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MAXIMIZATION OF DNA DAMAGE TO MGMT(+) EGFR(+) GBM CELLS USING OPTIMAL COMBINATION OF TEMOZOLOMIDE-ANTI EGFR MONOCLONAL ANTIBODY NIMOTUZUMAB

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Background: Glioblastoma multiforme (GBM) is the most aggressive primary brain tumor in adultswith dismal prognosis due to the unavailability of an effective therapy. Up to now, there had been no definitive studies published on EGFR inhibition therapy as a chemosensitizer for GBM therapy using Temozolomide (TMZ). This study aims to reveal the most effective method and timing to administer TMZ-anti EGFR targeted therapy which causes maximal DNA damage on GBM cells.
Methods: Various regimens of anti EGFR monoclonal antibody Nimotuzumab (NMZ) was administered in different combinations with TMZ, performed on U87MG MGMT(+) EGFR(+) cells. The effectiveness of the combinations were evaluated by measuring yH2AX levels which reflects the degree of DNA damage. One-way Anova and LSD tests were performed to determine the effects of each treatment with $p<0.05$. **Results and discussion:** the mean SD of yH2AX of each treatment was: 11,90±1,25 for the control group; 29.33±1.91 for NMZ alone; 28.13±1.58 for TMZ alone; 41.53±3.51 for concurrent use; 35.67 ±2.65 for NMZ after 24 hours TMZ; 31.87±2.94 for NMZ after 48 hours TMZ; 39.57±4.2 for TMZ after 24 hours NMZ; and 35.93 ±3.56 for TMZ after 48 hours NMZ. The administration of TMZ concurrent with or after 24 hours NMZ gives the highest amount of DNA damage to GBM cells. **Conclusion:** The administration of Nimotuzumab targeted therapy up to 24 hours before Temozolomide chemotherapy has been proven to be effective in maximizing the amount of DNA damage done to GBM cells in vitro.

Keywords: Glioblastoma; multiforme; Temozolomide; anti EGFR; Monoclonal;

INTRODUCTION

Glioblastoma multiforme (GBM) is the most frequently found and most aggressive glial cell tumor, associated with a dismal prognosis and mean survival time of one year after diagnosis.[1,2] This poor prognosis is caused by our incomplete understanding on this aggressive tumor's characteristics and the lack of an effective therapy.
The standard chemotherapy agent for GBM is Temozolomide (TMZ).[3] Many studies have been performed to overcome TMZ resistance, including modifications to administration dosage and mechanism, and the combination of TMZ with other agents or targeted therapies. Currently available targeted therapy for GBM include PI3-K/mTOR, PDGFR, VEGF/angiogenesis, Hedgehog GLI1 and EGFR/ EGFRvIII.[4-7]

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Overexpression and amplification of epidermal growth factor receptors (EGFR) is a dominant mutation of GBM cells, compared to other genetic mutations, and is linked to increased GBM cell resistance to radiotherapy and chemotherapy.[8] Chen, et al in 2007 have identified the radioprotective function of EGFR, through intranuclear translocation and its interaction with DNA-dependent protein kinase (DNA-PK), a key component of non-homologous end-joining pathway in DNA repair.[9] Even though Bao et al did not evaluate the pathways of DNA repair caused by induction of cytotoxic chemotherapy,[10] an analogous mechanism may be at work here. By attempting to interrupt the DNA repair mechanisms of EGFR at an early stage, anti EGFR Monoclonal Antibody Nimotuzumab (NMZ) was given before TMZ therapy, in hope of achieving a synergistic effect as a model of TMZ therapy for GBM cells. This study hopes to discover the effect of combination TMZ-NMZ therapy to find out the most effective chemotherapy regiment for MGMT methylated (+) and EGFR overexpression (+) GBM cells, especially in its DNA damage activity. Another goal for this study is to find the most effective administration order and interval.

MATERIALS AND METHODS

U87MG cell line culturing

Expansion and maintenance of U87MG cells were done on the bottom surface of 150 cm2 TC flasks, submerged with 30 ml of growth medium. The growth medium consisted of Dulbeco's Modified Eagle's Medium (DMEM) [Gibco], 10% Fetal Bovine Albumin (FBS) [Invitrogen], 0.5% L-Glutamine [Gibco], and 0,5% Gentamycin [Gibco]. After confluence some cells were transferred into new flasks for further expansion or experiment treatments. Some were added 10% Dimethyl Sulfoxide (DMSO) [Sigma] into its medium and frozen in cryovials submerged in liquid nitrogen as future usage stocks. Upon usage, DMSO were cleared off the cells by pelleting and replacing the medium as soon as it thawed.

Drug dosage determination

Cells were planted 3 days prior to Nimotuzumab (NMZ) and Temozolomide (TMZ) treatments, on 24-wells plate, submerged in growth medium. Each well was given 0, 10, 50, 100, 500, 1000 ug/ml NMZ, and 0, 2, 10, 20, 100, 200 μg/ml TMZ. Each treatment was done in duplet. Cells were observed under microscope every 24 hours for number and viability.

After 24 hours, the other duplet had its medium aspirated dry, harvested by submerging with 0.1% trypsin [Gibco] for 5 minutes at 37C. Cells from each well then were suspended in 500 μl PBS and analyzed by flow cytometry as separate samples: the whole 500 μl PBS of each sample was run through flow cytometer and had its approximate total cell number recorded by the program. Optimal drug concentration was also determined by these cell numbers.

U87MG drug treatment

Cells were planted 3 days prior to Nimotuzumab (NMZ) and Temozolomide (TMZ) treatments, on 3x12-wells plates, submerged in growth medium. Then on the third day, cells were treated with 8 different treatments. 4 wells were allocated for each of these treatment groups: Non-treated (Control), NMZ only for 72h (N), TMZ only for 72h (T), NMZ and TMZ for 72h (NT), NMZ after 24h TMZ (N24T), NMZ after 48h TMZ (N48T), TMZ after 24h NMZ (T24N), and TMZ after 48h NMZ (T48N). NMZ was given at 1000 μg/ml and TMZ at 20 μg/ml in their respective treatment groups, both based optimal drug concentration determined beforehand. The rest 4 wells were reserved as spare wells in case anything unexpected happened to any of the allocated wells prior to cell treatment, to ensure the cells within all wells to be treated were in possible best conditions and closest to identical numbers. Unused spare wells were later on used as flow-cytometry unlabeled control.

U87MG flow cytometry

After 72 hours of treatments, cells were harvested by submerging with 0.1% trypsin [Gibco] for 5 minutes at 37C. Every treatment group had 4 wells available to stain with fluorescent tagged antibody. Each of these 4 wells was allocated to be stained with anti-yH2AX-APC [Cell Signaling] to analyze cell DNA damage of the samples.

After trypsination, each well content was put into a single 1.7 ml microtube and washed once with staining buffer (PBS [Invitrogen] + 1% BSA [Sigma]) to remove the trypsin. Each tube which was allocated for anti-CD133-APC staining were directly resuspended with 50 ul staining buffer and added with 1 ul anti-CD133-APC and incubated for 1 hour in a dark room at room temperature.

All other samples were next fixated by resuspending them with 1% formaldehyde inside each micro-tube and incubate them all for 10 minutes at 37^0C, and then were washed with staining buffer to remove the formaldehyde. For the wells allocated for anti-yH2AX-APC, permeabilization of the outer plasma membrane and nuclear envelope was done by resuspending the cell pellet with 1% Triton-X [Biorad] in staining buffer and incubate them for 30 minutes at room temperature. Soon after permeabilization step, each of the samples were washed twice with staining buffer and then resuspended in 50 μl staining buffer in their own respective microtubes. After that, each microtube was added with anti-yH2AX-APC. All tubes were incubated for 1 hour in a dark room at room temperature.

After all treatment groups had been incubated for an hour, each tube was added with 450 ul of staining buffer, making each sample 500 ul in volume. Finally all samples were analyzed with flow cytometer [BD Accuri C6]. Samples with FITC fluorescent marker were excited by 488 nm blue laser and read at 533-563 nm wavelength channel, PE by 488 nm blue laser at 585-625 nm wavelength channel, and APC by 640 nm red laser at 675-700 nm wavelength channel.

Statistical analysis

Statistical analysis was performed using the SPSS for Windows, version 21.0. The significance of differences between groups was compared using One Way Anova. The significance of differences in groups was compared using LSD. Differences were considered significant if $p < 0.05$.

RESULTS

The results of flowcytometer examination of each treatment was presented in table and graph form. Of each treatment group have CD133negative. yH2AX levels are significantly higher ($p < 0.05$) for all treatment protocols compared to the control group.

Table 1 The Effects of NMZ, TMZ and Their Combinations to yH2AX Levels.

Groups	N	yH2AX levels (%) Mean (SD)	yH2AX levels (%) Difference in Mean from Control
Control	3	11.90 (1.25)[a]	0.00
Nimotuzumab (NMT)	3	29.33 (1.91)[b]	17.43[p]
Temozolomide (TMZ)	3	28.13 (1.580[b]	16.23[p]
N and T combinations	15	36,91 (4.53)[c]	25.01[q]

Superscripted letters in the same column shows LSD results after One Way Anova test showing $p > 0.05$; and its significant difference $p < 0.05$. The group with combination therapy resulted in significantly higher yH2AX levels when compared to the mono-therapy groups. There are no significant differences between single therapy NMT or TMZ groups.

Data of yH2AX levels grouped by order of and interval of drug administration were presented in Table 2.

Table 2
yH2AX levels grouped by order of and interval of drug administration

Groups	yH2AX levels (%) Mean (SD)	yH2AX levels (%) Different in mean from control group
Control (C)	11.90 (1.25)[a]	0.00
NMZ after 48 hours TMZ (N48T)	31.87 (2.94)[b]	19.97[p]
TMZ after 48 hours NMZ (T48N)	35.93 (3.56)[c]	24.03[q]
NMZ after 24 hours TMZ (N24T)	35.67 (2.65)[c]	23.77[q]
TMZ after 24 hours NMZ (T24N)	39.56 (2.06)[d]	27.66[r]
Concurrent TMZ and NMZ use	41.53 (3.51)[d]	29.63[r]

Superscripted letters in the same column shows LSD results after One Way Anova test showing $p > 0.05$; and its significant difference $p < 0.05$.

Table 2 shows the results of various combinations of NMZ and TMZ therapy, by order and treatment interval. The mean yH2AX levels in the combined NMZ and TMZ groups vary depending on their administration order and administration interval. The highest yH2AX levels are found in the concurrent therapy group (mean 41.53±3.51), and lowest in the NMZ after 48 hours TMZ group (mean 31.87±2.94). The differences are significant between the concurrent therapy group and the N24T, N48T and T48N groups; but not significant between the concurrent group and the T24N group.

DISCUSSION

Repair of DNA damage in GBM cells

This is a pioneer study in investigating the effect of the administering Nimotuzumab/NMZ (N) on the effectiveness of Temozolomide/TMZ (T), assessing the effects of the order of administration and the interval between administrations on the degree of DNA damage as represented by yH2AX levels. Higher yH2AX levels are interpreted as a higher degree of DNA damage. The highest significant yH2AX levels were identified when Temozolomide and Nimotuzumab were given concurrently, or when Temozolomide was given after 24 hours Nimotuzumab; compared to the yH2AX level in the control group. yH2AX levels increase by degrees between the T, N, N48T, N24T, T48N, T24N and NT groups. The combined use of Temozolomide and Nimotuzumab is proven to increase the degree of DNA damage significantly, when compared to the control group and monotherapy groups.

The repair of double-stranded DNA damage (*DNA double strain breaks, DNA DSBs*) is achieved through two pathways. The first pathway is to combine a sequence of DNA with a homologous template (*homologous recombinant, HR*), and the second pathway is to combine the end sequence of damaged DNA based on the presence of proteins and sequential systems (*non homologous-end joining, NHEJ*). NHEJ is the dominant pathway in repairing DNA DSBs, with the HR pathway as a supporting pathway.[9] The NHEJ pathway is active during the cell cycle, and occurs mostly at the G1 phase; the HR pathway happens after DNA replication was performed, where identical chromatins are used as a template in the repair process.[11]

In the NHEJ pathway, recombination the damaged DNA chains depend on the activity of sub unit Ku70 dan Ku80, which is the main mechanism for DNA recombination. They are tied to the DNA end chains, which activates the catalytic subunit of DNA protein kinase (DNA-PK) and Artemis, which interacts with the proteins between the DNA-PK molecules and forms a bridge between the DNA end chains. The combination of DNA-PK and Artemis becomes phosphorylated and activates other enzymes, such as Ligase IV/XRCC4 and polynucleotide kinase (PNK). Outside of the aforementioned process, protein complexes Mre11, Rad 50, and Nbs1(MRN) are also able to recombine and repair DNA fragments. Therefore, the DNA-PK enzyme plays a key role in repairing DNA DSBs.[12] EGFR also is one of themain keysin inhibiting of DNA DSBs repair.[9] In NHEJ pathway, interactions of EGFRwith DNA-PKwill control the disassembly of DNA-PK and the physical rejoining of DNA DSBs. EGFRbinds to the catalytic sub unit of DNA-PK and controls regulatory subunits Ku70 of DNA-PK.[15] By block of EGFR translocation into the nucleoplasm, the interactions of EGFR-DNA PK will interupted.[13,14]

Optimal Combination for DNA Damage

The LSD statistical test was performed to investigate the difference between the order of administration and the interval between the administration of Nimotuzumab and Temozolomide, and it shows that concurrent administration is significantly better than other drug regiments, except the administration of Nimotuzumab within 24 hours before the administration of Temozolomide. This proves that giving Nimotuzumab before Temozolomide can increase the degree of DNA damage caused by Temozolomide. This effect is thought to be caused by the effects of Nimotuzumab in inhibiting intracellular translocation of EGFR, and inhibiting the effect of DNA repair enzymes (DNA-PK) in repairing DNA double strain breaks.[13]

Similar drug administration order, with different administration interval, was shown to have a different effect; T24N has higher yH2AX levels than T48N, and N24T has higher yH2AX levels than N48T. This indicates that the timing of administration has an effect on the increased DNA damage mechanism. This study shows that the administration of Temozolomide or Nimotuzumab within 24 hours before the next drug can increase DNA damage compared to 48 hours. This is thought to be caused by a very fast reaction phase by the defensive mechanism of GBM cells towards radiochemotherapy; within 1-4 hours of drug administration, intranuclear EGFR translocation and DNA-PK already begins to repair the DNA damage caused by Temozolomide.[9] The administration of Nimotuzumab within the first 24 hours is effective in inhibiting the DNA repair process, while Temozolomide continues to cause DNA damage and DNA double strain breaks.[16] The administration of Nimotuzumab in the first 24 hours will inhibit the interaction and activity of EGFR-DNA-PK enzyme, increasing DNA damage in vitro. The inhibition of DNA repair through the main NHEJ pathways by EGFR-DNA PK interaction will increase the ability of Temozolomide in causing damage to GBM cell DNA.

CONCLUSION

The administration of Nimotuzumab, concurrently or within 24 hours before the administration of Temozolomide, is an effective combination in maximizing DNA damage to the DNA of GBM cells in vitro. The initial inhibition of DNA repair enzymes (DNA PK) through the mechanism of EGFR blockage will synergize with the effects of Temozolomide in causing DNA damage.

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