The Effect of Temozolomide Dose and Erythropoietin Combination On Survivin Gene Expression in Human Glioblastoma Cell Lines

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Summary

Glioblastoma (GBM) is an aggressive primary brain tumor and it is known that prognosis is poor. Because of the infiltration of the tumor cells to the whole brain tissue, surgery is done for decompression and for histopathological diagnosis. Radiotherapy is mandatory after surgery but survival period is not sufficient. In recent years there are many researches for chemotherapeutical agents to use for treatment of GBM but most of the results of the researches are controversial. Temozolomide (TMZ) is a new agent which is effective in prolonging survival of GBM patients. Erythropoietin promotes red blood cell maturation and used to prevent or treat anemia. It is also known that EPO has neuroprotective effect. In this study we used GMS-10 human glioblastoma cell lines. We applied TMZ with different doses and TMZ + EPO combination to check the efficacy on the survivin gene expression in human glioblastoma cell lines. As a result we observed that increasing the dose of TMZ and combination with EPO results over expression of survivin gene which means drug resistance.

Key words: Erythropoietin, Glioblastoma, Survivin, Temozolomide

Özet

Glioblastoma (GBM) çok agresif bir beynin tümörüdür ve prognozu kötüdür. Tümör hücrelerinin beynin dokusuna yaygın infiltrasyonu nedeniyle cerrahi girişim sadece dekompresyon ve doku tanısı amacıyla uygulanabilir. Cerrahi sonrası radyoterapi mutlak gerekçidir ancak ortalama yaşam süresi yeterli değildir. Son yıllarda GBM tedavisinde kullanılmak üzere kemoterapötik ilaç araştırmaları yoğunlaşmıştır ancak çoğu çalışma sonuçları tartışma konusu olmaktadır. Temozolomide (TMZ) GBM hastalarında yaşam süresini uzatabilen yeni bir ajandır. Eritropoietin (EPO) ise anemi korunmasında veya tedavisinde kullanılarak bir ajan olmakla beraber nöroprotectif özelliği de bulunmaktadır. GMS-10 GBM hücre kültürlerinde TMZ’ nin iki farklı doz ve Eritropoietin kombinasyonu ile survivin geni üzerindeki etkilerini araştırdık. Sonuçta TMZ dozu artırılması ve EPO kombinasyonunun survivin geni ekspresyonunu artırığı, yani ilaç direncine neden olduğunu saptadık.

Anahtar Kelimeler: Erythropoietin, Glioblastoma, Survivin, Temozolomide
INTRODUCTION
Primary intracranial tumors originates from the glial tissue, neurons, meninges, vessels or endocrine cells that are located at the intracranial space. According to the World Health Organisation (WHO)'s classification, gliomas are neuroepithelial tumors that originate from the supportive glial tissue and they are defined as astrocytoma, oligodendroglioma, ependymoma, unclear sourced glial tumor and mixed glioma. GBM (grade IV astrocytoma), unfortunately is the most common and the most malignant glioma arising in adults and it represents 12-15% of all intracranial tumors and 50-60% of all astrocytic tumors. They are usually unilateral, solitary tumors of the cerebral hemispheres. In many cases the tumor invades across the corpus callosum or appears precisely within the bilateral extension. Most of the patients are over 45 years of age, but it can also rarely arise in young people.

When the various types of mutations and alterations observed in glioblastomas are analyzed, it is apparent that they are not scattered randomly; in fact, two apparent subtypes of glioblastomas are observed. One subtype appears to present in older patients de novo as glioblastoma and tends to possess deletions in the cell-cycle related INK4a-ARF genes p16 and p19/p14ARF. At the signal transduction level, these tumors tend to have epidermal growth factor receptor (EGFR) gene amplifications, making them constitutively active. The second subtype tends to arise in younger patients, usually as a lower-grade glioma that progresses eventually to glioblastoma. Rather than INK4a-ARF deletions, these tumors are characterized by mutations in the tumor suppressor gene p53, along with either amplification of CDK4 or loss of RB, all of which affect cell cycling downstream of the INK4a-ARF locus. Tumors in this younger group of patients also tend to express a different signal transduction alteration-amplification or overexpression of platelet derived growth factor (PDGF).

Standard treatment modality of GBM is radiotherapy after surgery. Complete resection of the tumor is not possible because of the invasion of the tumor cells through the whole of the brain tissue. Surgery is planned for decompression and true histopathological diagnosis. Radiation therapy, age and performance status have been demonstrated to be the three most significant prognostic factors in patients with malignant gliomas. Of the three, radiotherapy is the only factor amenable to modulation by the clinician. Manipulation of total dose, fractionation, particle size and type, timing, and combination therapy with chemotherapy, hyperbaric therapy, cryotherapy, and interstitial brachytherapy have been tried to improve on the standard 5400 cGy administered over 5 weeks without significant gain in length of survival. Compared with the significant impact of radiotherapy on malignant gliomas, chemotherapy has had limited impact on these lesions. Alkylation agents are one of the mainstays of current chemotherapy regimens. They have been demonstrated to be quite efficacious in the management of a variety of neoplasms. In the early 1970s, two groups reported partial success with 1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU) in the treatment of recurrent GBM. Responses were judged as stable or the tumor burden decreased based on images studied. The response rate was approximately 40%.

Temozolomide (TMZ; 3-methyl-4-oxo-3,4-dihydroimidazo 5,1-d 1,2,3,5 tetrazine-8 carboxamide) is an oral prodrug that is rapidly absorbed and undergoes spontaneous hydrolysis at physiologic pH to form the active metabolite 3-methyl-(triazen-1-yl) imidazole-4-carbox-Gamide. TMZ in addition to standard postoperative radiotherapy as the first line treatment for glioblastomas demonstrated an increase in median survival from 12.1 to 14.6 months and an increase in 2-year survival rate from 10 to 26% as compared...
to postoperative radiotherapy alone. TMZ has therefore received much attention and become a current standard chemotherapy agent, notably in the treatment of malignant gliomas. Clearly, concomitant radiation therapy with TMZ chemotherapy followed by adjuvant TMZ treatment can yield meaningful results in glioblastoma patients, but their prognosis remains unsatisfactory. The limited efficacy of chemotherapy can be attributed largely to both inherent and acquired tumor drug resistance mechanisms. In addition to O⁶ – methylguanine-DNA methyltransferase (MGMT), base excision repair and mismatch repair are thought to be involved in the principal mechanisms contributing to TMZ resistance. The resistance of glioblastoma to TMZ appears to follow a more complex pattern than simple dependence on MGMT levels, although its detailed regulation at the genomic level remains poorly understood. The mechanism of cytotoxicity is alkylation of DNA. Alkylation of the O⁶ position of guanine is the lesion considered responsible for cytotoxicity. 40% of plasma concentration level passes to the cerebrospinal fluid and reaches to the efficient concentration level. TMZ is lipophylic and stable at the acidic medium. Its half-life is about 1.8 hours and eliminated mainly by kidneys. After oral intake, 5-10% of the unchanged drug is excreted with urine in 24 hours. The rest of the drug is excreted as 5-aminoimidazole-4-carboxamide or as undetermined polar metabolites. The initial studies with TMZ were with anasplastic astrocytoma (AA) and GBM mixed groups and mainly about phase 2 and most of the patients had progression after surgery, radiotherapy and/or chemotherapy. But the recent studies are done specifically with AA or GBM patients. The dosage of TMZ and the schedule is similar in all of the studies. Every fourth week, the patients intake TMZ orally 150-200 mg/m2/day for five days. If the patient has taken an initial dose or another chemotherapeutic agent before the cure, the dose of TMZ is planned as 150 mg/m2/day. Bone marrow functions should be checked at the 22nd day of the cycles, and any sign of bone marrow function depression is the indication to decrease the dose of TMZ to 150 mg. Understanding the molecular basis of TMZ sensitivity/resistance is necessary for improving the treatment outcome by devising strategies that are able to circumvent primary drug resistance. Yoshino et al published gene expression profiling predicts response to TMZ in malignant gliomas and reported that most highly up-regulated and down-regulated genes which may be involved in conferring TMZ sensitivity/resistance in malignant gliomas have not been implicated as a casual factor in the TMZ response except MGMT. An early study noted that patients with malignant glioma had an increase in the size of contrast enhancement, immediately after radiotherapy, with subsequent improvement without any further treatment. This occurrence, which mimics tumor progression, has been labelled pseudoprogression. Moreover, a high incidence of radionecrosis has been noted in patients who underwent surgery for progressive brain lesions within the first 6 months after combined chemotherapy with TMZ. Both findings could be consistent with the increased tumor-cell killing caused by the chemoradiotherapy. These progressive lesions have important consequences on the management of patients with progressive lesions immediately after TMZ chemoradiotherapy. In order to protect the neural tissue some additional treatment modalities should be considered during TMZ chemotherapy. Recombinant human erythropoietin (rEPO) is one of the most promising neuroprotective agents under investigation. Erythropoietin (EPO) is the primary endogenous cytokine that
promotes red blood cell maturation, so rEPO is widely used to prevent or treat anemia. In addition to its role in erythropoiesis, research has established that rEPO can also mediate neuroprotection in vitro and in vivo. The tissue protective functions of EPO are independent of its action on erythropoiesis. EPO and its receptors (EPOR) are expressed in multiple brain cells during brain development and up-regulated in the adult brain after injury. Peripherally administered EPO crosses the blood-brain barrier and activates in the brain anti-apoptotic, anti-oxidant and anti-inflammatory signaling in neurons, glial and cerebrovascular endothelial cells and stimulates angiogenesis and neurogenesis. These mechanisms underlie its potent tissue protective effects in experimental models of stroke, cerebral hemorrhage, traumatic brain injury, neuroinflammatory and neurodegenerative disease. The preclinical data in support of the use of EPO in brain disease have already been translated to first clinical pilot studies with encouraging results with the use of EPO as a neuroprotective agent.

Survivin is a member of the inhibitor of apoptosis (IAP) family(11). It includes only one Baculovirus IAP Repeat (BIR) domain and elongated –COOH terminal alpha – helix spiral region. The survivin protein functions to inhibit caspase activation, thereby leading to negative regulation of apoptosis or programmed cell death. This has been shown by disruption of survivin induction pathways leading to increase in apoptosis and decrease in tumour growth. The survivin protein is expressed highly in most human tumours and fetal tissue, but is completely absent in terminally differentiated cells. This fact therefore makes survivin an ideal target for cancer therapy as cancer cells are targeted while normal cells are left undisturbed(22). Survivin expression is also highly regulated by the cell cycle and is only expressed in the G2-M phase. It is known that survivin localizes to the mitotic spindle by interaction with tubulin during mitosis and may play a contributing role in regulating mitosis. The gene of survivin takes place at the 17q25 human chromosome and it encodes the 16.5 KD protein. This gene is founded by Altieri in Yale University by hybridisation and filtration of human genomes. During interphase, it separates by ubiquitination and proteosome dependent destruction and its expression contributes to cellular cycles periodicity. During G2/M, it locates to mitotic apparatus with more than 40 times gene expression. These are centrosomes (also called microtubule organising centers), metaphase and anaphase spindle microtubules and remnants of mitotic apparatus, ie midbodies at telophase. Recent studies showed that cellular survivin pool is localised at central spindle region and kinetochores of the metaphase chromosomes. Various type of survivin pools are biochemically different and they reflect posttranslational modifications that arrange epitope accessibility and input-output of mature proteins. Two isoforms of survivin; Survivin 2-B is produced by addition of alternative exon and Survivin-Δex-3 is produced by removal of exon 3. It is known that survivin and survivin-Δex-3 are associated with the inhibition of apoptosis. The unique –COOH terminal which is produced by the frameshift of Survivin-Δex-3 has been shown that it mediates the blocking of this isoform of survivin's nuclear collectings.

In this study, we investigated the relationship of TMZ with the survivin gene at the human GBM cell and the role in drug resistance interaction with the other routine chemotherapeutic agents. The effect on survivin gene expression level with the standard dose of TMZ and the drug resistance after Erythropoietin (EPO) application are evaluated in GMS-10 human glioblastoma cell line.

MATERIAL AND METHODS
In this study we used commercially available GMS-10 (DSMZ No: ACC 405) human glioblastoma cell line. These cells
were obtained from a 49 years old male patient with malignant glial tumor. They confluent in a single layer like fibroblasts. Duplication time is 50-70 hours. The cells uncoiled in a waterbath at 37°C and implanted in a complete cell culture medium within DMEM+Penicillin (10 U/ml), Streptomycin (10 mg/ml) + 10 % FBS + 1% Glutamine in 75 cm² flasks. After then they were incubated at the CO₂ incubator. After the culture reached to 90% density, we removed them from the flasks with tripsin/EDTA to implant them in 25 cm² flasks after counting and checking the viability of the cells with trypan blue. We created 6 groups and G1 remained as the control group. The other groups were; G2: TMZ 75 mg/m²/day, G3:TMZ 200 mg/m²/day, G4:EPO 30 IU, G5: TMZ 75 mg/m²/day + EPO 30 IU, G6: TMZ 200 mg/m²/day + EPO 30 IU.After 18 hours of incubation period, mRNA was isolated by Roche High Pure RNA Isolation Kit® (Cat Nr: 11 828665001). cDNA was synthesized from the isolated mRNA by Roche Transkiptae First Strand cDNA Synthesis Kit® (Cat Nr: 04 379012001). Human Survivin Light Cycler set (Lot:170608) used for real time PCR reactions with the cDNA by using the protocols of 10 min 95 Co, 45 cycles 10 sec 95 C°, 10 sec 68 C°, 16 sec 72 C° and 30 sec 40 C°.
To obtain 0.125 mg in 10 µl solution, the content of 250 mg Temozolomide capsule (Temodal® 250 mg capsule, Schering-Plough, Serial Nr: 124400907) was dissolved in 20 ml sterile saline. 45 µl solution was used for 75 mg/m²/day, and 120 µl solution was used for 200 mg/m²/day dose. There was 4000 IU of EPO in 0.4 ml Eprex 4000®(Cilag) and 3 µl (30 IU) was used for EPO dose.

RESULTS
At the cells in group 3 (G3) which TMZ had used 200 mg/m²/day, survivin gene expression levels were significantly higher than the cells in control group (G1) and TMZ 75 mg/m²/day group (G2) (p=0.008; p=0.017). Also levels are higher in TMZ 75 mg/m²/day + EPO group cells (G5) and TMZ 200 mg/m²/day + EPO group cells (G6) than in G1 (p=0.0008;p=0.003). Survivin gene in G6 cells were expressed higher than in G3 cells (p=0.03) and similarly G5 cells were expressed higher than G2 cells (p=0.018). Survivin gene expression level in G6 cells were higher than in G2 cells (p=0.004).

DISCUSSION
Malign gliomas are the most aggressive tumors among the human neoplasms. These are resistant to the standard therapeutical modalities such as surgery, radiotherapy and chemotherapy. Even though the improvement of the therapeutical modalities, results are unchanged. Owing to infiltration of the high grade tumor cells to the normal brain tissue, it is not possible to resect the tumor completely and radiotherapy is a standard therapy after surgery. Despite of improvement of neuroradiology, neurosurgery and radiotherapy technics, prognosis of the GBM patients are still poor. As a result, research of effective chemotherapeutical agents is very important.
Initially TMZ studies were done with AA and GBM patients, but recent researches are separated as AA or GBM. In 2004, a group of clinical studies showed the effectiveness of TMZ on recurrent malign glioma patients. Surviving period without progression and the response ratio to TMZ treatment are variable related to patient selection and the number of GBM patients. Many studies indicated that the period through progression was 3-5 months. Table 2 summarizes the results of those studies and it is clear that combination of cytotoxic agents with TMZ didn't improve the results of the treatment.
In 2008, Hassouna I. treated human derived GBM cell lines U87, G44, G112 and the gliosarcoma derived cell line G28 with EPO with and without combination of radiotherapy or TMZ(16). Response of glioma cells to EPO therapy was measured by cell migration from spheroids, cell proliferation and clonogenic survival. Implantation of U87 cells into the brain of nude mice after 5 days later than EPO treatment (5.000 U/kg intraperitoneal every day for 2 weeks) should reveal effects of EPO on tumor growth in vivo. EPO did not modulate basal glioma cell...

Table 1: RT-PCR results.

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<td>18.98</td>
<td>0.69</td>
</tr>
</tbody>
</table>

(CP: Crossing point; Cons.Ratio: Constant Ratio)

Table 2. Groups (Expression Levels)

In 2008, Hassouna I. treated human derived GBM cell lines U87, G44, G112 and the gliosarcoma derived cell line G28 with EPO with and without combination of radiotherapy or TMZ(16). Response of glioma cells to EPO therapy was measured by cell migration from spheroids, cell proliferation and clonogenic survival. Implantation of U87 cells into the brain of nude mice after 5 days later than EPO treatment (5.000 U/kg intraperitoneal every day for 2 weeks) should reveal effects of EPO on tumor growth in vivo. EPO did not modulate basal glioma cell...
migration and stimulated proliferation in only one of four cell lines. After 3 hours of preincubation of the cells with EPO, irradiation and TMZ used for 24 hours. As a result, he mentioned that the three cell lines were protected against chemotherapy + radiotherapy induced cytotoxicity and EPO induced a dose dependent decrease in survival of G28 gliosarcoma cells. So he did not advise concomitant use of EPO with radiotherapy and/or chemotherapy.

We combined EPO to TMZ treatment to obtain the neuroprotective effect and avoid post-chemotherapy side effects of TMZ such as pseudoprogression. Unfortunately, opposing the expectation of benefits of EPO, we found that combination resulted an increase on drug resistance to TMZ. TMZ treatment for malignant gliomas in patients who get also EPO treatment for anemia will be least effective because of this resistance.

In our study we investigated the intercourse between TMZ and the survivin gene of the human GBM cell line, the role of this intercourse on the resistance to routine chemotherapeutical agents by interaction with TMZ, and the effect of apoptosis related treatment on tumor prognosis. At the TMZ 200 mg/m²/day group survivin gene expression level was higher than TMZ 75 mg/m²/day group. At the TMZ 200 mg/m²/day +EPO and TMZ 75 mg/m²/day groups, expression levels were higher than the control group. Survivin gene expression level at the TMZ 200 mg/m²/day + EPO group was significantly higher than the TMZ 200 mg/m²/day group, similarly TMZ 75 mg/m²/day + EPO group had higher expression level than the TMZ 75 mg/m²/day group. Finally TMZ 200 mg/m²/day group had higher gene expression level than TMZ 75 mg/m²/day group. The findings mean that; increasing the dose of TMZ and combining with EPO results over-expression of survivin gene.

CONCLUSION

There was a direct correlation between the dose of TMZ and the survivin expression level. When EPO was used alone, there was no difference at the gene expression level but when it was combined with TMZ, expression level became higher than the TMZ alone group. So, we believe that increasing the dose of TMZ and/or combination of TMZ and EPO will be more efficacious in expressing survivin gene at GBM patients. So, TMZ should be used with least effective dose for the chemotherapy protocol of GBM, and patients who also get EPO treatment for anemia should be considered to stop the EPO treatment or change TMZ chemotherapy with another alkylating agent.

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