Glycogen synthase kinase 3 β inhibition sensitizes human glioblastoma cells to temozolomide by affecting O6-methylguanine DNA methyltransferase promoter methylation via c-Myc signaling

【総説】

第12回　高安賞最優秀論文賞受賞

論文　「Glycogen synthase kinase 3β inhibition sensitizes human glioblastoma cells to temozolomide by affecting O^6-methylguanine DNA methyltransferase promoter methylation via c-Myc signaling」
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グリコーゲンシンターゼキナーゼ-3βの阻害によるc-Mycを介したMGMT promoterメチル化促進に起因する膠芽腫細胞のテモゾロミド感受性増強効果

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背景：セリン・スレオニンキナーゼの1種であるグリコーゲンシンターゼキナーゼ-3β (GSK3β) は膠芽腫を含めたヒト癌の悪性形質に関与すると報告されている．現在，膠芽腫に対しては標準治療薬としてテモゾロミドが使用され，その有効性は腫瘍組織内の薬剤抵抗分子O6-methylguanine DNA-methyltransferase (MGMT) の有無によって予測可能である．本研究ではGSK3β 阻害によるテモゾロミドに対する膠芽腫細胞の感受性増強作用とそのメカニズムを解析した．

方法：GSK3β 阻害医薬品を使用する再発膠芽腫を対象とした臨床試験で得られた初発時，剖検時の摘出検体を用いてMGMTの免疫染色とMGMT promoterのメチル化解析にmethylation-specific polymerase chain reaction (MSP) を行った．4種類の膠芽腫細胞株（T98G, U138, U251, U87）を用いてGSK3β の特異的阻害剤およびsmall interfering RNA (siRNA) によるTMZ感受性増強効果を増殖アッセイによって調べた．そのメカニズムをMSP, メチル化の定量のためにMethyLight assay, MGMT発現量の定量のためにQRT-PCR, MGMT promoterに結合する分子を同定するためにCHIP assayを行った．

結果：GSK3β 阻害医薬品の投与を受けた剖検検体でGSK3β の基質であるグリコーゲンシンターゼのリン酸化の抑制が認められるとともにMGMTの発現が低下していた．膠芽腫細胞株においてテモゾロミドとGSK3β 阻害剤の併用は相加的，相乗的な細胞増殖抑制効果を示した．GSK3β 阻害によりMGMT promoterのメチル化は促進しMGMTの発現は抑えられた．そのメカニズムとしてc-MycがMGMT promoterに結合しDANメチル化酵素DNMT3Aがリクルートされることが明らかとなった．

結論：GSK3β 阻害によるテモゾロミドに対する膠芽腫細胞の感受性増強作用は，c-Mycを介してMGMT promoterのメチル化が促進されMGMTの発現が抑えられることに起因すると考えられた．これは膠芽腫に対してテモゾロミドと併用するGSK3β 阻害療法の正当性を支持する基礎基盤となる結果である．

I. Introduction

Glioblastoma (GBM) is the most frequent malignant tumor of the brain and is highly unresponsive to the currently available anticancer treatments. The proliferative and invasive activity of GBM [1] hinders curable surgical intervention, and makes GBM highly resistant to radiation and chemotherapy [2]. Consequently, there is an urgent need to develop new treatment modalities represented by molecular target-directed therapies.

O^6-methylguanine DNA methyltransferase (MGMT) promoter methylation is an independent favorable prognostic factor in patients with GBM [3]. The levels of MGMT gene promoter methylation are associated with MGMT expression and are important in setting the transcriptional state of the MGMT promoter through changes in chromatin structure. Methylation status of the promoter is implicated in chemosensitivity to temozolomide (TMZ) [3].

Glycogen synthase kinase 3β (GSK3β) is a serine/threonine protein kinase involved in human cancers including glioblastoma. We have previously demonstrated that GSK3β inhibition enhances temozolomide effect in glioma cells. In the report, we have investigated the molecular mechanisms of sensitization of glioblastoma cells to temozolomide by GSK3β inhibition, focusing on O6-methylguanine DNA methyltransferase (MGMT) gene silencing. Glioblastoma tissues from patients treated with the GSK3β-inhibiting drugs were subjected to immunohistochemistry and methylation-specific PCR assay. Human glioblastoma cell lines T98G, U138, U251 and U87 were treated with a small-molecule GSK3β inhibitor, AR-A014418 or GSK3β-specific small interfering RNA. The combined effect of

temozolomide and AR-A014418 on cell proliferation was determined by AlamarBlue assay and an isobologram method. MGMT promoter methylation was estimated by methylation-specific PCR and MethyLight assay. MGMT gene expression was evaluated by real-time quantitative reverse transcriptase-PCR. c-Myc and DNA (cytosine-5)-methyltransferase 3A binding to the MGMT promoter was estimated by chromatin immunoprecipitation assay. GSK3β inhibition decreased phosphorylation of glycogen synthase and reduced MGMT expression and increased MGMT promoter methylation in clinical tumors. In glioblastoma cell lines, GSK3β inhibition decreased cell viability, enhanced temozolomide effect and downregulated MGMT expression with relevant changes in the methylation levels of the MGMT promoter. Here, we showed for the first time that c-Myc binds to the MGMT promoter with consequent recruitment of DNA (cytosine-5)-methyltransferase 3A, regulating the levels of MGMT promoter methylation. The results of this study suggest that GSK3β inhibition enhances temozolomide effect by silencing MGMT expression via c-Myc-mediated promoter methylation.

II. Results and discussion

1) *GSK3β inhibition and glioma cell viability*

To determine the function of GSK3β in the glioma cell lines, we used siRNA to specifically knockdown endogenous GSK3β. Depletion of GSK3β, confirmed by Western blot analysis, significantly decreased cell viability in T98G and U251 cells. To further assess the effect of inhibition of GSK3β activity, we treated the cells with dose-escalating AR-A014418. Cell viability was decreased by AR-A014418 treatment in T98G and U251 cells. Depletion of total and tyrosine 216-phosphorylated GSK3β by AR-A014418 was observed in glioma cells by Western blot analysis.

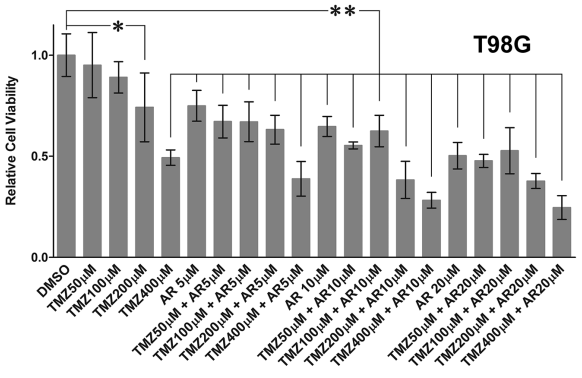

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Fig. 1. The combined effect of TMZ and a GSK3β inhibitor (AR-A014418) on glioblastoma cells. The relative cell viability of T98G cells after treatment for 144 h with temozolomide (TMZ; 50, 100, 200, and 400 μmol/L) or AR-A014418 (5, 10, and 20 μmol/L), alone or in different combinations shown below the panel, measured by the AlamarBlue assay. The relative viability of the cells treated with DMSO alone was scored as 1.0. *, $p < 0.05$; **, $p < 0.01$; AR, AR-A014418; Mann-Whitney U Test, compared to DMSO-treated cells; bars show SD.

2) *Combined effect of GSK3β inhibitor and TMZ in GBM cells*

To investigate whether GSK3β inhibition enhances TMZ effect, T98G, U138 and U87 cells were treated with different doses of TMZ or AR-A014418, alone or in combination. In our study, T98G and U138 cells appeared to be highly resistant to TMZ treatment, and this can be attributed to high MGMT gene expression level in these cell lines. The combination of low-dose AR-A014418 and TMZ significantly reduced cell viability compared to the treatment with TMZ or low-dose AR-A014418 alone (Figure 1).

We showed that GSK3β inhibition enhanced TMZ effect in T98G cells to sensitivity levels seen in TMZ-sensitive cell lines, whereas U138 demonstrating lower basal MGMT expression showed additive and moderate synergistic effect, while mainly additive effect was noted in U87 and in U251 with undetectable and low MGMT expression, respectively.

3) *Effect of GSK3β inhibition on MGMT promoter methylation status and gene expression*

To investigate the influence of GSK3β inhibition on MGMT promoter methylation, a MSP assay was performed in T98G, U251 and U138 cells expressing MGMT. AR-A014418 treatment decreased or ablated unmethylated MGMT promoter in T98G, increased methylated MGMT promoter in U251 and decreased unmethylated MGMT promoter in U138 cells. MSP assay data were confirmed by the MethyLight assay showing that GSK3β inhibition increased methylation of CpG islands in the MGMT promoter in T98G, U251 and U138 cells after treatment with AR-A014418. Consistent with the changes in methylation levels, GSK3β inhibition by AR-A014418 decreased the levels of MGMT gene expression in T98G, U138 and U251 cells as measured by QRT-PCR. Consistently, MGMT protein expression was decreased by AR-A014418 treatment in T98G and U138.

4) *Effect of GSK3β inhibition on c-Myc and DNMT3A binding to the MGMT promoter*

To investigate the molecules that are responsible for the changes in MGMT promoter methylation status, we focused on c-Myc signaling. c-Myc is a good candidate

since GSK3β inhibition increases c-Myc expression in U251 [4], T98G, U138 and U87 cells, as shown by Western blot analysis and QRT-PCR.

Concomitant treatment with the c-Myc inhibitor and AR-A014418 maintained the fraction of unmethylated MGMT promoter in T98G, U251 and U138 cells as shown by MSP assay, suggesting that c-Myc plays a role in MGMT promoter methylation in the GBM cells. MethyLight assay demonstrated that c-Myc inhibitor abrogated the AR-A014418-induced increase of MGMT promoter methylation in all cells. The results of MSP and MethyLight assays collectively suggest that c-Myc-mediated signaling is responsible for MGMT promoter methylation induced by the GSK3β inhibitor in the GBM cells.

c-Myc is an important factor in cell proliferation that enables G1/S cell cycle progression [5]. A previous report demonstrated that c-Myc has a suppressive effect in human GBM cells, as c-Myc upregulation is associated with increased apoptosis [4]. c-Myc expression is increased in GBM cells by GSK3β inhibition via its effects on c-Myc phosphorylation and in some cell lines its effect on β-catenin-mediated gene transcription.

c-Myc is known to selectively target DNMT3A to the promoter of the gene, resulting in DNA methylation *de novo* and silencing of the targeted promoter [6]. However, there are no studies showing binding of c-Myc to the MGMT promoter, despite the presence of an E-box transcription factor-binding site in the MGMT promoter. In this study, we hypothesized that a high expression level of c-Myc in GBM cells induced by GSK3β inhibition induces c-Myc binding to the E-box, resulting in transcriptional repression via recruitment of DNMT3A.

We examined possible interaction between c-Myc and DNMT3A by the ChIP assay. In ChIP assay, c-Myc binding to the E-box transcription factor binding site in the MGMT promoter was on detectable level in U138 and below the limit of quantitation in T98G in DMSO-treated control, whereas AR-A014418 treatment increased c-Myc binding to the same site in the MGMT promoter in both cell lines. This finding was accompanied by increased DNMT3A binding to the same region in the MGMT promoter in T98G and U138 cells (Figure 2A, B). These data suggest that, in response to GSK3β inhibition, c-Myc recruits DNMT3A to the MGMT promoter, which increases its methylation level in T98G and U138 cells. Our ChIP assay demonstrated for the first time that c-Myc binds to the MGMT promoter in intact cells and that c-Myc and DNMT3A binding to the MGMT promoter increases after AR-A014418 treatment. The mediation of c-Myc for

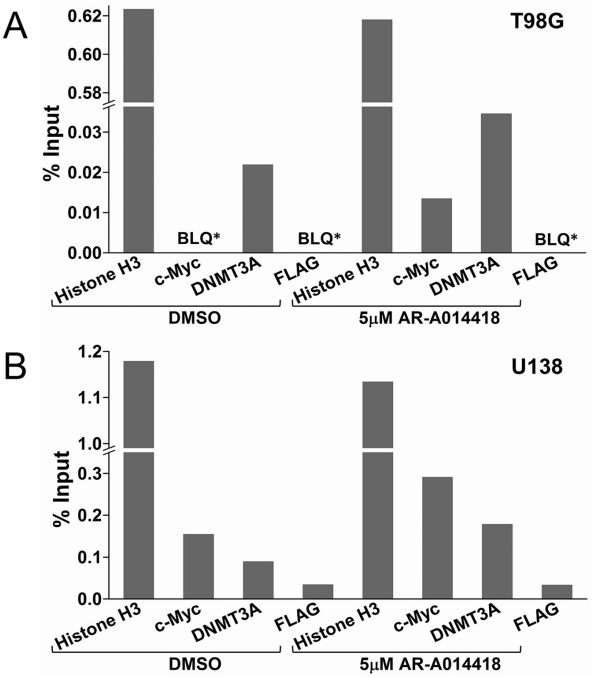

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Fig. 2. Epigenetic silencing of MGMT expression by GSK3β inhibition. (A, B) Comparison by ChIP assay of the binding of histone H3, c-Myc, DNMT3A, and FLAG to the E-box site in the MGMT promoter between the GBM cells treated with DMSO or with a GSK3β inhibitor (AR-A014418). T98G and U138 cells were treated for 72h with DMSO or 5 μmol/L of AR-A014418. The amount of DNA coprecipitated with the antibody to each molecule was measured by qPCR with a set of primers specific to the E-box transcription factor-binding site in the MGMT promoter. *BLQ, below the limit of quantitation. The figure shows the representative data from two independent immunoprecipitations.

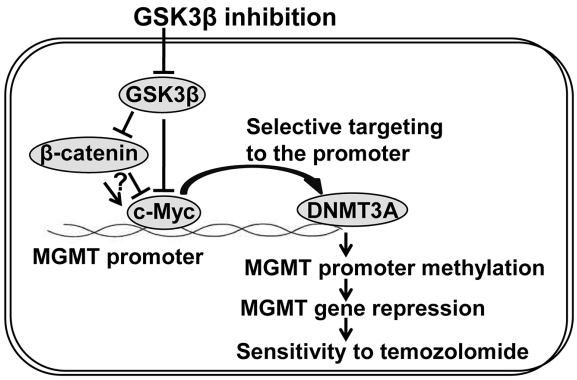

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Fig. 3. Regulation of MGMT expression by GSK3β signaling. GSK3β inhibition results in c-Myc activation directly and via activation of β-catenin-mediated signaling, which consequently increases recruitment of DNMT3A by c-Myc to the MGMT promoter, thus increasing *de novo* DNA methylation in the MGMT promoter. The methylated status of the MGMT promoter increases the sensitivity of GBM to TMZ.

recruitment of DNMT3A to the MGMT promoter was confirmed by comparison of data from the conventional ChIP assay, which includes a one-step protein and chromatin fixation with formaldehyde, and data from the ChIP assay with a two-step protein and chromatin fixation. Conventional ChIP assay, which effectively detects direct protein/DNA binding, did not reveal detectable DNMT3A binding to the MGMT promoter. In contrast, ChIP assay with a two-step protein and chromatin fixation provides effective detection of both direct protein/DNA binding, and indirect protein/protein/DNA binding. Consequently, our ChIP assay with a two-step protein and chromatin fixation showed detectable DNMT3A binding to the MGMT promoter with an increased binding level in GBM cells treated with AR-A014418. This was consistent with an increase in c-Myc binding after AR-A014418 treatment, suggesting recruitment of DNMT3A by c-Myc to the MGMT promoter (Figure 2A, B).

Based on our data, we speculate that GSK3β inhibition eliminates the suppressive effect of GSK3 β on c-Myc expression by the effects on c-Myc phosphorylation, which subsequently increases recruitment of DNMT3A by c-Myc to the MGMT promoter in GBM cells. The interaction between c-Myc and DNMT3A would then activate DNA methyltransferase, which increases local *de novo* DNA methylation in the CpG islands of the MGMT promoter. MGMT promoter methylation silences MGMT gene expression, and this finally sensitizes GBM cells to TMZ (Figure 3).

III. Conclusion

In our clinical research, the GSK3β-inhibiting drugs inhibited glycogen synthase phosphorylation and decreased MGMT expression, a predictor for resistance to TMZ, in tumor cells in patients with recurrent GBM. Consistent with these findings, the status of the MGMT promoter changed from unmethylated to methylated after treatment with GSK3β-inhibiting drugs. These data suggest that GSK3β inhibition decreases MGMT production in GBM affecting the methylation status of the MGMT promoter. This observation in patient tumors was extrapolated by novel findings in this study, demonstrating that GSK3β inhibition in GBM cells induced c-Myc-dependent recruitment of DNMT3A, leading to MGMT promoter methylation and consequent silencing of MGMT expression (Figure 2A and B). The provided data will facilitate the development of an optimal combination of GSK3β inhibitor and alkylating agents for their use in the treatment of patients with GBM.

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