF expression in C6 glioma cells treated with ellagic acid (EA) and temozolomide (TEM). 2^-ΔΔCt, fold difference in ΔCt treated relative to ΔCt untreated (control); C, control group; E, EA group; E+T, TEM and EA group; T, TEM group; VEGF, vascular endothelial growth factor. $***P < 0.001$ versus all groups.

Progress in glioma therapy could be provided by improved comprehension of glioma biology, identification of relevant molecular targets and signaling pathways for treatment interventions, and development of personalized medicine. The targeted approaches currently in clinical trials or in laboratory development include drugs, monoclonal antibodies, immunotherapy, small molecules inhibiting specific proteins, and specific targeting of cancer cells. Therefore, there is a need to reveal the potential clinical impacts by giving the current roles of molecular therapy of GBM. Strategies such as gene therapy, microRNA therapy, stem cells, and immunotherapy may potentially lead to effective GBM treatments.[36] Recently, the use of inhibitors of PI3K/Akt and Notch signaling to treat patients with cancer clinically is currently being assessed in clinical trials. In an in vitro and in vivo study by Wang et al.,[19] EA was revealed to inhibit glioblastoma U87 xenografted tumor growth, which was

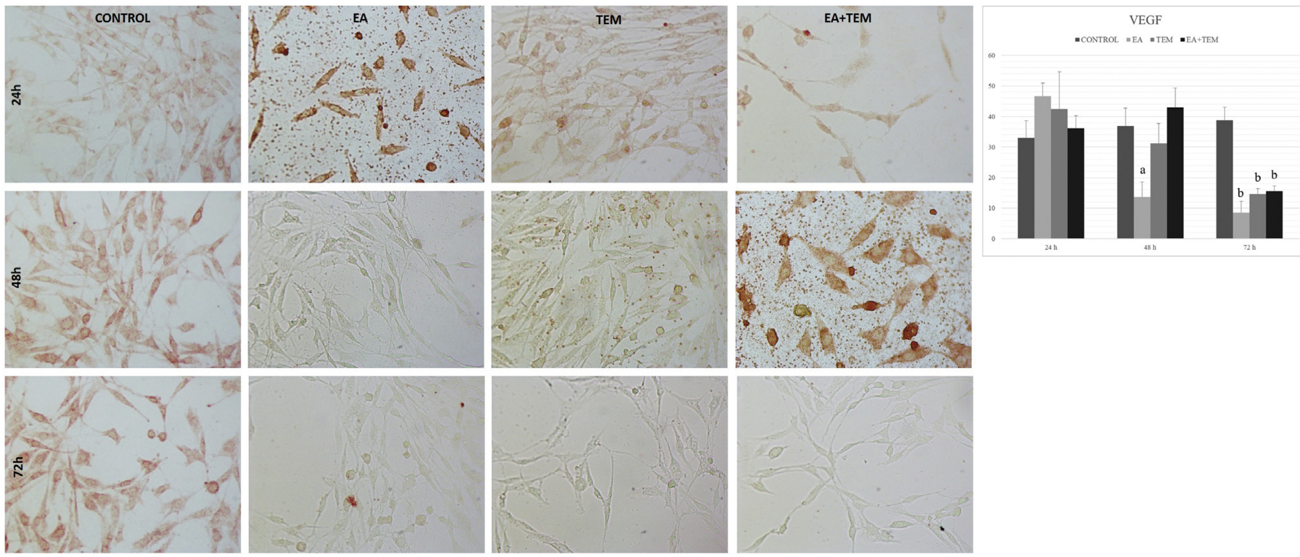

Figure 5. Immunohistochemical analysis of VEGF in C6 glioma cells treated with EA and TEM. Magnification: 400×. EA, ellagic acid; TEM, temozolomide; VEGF, vascular endothelial growth factor. $^{a}P < 0.05$, $^{b}P < 0.001$ versus control group.

involved in the inhibition of the Akt and Notch signaling pathways. The Akt and Notch signaling pathways play an important role in tumor carcinogenesis. In this study, in vitro EA treatment with TEM selectively increased E-cadherin but decreased N-cadherin expression in a time-independent manner, which might be correlated with a mesenchymal—epithelial transition in glioma cells. Moreover, EA reduced VEGF expression alone, suggesting an inhibitory role of EA on angiogenesis in these cells.

In a study by Chung et al.,[37] human ovarian carcinoma cell line ES-2 cells were treated with doxorubicin and EA sequentially, and a synergistic effect of EA was found in ES-2 cells. A similar effect was also observed after sequential treatment of another human ovarian carcinoma cell line, PA-1, with doxorubicin and EA. They also found the synergistic effects of pretreatment of paraplatin or paclitaxel followed by EA treatment in both ovarian carcinoma cells. They suggest that pretreatment of chemotherapeutic drugs followed by EA treatment could overcome the chemoresistance of ovarian carcinoma.[37] The present study is lacking to show the effect of sequential treatment with EA and TEM in GBM cells.

Navone et al.[34] used human primary GBM endothelial cells to ascertain whether ASA could inhibit angiogenesis and improve cell sensitivity to drugs. They reported that ASA affected GBM—endothelial cell viability, tube-like structure formation, cell migration, and VEGF releasing in a dose-dependent manner and that combined treatments with TEM synergized to counteract proangiogenic cell ability. The expression analysis displayed a marked effect of ASA in reducing VEGF, VEGF receptor, HIF-1α, Ras/mitogen-activated protein kinase kinase, mitogen-activated protein kinase kinase, AKT, and Bcl-2-associated X protein/B-cell lymphoma 2, and a combined anticancer effect of ASA together with TEM. They concluded that ASA and antiangiogenic therapies showed synergetic anticancer efficacy in human primary GBM endothelial cells.[34]

Because glioma cells sustaining the EMT process are able to induce metastasis and invasion, cancer progression seems to be involved in upregulated N-cadherin expression, resulting in therapeutic resistance. In contrast, the microenvironment of a tumor, including the hypoxic conditions and microvascular proliferation, highly influences the EMT process. Tissue hypoxia directly induces the EMT and VEGF expression in migrating cells.[38] Therefore, we maximized the efficacy of chemotherapeutic TEM by combining it with EA and suggest an inhibitory role of EA on VEGF expression in C6 glioma cells. However, further studies are needed to demonstrate the exact effects and mechanisms of EA in GBM by using more complex experimental designs such as 3-dimensional cell culture or animal studies, and even human studies. However, this in vitro study provides baseline evidence in favor of combining EA with TEM, resulting in a synergistic effect. Although the results of this study are preliminary and needs to be evaluated on different cell lines and subsequently in clinical translational models, these should be considered to be encouraging to stimulate further research on neurosurgical glioma treatment.

CONCLUSIONS

The findings of this study suggest potential targets for further evaluation of combined EA and TEM therapy in future clinical trials for resistant and incurable brain tumors. Further data from in vitro and clinical studies may indicate that a combined strategy exerts much higher efficacy in treating gliomas than single administration of a chemotherapeutic drug, therefore reducing the dosage and minimizing the side effects of cytotoxic therapy.

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